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Effects of different light quality and biofertilizers on structural and physiological traits of spinach plants

L. VITALE[*,†,+], E. VITALE[**,†], G. GUERCIA[**], M. TURANO[**], and C. ARENA[**]

National Research Council (CNR), Institute for Agricultural and Forestry Systems in the Mediterranean (ISAFoM), Via Patacca 85-80056, Ercolano (Na), Italy[*]
University of Naples Federico II, Department of Biology, Via Cinthia, 80126 Naples, Italy[**]

Abstract

In this work, the effects of light quality and beneficial microbes (biofertilizer) supply on structural and ecophysiological traits of spinach were investigated. Plants were grown under four light quality regimens: white light (WL), red-blue (RB), red-green (RG), and red (R) light, with or without the addition of biofertilizer. RG and R plants without biofertilizer showed morphological traits typical of shaded plants as wide leaf lamina and high photosynthetic pigment content. These plants also exhibited a higher photosynthetic capacity compared to WL and RB plants. The improved photosynthesis in RG plants was due to both morphological and physiological adjustments allowing a better utilisation of light energy, whereas in R plants it has been attributed to a reduced photorespiration rate. Biofertilizer application under WL improved plant performance enhancing photosynthesis. The high carbon gain compensates the costs of symbiosis. Biofertilizer application under R light favouring too much the microbial root colonisation, removed the benefits of symbiosis. The interaction of light quality and biofertilization significantly affects the root–microbe relationship.

Additional key words: antioxidants; gas exchange; light manipulation; photochemistry; plant–microbe interaction.

Introduction

Agroecosystems require high-energy inputs to reach a high level of productivity, deeply affecting climate and environment (Clark and Tilman 2017). For this reason, it is crucial to shift towards a sustainable agriculture to preserve natural resources and reduce the impact on the environment. Indoor cultivation by sustainable innovative tools might represent a promising solution to reduce the deleterious effects of extensive crop production on the ecosystems.

Light manipulation, through light-emitting diode (LED) technology, is becoming one of the most valuable approaches in controlled-environment agriculture. The LED technology offers many advantages over traditional forms of lighting including high luminous efficiency, reduced energy consumption and cost, and low heat production (Singh *et al*. 2015, Izzo *et al.* 2019, Paradiso *et al.* 2019). Moreover, the LED light systems allow managing the light spectrum composition defining specific light regimes useful for plant growth and development.

Light spectrum composition affects plant growth influencing plant anatomy, morphology, and physiology (Ye *et al.* 2017, Zheng and Van Labeke 2017). In particular, red and blue wavelengths are efficiently used by photosynthetic apparatus and are fundamental for the plant healthy growth. Red light determines changes in shoot/stem ratio or shoot/root ratio, plant structure, and photosynthesis (Schuerger *et al.* 1997, Amitrano *et al.* 2018). Blue light is essential for chlorophyll biosynthesis, stomatal opening, chloroplast development and maturation, as well as synthesis of photosynthetic enzymes (Heo *et al.* 2002, Urbonaviciute *et al.* 2007, Hernández and Kubota 2016, Wang *et al.* 2016). The addition of green light can further increase plant biomass under certain circumstances (Kim *et al.* 2004, Johkan *et al.* 2012). Some studies have demonstrated that also green light has an essential role in controlling plant development and photosynthesis, because it penetrates deeper into the leaf mesophyll and canopy layers, driving photosynthesis where other wavelengths (*i.e.*, red and blue) are limiting (Terashima *et al.* 2009, Folta 2005, Smith *et al.* 2017). Green light has been rarely mixed to red and blue wavelengths for leafy vegetable production (Arena *et al.* 2016, Hristozkova *et al.* 2017). For this reason, further investigations are needed to assess if the beneficial effects of green light are the result of a

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[+]Corresponding author; phone: +390817886701, fax: +390817718045, e-mail: luca.vitale@cnr.it
Abbreviations: Anth – anthocyanin; Car – carotenoid; C_c – chloroplast CO_2 concentration; DAS – days after sowing; ETR – electron transport rate; ETR/P_{gmax} – electron sink processes other than carbon assimilation; FRAP – ferric reducing antioxidant power; g_m – mesophyll conductance; g_s – stomatal conductance; I – inoculated; LDMC – leaf dry mass content; LMA – leaf mass per area; LT – leaf thickness; NI – noninoculated; P_{gmax} – light-saturated gross photosynthetic rate; P_{Nmax} – light-saturated net photosynthetic rate; TP – total polyphenols; TSC – total soluble carbohydrates; Φ_{NO} – quantum yield of nonregulated energy dissipation; Φ_{NPQ} – quantum yield of regulated energy dissipation; Φ_{PSII} – effective quantum yield of PSII photochemistry.
[†]Co-first author.

direct effect on photosynthesis or rather is responsible for other light-mediated morphogenic mechanisms.

Light quality may also enhance the production of bioactive compounds, improving the nutraceutical properties of some crop species. More specifically, the selection of specific wavelengths influences the biosynthesis of polyphenols (*e.g.*, phenols, flavonoids, anthocyanins, *etc*.) (Victório *et al.* 2015, Ye *et al.* 2017) and other antioxidant compounds (*e.g.*, ascorbic acid, tocopherols, carotenoids, *etc*.) (Samuolienė *et al.* 2016) with valuable effects on human health.

Besides light quality, the addition of beneficial microorganisms to soil is a conventional practice to improve plant productivity as it influences the availability and the uptake of macro and micronutrients (Ahemad and Kibret 2014, Nascente *et al.* 2017) or the synthesis of natural growth regulators (*i.e.*, hormones) (Spaepen and Vanderleyden 2011). Furthermore, microorganisms offer to plant the protection from pathogens through antimicrobials production, trigger the accumulation and/or release of secondary metabolites, and stimulate the induction of systemic resistance (Compant *et al.* 2005, Mhlongo *et al.* 2018). All these aspects contribute to the overall plant health status and represent an attractive alternative to the use of synthetic chemicals for sustainable agriculture with benefits on both human health and the environment.

It is noteworthy that light extent and quality directly or indirectly influence microbial growth. Bacteria and fungi perceive the environmental light conditions through light-sensing proteins and modulate their growth in response to light (Purschwitz *et al.* 2007, Hristozkova *et al.* 2017). Some studies show that blue wavelengths inhibit bacteria and fungi growth (De Lucca *et al.* 2012) whereas red wavelengths promote the formation of arbuscular mycorrhizal fungi (AMF) (Cruz 2016). Light may also indirectly affect microbial growth as it stimulates the production of photosynthetic exudates that represent a source of readily available nutrients for microorganisms (Doornbos *et al.* 2012). Thus, any influence of light on plant metabolism or microorganism growth may influence plant–microorganism interactions. These relationships are species-specific for plants and microorganisms and might depend on the applied light quality regimen (Hristozkova *et al.* 2017). Current knowledge on the combined effects of light quality and beneficial microorganisms on plant growth is limited (Alsanius *et al.* 2019); more research on this topic might help to maximise the plant productivity for food provisioning by setting-up specific growth protocols.

Among crops widely utilised in human nutrition, spinach (*Spinacia oleracea* L.) responds to different light quality regimens with changes in plant development and nutritional properties (Matsuda *et al.* 2007, 2008; Ohashi-Kaneko *et al.* 2007, Agarwal *et al.* 2018). This leaf vegetable is also sensitive to the beneficial microorganism biofertilisation as increases edible biomass, bioactive compound content, and resistance to stress (Çakmakçı *et al*. 2007, Zuccarini and Savé 2016, Khalid *et al.* 2017).

In this paper, we assessed the relationship between different light quality regimes and the application of beneficial microorganism in spinach plants. In particular, we analysed plant growth, photosynthetic behaviour, functional leaf traits and bioactive compound production under different light quality treatments, with or without the addition of plant growth-promoting microorganisms (PGPM) on the soil. The information acquired from this study will contribute to the knowledge on the light–plant–microbe interaction and can be used to develop sustainable growth protocols for leafy crops by maximising the indoor cultivation.

Materials and methods

Plant material and experimental set-up: Seeds of spinach plants (*Spinacia oleracea* L.) were sown in 0.5-L plastic pots filled with a mixture of sterilised sandy soil and perlite substrate (3:1, v/v) and placed inside a growth chamber, equipped with a LED lighting system, under four different light regimes: broad-spectrum white light (WL), red-blue (RB, emission peaks at 620 and 660 nm, emission peak at 460 nm, 60:40), red-green (RG, emission peaks at 620 and 660 nm, emission peaks at 500 and 530 nm, 60:40), and 'pure' red (R, emission peaks at 620 and 660 nm) light. All plants were subjected to the same growth conditions: PPFD of 350 μmol(photon) m^{-2} s^{-1} at the top of the canopy, 25/15°C day/night temperature, 50/70% day/night relative humidity, and photoperiod of 12 h. Temperature and humidity were monitored by a digital thermo-hygrometer (*HC520 Digital Thermo-Hygrometer*, *Cheerman*, Guangdong, China), and the irradiance was measured by the *Li-Cor190R* quantum sensor (*Li-Cor*, Lincoln, Nebraska, USA). Plants were watered to field capacity to reintegrate water lost by evapotranspiration and fertilised every week with a complete nutritive solution composed by micronutrients, nitrogen, phosphorus, and potassium ($N:P_2O_5:K_2O$, 20:20:20 g L^{-1}) (*Poly-Feed GG*, *Haifa Italia*, Bologna). A commercial biofertilizer (*RadiNET*, *Micosat F®*, *C.C.S. Aosta s.r.l.*, Aosta, Italy) containing mainly arbuscular mycorrhizal fungi (AMF) (*Glomus* genus, *Rhizophagus irregularis*), saprophytic fungi (*Pochonia chlamydosporia*, *Tricoderma* genus), and a reduced amount of rhizosphere bacteria (*Bacillus* and *Streptomyces* genus) was applied to soil at sowing and every week for three weeks. In each application, 0.6 g of biofertilizer was dissolved in 10 ml of deionized water. For each light regime (WL, RB, RG, R), five plants were treated with biofertilizer (inoculated plants – I) and five plants without (noninoculated plants – NI); ten plants for each light regime in total.

Biometrical measurements and functional leaf trait determinations: Green leaf area per plant was measured every 20 d, acquiring the images by a digital camera and measuring leaf expansion by *Image J* software (Wayne Rasband NIH, http://imagej.nih.gov/ij/index.html).

Plant biomass was determined at 100 d after sowing (DAS) drying roots and shoots in a forced-air oven at 75°C up to constant mass. Functional leaf traits were monitored at harvest time (100 DAS) on five noninoculated and five inoculated plants by each light growth treatment. The leaf area – LA [cm^2], leaf mass per area – LMA [g cm^{-2}], leaf

dry mass content – LDMC [g g^{-1}], and leaf thickness – LT [μm], were determined according to Cornelissen *et al.* (2003).

Photosynthetic pigment content: Total chlorophylls and carotenoids were determined at 100 DAS on five different leaf samples from each light treatment according to Lichtenthaler (1987). Pigments were extracted from samples using mortar and pestle in ice-cold (4°C) 100% acetone and centrifuged at 5,000 rpm for 5 min (*Labofuge GL*, *Heraeus Sepatech*, Hanau, Germany). The absorbance of supernatants was quantified by a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Santa Clara, CA, USA) at 470, 645, and 662 nm and pigment concentration expressed in mg 100 g^{-1}(FM).

Polyphenols, anthocyanins, antioxidant capacity and carbohydrates determination were carried out on five different leaves (one leaf per plant) collected at 100 DAS. Polyphenols were determined as reported in Arena *et al.* (2019). Samples (0.02 g) were ground in liquid nitrogen, incubated with methanol at 4°C, and centrifuged at 11,000 rpm for 5 min. The supernatant was extracted and mixed with 1:1 (v/v) 10% Folin-Ciocâlteu and 1:5 (v/v) 700 mM Na_2CO_3 solution. Samples were incubated at 4°C for 2h. The absorbance was quantified by a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA) at 765 nm. The total polyphenols concentration was calculated and expressed as gallic acid equivalents [mg(GAE) 100 g^{-1}(FM)] using a regression equation between gallic acid standards and A_{765}.

Total anthocyanins content was determined on 0.05-g sample leaves ground in liquid nitrogen, treated with methanol 1% HCl solution, and stored overnight at 4°C. After the addition of 1:0.6 (v/v) ultra-pure water and 1:1.6 (v/v) chloroform, samples were centrifuged at 11,000 rpm for 5 min. Supernatant was extracted from each sample adding 1:1 (v/v) 60% methanol 1% HCl 40% ultra-pure water solution. The absorbance was measured spectrophotometrically (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA) at 530 and 657 nm. The relative amount of anthocyanin was expressed as [($A_{530} - 0.33A_{657}$) 100 g^{-1}(FM)] (Mancinelli *et al.* 1975).

The ferric reducing antioxidant power (FRAP) assay was performed to determine the total soluble antioxidant capacity according to method described by George *et al.* (2004) and modified by Motta *et al.* (2019). Samples (0.250 g) were ground in liquid nitrogen, treated with 60:40 (v/v) methanol/water solution and centrifuged at 14,000 rpm for 15 min (4°C), collecting supernatants for the assay. The FRAP reagent (1:16 300 mM acetate buffer pH 3.6; 1:1.6 of 10 mM TPTZ in 40 mM HCl; 1:1.6 of 12 mM $FeCl_3$) was added to each sample extract and the mixture incubated in the darkness for 1 h. The sample absorbance was read by a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA) at 593 nm. Total antioxidant capacity was quantified and expressed as mmol Trolox equivalents [mmol(TE) 100 g^{-1}(FM)] using a Trolox standard curve.

Total soluble carbohydrates content was determined on five different leaf samples (0.01 g) of each treatment following the anthrone method reported by Hedge and Hofreiter (1962). The absorbance was measured at 630 nm by a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA). The amount of total soluble carbohydrates in the extracts was calculated using a glucose standard curve and expressed as glucose equivalents [g(GE) 100 g^{-1}(FM)].

Photosynthetic characteristics and Chl fluorescence parameters: Gas exchange and fluorescence measurements were simultaneously performed on fully expanded leaves by means of *LI-6400* (*Li-Cor*, Lincoln, Nebraska, USA) integrated with *LI-6400-40* leaf chamber fluorometer. Light-response curves (LRC) were carried out illuminating the leaves with red plus blue LEDs at 25°C, 360 μmol(CO_2) mol^{-1}, and 50% air relative humidity (RH) to determine the light-saturated net photosynthesis. Net photosynthetic rate (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981). At each irradiance level, the steady-state fluorescence yield (F_s) and the maximal fluorescence yield in the light-adapted state (F_m') were measured applying a 0.8 s-saturating flash of 8,000 μmol(photon) m^{-2} s^{-1}, and the effective quantum yield of PSII photochemistry (Φ_{PSII}) (Genty *et al.* 1989), the regulated (Φ_{NPQ}) and the nonregulated (Φ_{NO}) energy dissipation (Kramer *et al.* 2004) were calculated. Electron sink processes other than carbon assimilation (ETR/P_{gmax}) were evaluated by the ratio between the electron transport rate (ETR) and light-saturated gross photosynthetic rate (P_{gmax}) (Krall and Edwards 1992). Maximal quantum yield of PSII photochemistry (F_v/F_m) was measured at the end of each LRC on 30-min dark-adapted leaves measuring the minimal fluorescence of the dark-adapted state (F_0) and maximal fluorescence yield of the dark-adapted state (F_m), applying a saturation pulse of 8,000 μmol(photon) m^{-2} s^{-1}. Mesophyll conductance (g_m) was determined at 360 μmol(CO_2) mol^{-1} by the variable *J* method (Loreto *et al.* 1992), assuming that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration and that chlorophyll fluorescence gives a reliable estimation of the quantum yield of electron transport. Mitochondrial respiration in the light (R_L) and the CO_2-compensation point in the absence of day respiration (T^*) were estimated according to Laisk and Oja (1998) by performing P–C_i response curves at three different light intensities and using only the points on the linear portion of the curves. g_m was used to calculate the concentration of CO_2 at the sites of carboxylation (C_c).

Arbuscular mycorrhizal fungi (AFM) colonization assay: A set of plants was used for AMF colonization assay in WL and R plants. Pieces of roots were cleared in 10% KOH and stained with 0.05% aniline blue in vinegar 5% (v/v), according to Vierheilig *et al.* (1998) and Vierheilig and Piché (1998). Images were acquired by light microscopy (*Nikon Eclipse E1000*, *Nikon Instruments Inc.*, Melville, New York, USA) using a digital camera (*Nikon DXM1200F Microscope Camera*, *Nikon Instruments Inc.*,

Melville, New York, USA). The root colonization was expressed as % considering the ratio between the number of root fragments showing colonization and the total number of root fragments observed; the root infection was expressed as number of vesicles presenting on a cm of root fragment.

Statistical analysis was performed by *Sigma-Plot 12.0* software package (*Jandel Scientific*, San Rafael, CA, USA). Data were analysed by two-way *ANOVA* followed by the *Duncan*'s test for multiple comparison procedures. The results are reported as mean (n = 5) ± standard deviation. Differences were considered statistically significant at $p \leq 0.05$. *Shapiro-Wilk*'s and *Kolmogorov-Smirnov*'s tests were performed to check for normality. The correlations between selected parameters were investigated using *Pearson*'s correlation test.

Results

Root colonization by AMF: Spinach roots resulted colonized by AMF (Fig. 1). Roots of plants grown under monochromatic red light showed a higher microbe infection compared to plants grown under white light (Table 1).

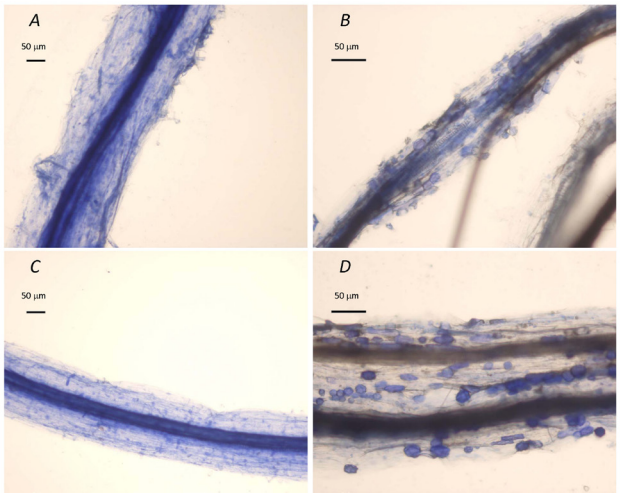

Fig. 1. Microbe infection in spinach roots. Noninoculated plants under white light, WL (*A*); inoculated plants under WL (*B*); noninoculated plants under pure R light, R (*C*); inoculated plants under pure R light, R (*D*). 10× magnification – *A*,*C*; 20× magnification – *B*,*D*. Scale bar = 50 µm.

Plant growth and leaf functional traits: The diverse light treatments significantly affected the plant morphology (Fig. 1S, *supplement*). The total biomass did not change under different light treatments compared to WL in both NI and I plants (Fig. 2*A*). The combination light treatments × biofertilisation produced a rise of the biomass in I compared to NI plants only under RG light treatment. Conversely to dry shoot mass (Fig. 2*B*), the interaction light quality × biofertilizer affected root dry mass production (Fig. 2*C*). Among all light treatments, RB light promoted root biomass accumulation in noninoculated plants. The addition of biofertilizer to light treatments induced a significant rise of root biomass in WL and RG plants compared to respective noninoculated samples. The shoot/root ratio was the lowest in RB noninoculated plants compared to other light treatments. The biofertilizer application under different light quality treatments did not induce an increase of the root/shoot ratio, compared to noninoculated plants, except for RB plants that show a slight significant increase (Fig. 2*D*). As regards leaf functional traits, plants grown under RG and R light treatments showed leaves with greater area, lower LMA and LT compared to WL and RB plants (Table 2). These latter were characterised by high values of LMA and LDMC. The interaction biofertilizer × light treatment affected only R plants (I-R) where leaves with lower LA and higher LMA were found compared to noninoculated ones.

Photosynthetic and Chl fluorescence parameters: Light quality influenced leaf gas exchanges in both noninoculated (NI) and inoculated (I) plants (Fig. 3). The light-saturated net photosynthetic rate (P_{Nmax}), stomatal (g_s) and mesophyll (g_m) conductance to CO_2 were higher in RB, RG, and R compared to WL plants, reaching a maximum under pure R light treatments (Fig. 3 *A*–*C*). In inoculated plants, the highest values were observed under RG treatment. The addition of biofertilizer significantly increased P_{Nmax}, g_s, and g_m in WL and RB plants, whereas significantly reduced these parameters under pure R treatment. The concentration of CO_2 at carboxylation sites (C_c) was higher under RG and R compared to WL and RB light growth regimes in both NI and I plants (Fig. 3*D*). The noninoculated R plants showed the highest C_c value. The interaction light × biofertilizer was significant only for R plants showing a reduction of C_c in I compared to NI plants.

The effective quantum yield of PSII photochemistry (Φ_{PSII}) increased under RB, R, and RG light treatment

Table 1. Root colonization [%] and root infection [number of vesicles per cm] in noninoculated (NI) and inoculated (I) plants. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. *Capital letters* indicate significant differences between WL and R light treatments in inoculated (I) plants ($p \leq 0.05$). *Asterisks* represent different levels of significance ($^{***}p \leq 0.001$). WL – white light; R – pure red light; L – light treatment; B – biostimulant; L × B – interaction light × biostimulant.

Parameters	WL		R		*ANOVA*		
	NI	I	NI	I	L	B	L × B
Colonization [%]	0	72.00 ± 4.80^{B}	0	94.00 ± 7.17^{A}	***	***	***
Infection [vesicles cm^{-1}]	0	33.95 ± 5.30^{B}	0	111.27 ± 37.93^{A}	***	***	***

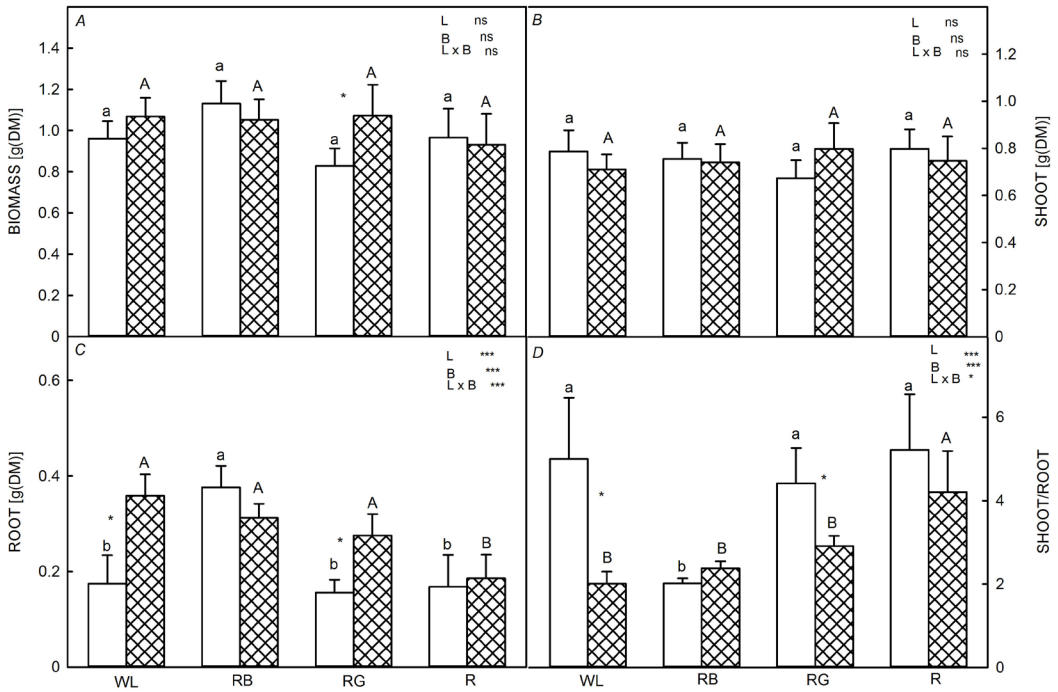

Fig. 2. Total biomass (*A*), shoot (*B*), and root (*C*) biomass, and shoot/root ratio (*D*) in noninoculated (*white bar*) and inoculated (*full bar*) plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences ($p \leq 0.05$) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences ($p \leq 0.05$) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represents different levels of significance ($^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B); ns – not significant.

compared to WL (Fig. 4*A*). The interaction light × biofertilizer determined a remarkable increase of Φ_{PSII} in WL plants and a significant reduction in R plants. The quantum yield of regulated energy dissipation (Φ_{NPQ}) showed an opposite trend, inducing a decrease of Φ_{NPQ} in WL and an increase in R plants (Fig. 4*B*). A significant increase of nonregulated energy dissipation (Φ_{NO}) was observed only under R treatment regardless of biofertilizer application (Fig. 4*C*). The pure R treatment determined a reduction of ETR/P_{gmax} ratio compared to the other light regimes (Fig. 4*D*). An interaction light × biofertilizer was found only for Φ_{PSII}. In particular, under WL the application of biofertilizer increased Φ_{PSII}, while it decreased Φ_{PSII} under R light.

Bioactive compounds: Plants grown under RG and R light treatments showed a significant increase of total chlorophyll and carotenoid content, compared to WL and RB plants, with the highest value in R plants. Within R light treatment, the application of biofertilizer induced a reduction of photosynthetic pigment content in I compared to NI plants (Table 3). The total polyphenol content was significantly reduced in RB, RG, and R compared to WL plants (Table 3); the application of biofertilizer increased polyphenol content only in RG inoculated plants compared to noninoculated but did not affect the anthocyanins content that was the same for both NI and I RG plants. Conversely, the addition of biofertilizer decreased anthocyanin amount in WL and RB plants compared to noninoculated samples (Table 3). The soluble antioxidant capacity was higher in RG and R compared to WL and RB plants in both inoculated and noninoculated plants. The total soluble carbohydrate content was lower in RG and R compared to WL and RB plants in both inoculated and noninoculated plants, with the lowest values for R plants (Table 3).

Correlation among the investigated leaf parameters: P_{Nmax} was positively correlated to g_s (r = 0.891) and g_m (r = 0.799), Φ_{PSII} (r = 0.841), photosynthetic pigments (r = 0.560), and antioxidant capacity (r = 0.635), and was negatively correlated to Φ_{NPQ} (r = – 0.856) (Table 4). This latter was negatively correlated to LMA (r = – 0.610) and LDMC (r = – 0.653). g_m was negatively correlated to LMA (r = – 0.838) and LMDC (r = – 0.783). Φ_{PSII} was negatively correlated to Φ_{NPQ} (r = – 0.826) and anthocyanins content (r = – 0.379), and positively correlated to soluble antioxidant capacity (r = 0.569) (Table 4). This latter was positively correlated to photosynthetic pigment content (r = 0.544) and negatively correlated to total polyphenol amount (r = – 0.403) and Φ_{NPQ} (r = – 0.759) (Table 4).

Discussion

Plant growth, photosynthesis, and bioactive compound production: The manipulation of the light spectrum allows to obtain specific physiological responses in spinach plants associated with the modulation of photosynthesis and the synthesis of bioactive compound. Our data indicate that

Table 2. Leaf area (LA), leaf mass per area (LMA), leaf dry mass content (LDMC), and leaf thickness (LT) determined in noninoculated (NI) and inoculated (I) spinach plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences ($p \leq 0.05$) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences ($p \leq 0.05$) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represent different levels of significance (*** $p \leq 0.001$, * $p \leq 0.05$) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B). ns – not significant.

Leaf traits	WL		RB		RG		R		*ANOVA*		
	NI	I	NI	I	NI	I	NI	I	L	B	L × B
LA [cm^2]	5.52 ± 1.32^{a}	5.47 ± 1.38^{A}	4.88 ± 0.74^{b}	4.55 ± 0.65^{B}	11.09 ± 0.54^{c}	10.49 ± 2.62^{C}	25.67 ± 5.20^{d*}	11.32 ± 6.38^{C}	***	*	ns
LMA [g cm^{-2}]	0.012 ± 0.002^{a}	0.011 ± 0.001^{A}	0.013 ± 0.001^{a}	0.013 ± 0.001^{A}	0.0048 ± 0.000^{b}	0.0055 ± 0.001^{B}	0.0020 ± 0.000^{c*}	0.0046 ± 0.001^{B}	***	*	ns
LDMC [g g^{-1}(DM)]	0.14 ± 0.02^{a}	0.14 ± 0.01^{A}	0.16 ± 0.02^{a}	0.15 ± 0.00^{A}	0.086 ± 0.01^{b}	0.091 ± 0.01^{B}	0.086 ± 0.01^{b}	0.093 ± 0.01^{B}	***	ns	ns
LT [µm]	0.082 ± 0.013^{a}	0.077 ± 0.001^{A}	0.085 ± 0.002^{a}	0.089 ± 0.000^{A}	0.056 ± 0.002^{b}	0.059 ± 0.005^{B}	0.023 ± 0.002^{c*}	0.049 ± 0.010^{B}	***	*	ns

the RB light treatment, without biofertilisation, induced a partitioning of biomass toward roots compared to the other light regimes, confirming the positive influence of blue light on root development (Canamero *et al.* 2006) and the requirement of blue light for the optimal growth of spinach plants (Yorio *et al.* 2001, Agarwal *et al.* 2018). Generally, the absence of blue light or its insufficient or excessive amount, determined shade-avoidance responses, causing a reduction of total biomass and an imbalance in plant development (Chang *et al.* 2016, Yorio *et al.* 2001, Hernández and Kubota 2016, Agarwal *et al.* 2018). In our experiment, plants grown without blue light (RG and R plants) developed typical traits of a shade-avoidance syndrome (*i.e.*, higher leaf area and elongated shoots and petioles) but did not reduce their biomass. These plants showed morphological, physiological, and biochemical adjustments favouring the carbon gain. Beside a greater leaf area per plant, the changes included thinner leaves characterised by lower LMA and LDMC values and high chlorophylls and carotenoid content compared to the other light treatments. These specific traits may be associated with a more efficient light harvesting and CO_2 distribution inside the leaf and may have favoured the photosynthesis.

Leaf structure is an important determinant in controlling photosynthesis because it influences the light distribution within leaf as well as the CO_2 diffusion at the carboxylation sites. The light wavelengths are selectively absorbed and distributed inside the leaf. Red or blue light is largely absorbed by chloroplasts near leaf surface, whereas green penetrating deeper than red or blue light, drives the photosynthesis deeply in the mesophyll (Terashima *et al.* 2009). We hypothesise that the development of thin leaves under RG and R light treatments allowed light to penetrate deeper in the leaf layers. The reduced content of anthocyanins and polyphenols in plants grown under green wavelengths also may be associated with a more light penetration within leaf tissues since these compounds act as a natural filter against the light (Steyn *et al.* 2002, Landi *et al.* 2015). The reduced LMA and LDMC in RG and much more in R leaves contributed to alleviation of the limitations to the CO_2 diffusion in the mesophyll (Niinemets *et al.* 2009). We assumed that the higher photosynthetic rate of RG and R plants was due to the higher stomatal and mesophyll conductance. In fact, the reduced leaf thickness and tissue density shortening the pathway of CO_2 diffusion toward chloroplasts, likely helped gas exchange. The plant growth under green light (RG) developed leaves with higher Φ_{PSII} and carbohydrate content compared to pure R leaves, indicating that the green wavelength drives a higher utilisation of radiant energy in photochemistry. The improved CO_2 diffusion in R compared to RG leaves was the main reason for the stimulation of photosynthesis in these plants. Such elevated CO_2 concentration in the chloroplasts significantly reduced the photorespiration, according to Φ_{PSII} decline and ETR/P_{gmax} ratio value near to the theoretical threshold of 4–5. The decrease of photorespiration determined the rise of Φ_{NO} and the decline of Φ_{NPQ}, exposing plants to the risks of reactive oxygen species (ROS) accumulation (Agarwal *et al.* 2018), this could be a reason by which in

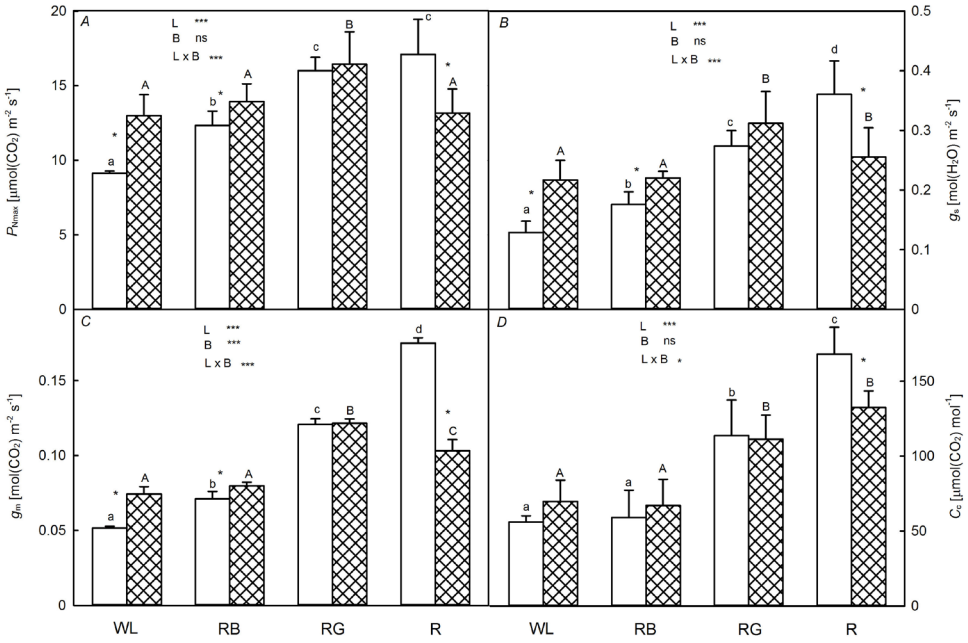

Fig. 3. Light-saturated net photosynthetic rate (P_{Nmax}) (*A*), stomatal conductance (g_s) (*B*), mesophyll conductance (g_m) (*C*), chloroplast CO_2 concentration (C_c) (*D*) in noninoculated (*white bar*) and inoculated (*full bar*) plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences (p≤0.05) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences (p≤0.05) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represents different levels of significance (***p≤0.001, **p≤0.01, *p≤0.05) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B); ns – not significant.

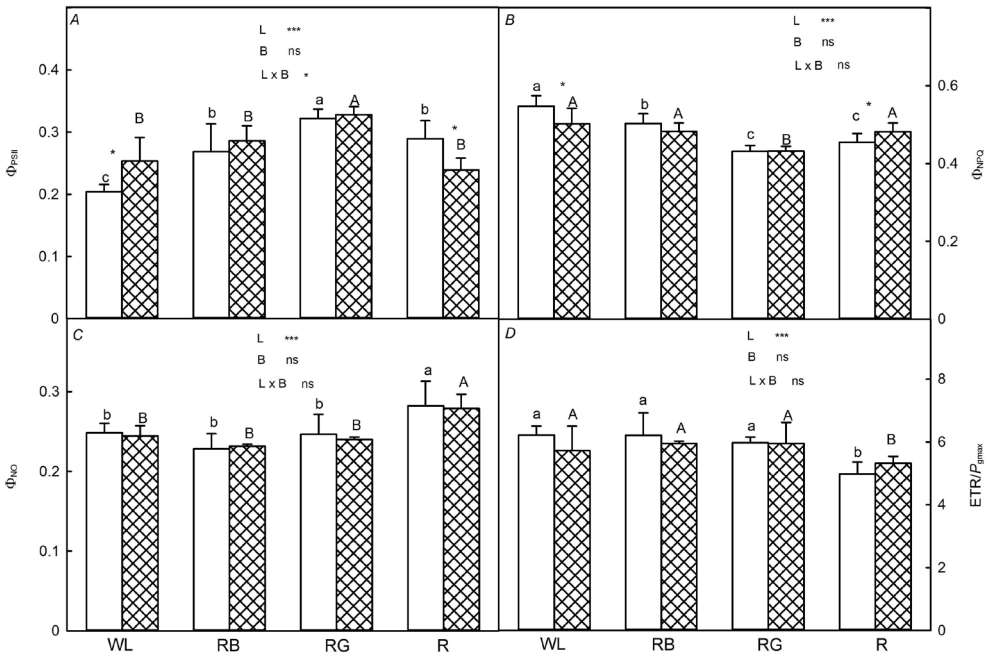

Fig. 4. Effective quantum yield of PSII photochemistry (Φ_{PSII}) (*A*), quantum yield of regulated energy dissipation (Φ_{NPQ}) (*B*), quantum yield of nonregulated energy dissipation (Φ_{NO}) (*C*), electron sink processes other than carbon assimilation (ETR/P_{gmax}) (*D*) in noninoculated (*white bar*) and inoculated (*full bar*) plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences (p≤0.05) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences (p≤0.05) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represents different levels of significance (***p≤0.001, **p≤0.01, *p≤0.05) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B); ns – not significant.

Table 3. Total chlorophyll, Chl (*a+b*); total carotenoids, Car (*x+c*); total polyphenols, TP; relative content of anthocyanins, Anth; soluble antioxidant capacity, FRAP; and total soluble carbohydrates, TSC; determined in noninoculated (NI) and inoculated (I) spinach plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences ($p \leq 0.05$) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences ($p \leq 0.05$) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represent different levels of significance (*** $p \leq 0.001$) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B). ns – not significant.

Parameter	WL		RB		RG		R		*ANOVA*		
	NI	I	NI	I	NI	I	NI	I	L	B	L × B
Chl (*a+b*) [mg 100 g^{-1}(FM)]	112.40 ± 11.91^{a}	103.84 ± 23.86^{A}	95.77 ± 25.24^{a}	101.74 ± 12.70^{A}	202.67 ± 25.33^{b}	195.71 ± 30.69^{B}	422.80 ± 86.30^{c*}	181.73 ± 32.43^{B}	***	***	***
Car (*x+c*) [mg 100 g^{-1}(FM)]	22.64 ± 3.65^{a}	21.80 ± 3.47^{A}	21.04 ± 4.30^{a}	21.74 ± 2.47^{A}	41.02 ± 5.75^{b}	38.65 ± 5.75^{B}	84.48 ± 18.97^{c*}	35.77 ± 4.66^{B}	***	***	***
TP [mg(GAE) 100 g^{-1}(FM)]	141.08 ± 20.54^{a*}	115.20 ± 9.92^{A}	106.06 ± 13.41^{b}	90.79 ± 14.71^{BC}	86.38 ± 10.32^{c}	98.56 ± 17.06^{AB}	87.69 ± 8.65^{c*}	53.68 ± 10.59^{D}	***	***	***
Anth [Anth 100 g^{-1}(FM)]	90.30 ± 10.12^{a*}	70.83 ± 9.72^{B}	87.44 ± 10.03^{a}	72.82 ± 2.93^{B}	37.75 ± 7.54^{c}	39.78 ± 19.24^{C}	77.56 ± 14.44^{a}	79.86 ± 16.46^{AB}	***	ns	ns
FRAP [mmol(TE) 100 g^{-1}(FM)]	0.27 ± 0.03^{a}	0.34 ± 0.06^{A}	0.31 ± 0.05^{a}	0.39 ± 0.02^{A}	0.65 ± 0.07^{b}	0.74 ± 0.14^{B}	0.60 ± 0.16^{b}	0.55 ± 0.10^{C}	***	ns	ns
TSC [g(GE) 100 g^{-1}(FM)]	23.96 ± 3.38^{a}	21.80 ± 1.34^{A}	26.76 ± 1.63^{a}	27.04 ± 4.97^{B}	7.63 ± 5.15^{b}	9.19 ± 2.57^{C}	3.14 ± 1.07^{c}	3.40 ± 1.36^{D}	***	ns	ns

RG and R plants increased significantly the antioxidant capacity in order to counteract efficiently the oxidative stress. Changes in the light spectrum are also responsible for changes in the amounts of polyphenols. The absence of blue wavelengths in RG and R treatments led to a drastic reduction of polyphenols content in spinach plants, emphasising the critical role exerted by blue light on the synthesis of the secondary metabolites.

Compared to WL, considered as control, the growth under red-blue (RB) light improved both stomatal and mesophyll conductance, stimulating photosynthesis. This result is consistent with previous studies on tomato and oriental plane (Arena *et al.* 2016). Other authors reported no benefits of blue light on photosynthesis in spinach (Yorio *et al.* 2001, Agarwal *et al.* 2018) or a reduced photosynthesis and mesophyll conductance in other species (Loreto *et al.* 2009, Pallozzi *et al.* 2013). As no difference was found between WL and RB in leaf functional traits affecting g_m (*i.e.*, LT, LMA, and LDMC) (Tomás *et al.* 2013), we supposed that other factors were responsible for the high photosynthetic performance of RB compared to WL plants.

The high Φ_{PSII} in RB plants may indicate an enrichment of electron transport, likely mediated by an enhancement of cytochrome *f* (Cyt *f*) complex. Matsuda *et al.* (2007) reported an increase in Cyt *f* content in spinach plants grown under a blue light intensity of 300 μmol(photon) m^{-2} s^{-1}, similar to that utilised in our study. From biochemical point of view, the plant growth under RB light induced a reduction of polyphenols content compared to WL. This result was in contrast with previous studies on spinach plants (Agarwal *et al.* 2018) and other crops (Tomás *et al.* 2013).

Generally, polyphenols contribute to the second antioxidant system and are engaged when primary antioxidants are exhausted, such as under stress circumstances. Their synthesis, driven by blue light, is mediated by cytochrome P_{450} and lead to ROS accumulation (Lobiuc *et al.* 2017). Based on this statement, we supposed no stress condition for spinach plants at blue light intensity utilised in our study. Also, the content of anthocyanins and total antioxidant capacity in RB plants comparable to WL control seems to suggest the absence of stress due to prolonged growth under blue light.

Light quality and plant-microorganism interaction: Beneficial microorganisms (PGPM) such as fungi and bacteria added to plant growth mean may improve productivity and tissue nutraceutical value eventually potentiating the effect of specific light wavelengths. At present, little information is available on the interplay between light quality and beneficial microbes on plant physiological performance. Our data demonstrated that arbuscular mycorrhizal fungi (AMF) infected the spinach roots. The results are in agreement with other studies demonstrating that AMF belonging to *Glomus* genus infect spinach roots (Zuccarini and Savé 2016, Khalid *et al.* 2017) and the higher root infection occurred with rhizobacterial inoculation (Khalid *et al.* 2017). The inoculation of spinach plant under white light regime

Table 4. Coefficients of correlation (*Pearson*'s test) among the leaf investigated parameters: light-saturated net photosynthetic rate, P_{Nmax}; stomatal conductance, g_s; mesophyll conductance, g_m; chloroplast CO_2 concentration, C_c; effective quantum yield of PSII photochemistry,Φ_{PSII}; regulated energy dissipation, Φ_{NPQ}; nonregulated energy dissipation, Φ_{NO}; electron sink processes other than carbon assimilation, ETR/P_{gmax}; total chlorophyll, Chl (a+b); total carotenoids, Car (x+c); leaf area, LA; leaf mass per area, LMA; leaf dry mass content, LDMC; leaf thickness, LT; total polyphenols, TP; relative content of anthocyanins, Anth; soluble antioxidant capacity, FRAP; and total soluble carbohydrates, TSC. Data are means ± SD (n = 5). The significant *Pearson*'s correlation coefficients are reported in bold (p<0.05).

	P_{Nmax}	g_s	g_m	C_c	Φ_{PSII}	Φ_{NPQ}	Φ_{NO}	ETR/P_{gmax}	Chl (a+b)	Car (x+c)	LMA	LDMC	LT	TP	FRAP	Anth	TSC
P_{Nmax}	1	**0.891**	**0.799**	**0.584**	**0.841**	**–0.856**	0.103	**–0.350**	**0.562**	**0.550**	**–0.610**	**–0.653**	**–0.537**	–0.181	**0.635**	–0.196	**–0.510**
g_s		1	**0.806**	**0.754**	**0.563**	**–0.727**	**0.394**	**–0.617**	**0.637**	**0.613**	**–0.696**	**–0.708**	**–0.645**	–0.224	0.537	–0.073	**–0.590**
g_m			1	**0.898**	**0.512**	**–0.671**	**0.507**	**–0.545**	**0.908**	**0.902**	**–0.838**	**–0.783**	**–0.824**	–0.302	**0.710**	–0.009	**–0.755**
C_c				1	0.192	**–0.491**	**0.737**	**–0.713**	**0.853**	**0.837**	**–0.869**	**–0.817**	**–0.831**	**–0.434**	**0.619**	0.089	**–0.797**
Φ_{PSII}					1	**–0.826**	–0.339	0.207	0.256	0.251	**–0.350**	**–0.402**	–0.266	–0.026	**0.569**	**–0.380**	–0.212
Φ_{NPQ}						1	**–0.153**	0.111	**–0.404**	**–0.386**	**0.584**	**0.630**	**0.458**	0.201	**–0.759**	0.231	**0.489**
Φ_{NO}							1	**–0.745**	**0.578**	**0.568**	**–0.586**	**–0.519**	**–0.583**	–0.330	0.269	0.296	**–0.654**
ETR/P_{gmax}								1	**–0.546**	**–0.535**	**0.457**	**0.463**	**0.465**	0.305	–0.163	–0.274	**0.523**
Chl (a+b)									1	**0.996**	**–0.781**	**–0.665**	**–0.832**	–0.296	**0.544**	0.191	**–0.713**
Car (x+c)										1	**–0.761**	**–0.636**	**–0.824**	–0.292	**0.521**	0.178	**–0.692**
LMA											1	**0.915**	**0.927**	**0.438**	**–0.702**	–0.118	**0.895**
LDMC												1	**0.714**	**0.424**	**–0.695**	–0.136	**0.794**
LT													1	**0.361**	**–0.589**	–0.090	**0.856**
TP														1	**–0.403**	–0.153	**0.477**
FRAP															1	–0.026	**–0.671**
Anth																1	–0.184
TSC																	1

promoted the growth enhancing the dry mass production mainly in the roots. The PGPM addition improved nutrient uptake, phosphorus solubility, and hormones production, while host plant sustained symbiotic costs by supplying photosynthates for microbe metabolism and growth. In WL inoculated plants (WL-I), P_{Nmax} was upregulated likely to compensate for the costs of symbiosis (Kaschuk *et al.* 2009). The improved CO_2 uptake was attributed to the rise of both g_s and g_m as well as to an increase of ETR. The enhanced electron transport capacity may be the consequence of a high nutrient availability promoted by beneficial microorganisms (Walker *et al.* 2014). Consistent with studies of Khalid *et al.* (2017) on spinach, we found in WL inoculated plants a lower polyphenol and anthocyanin content and a high antioxidant capacity compared to noninoculated samples. In our opinion, the high antioxidant capacity might balance the low polyphenol amount, increasing the scavenging potential of the inoculated plants.

The interaction with light quality changes the relationships between plants and microorganisms. Several studies demonstrated the importance of light for the symbiotic functioning of PGPM, and in particular for AMF. Hristozkova *et al.* (2017) showed for the first time the influence of light quality on mycorrhizal symbiosis formation in tomato, indicating how the phenotypic plasticity was affected by light spectral composition. In our study, a different phenotypic plasticity was found in several key traits of inoculated plants confirming that different light quality regimes strongly change the plant's plastic responses to beneficial microorganisms. In our experiment, red light strongly promoted the root colonization by microbes, enhancing the development of arbuscular mycorrhizal fungi, in particular *Glomus* (Cruz 2016). Conversely, blue wavelengths seemed to inhibit bacteria and fungi growth (De Lucca *et al.* 2012). If the interaction light quality × microorganisms is favourable under WL and RB light treatments, it becomes null or negative in RG and R plants, respectively. It is likely to suppose that in inoculated R plants the energetic costs of the symbiosis became too elevated for the high AMF colonisation. Thus, the decrease of photosynthesis was likely due to the strong microorganisms' carbon demand. Under RG treatment, the inoculated plants were able to pay for the energy cost of symbiosis, increasing photosynthesis thanks also to beneficial properties of the green wavelength. The RG plants, investing more carbon in aboveground biomass compared to shoots, improved the nutrient uptake by the roots. The interplay of RB light and microorganisms increased photosynthetic capacity maybe for the low symbiosis cost; we based our hypothesis on the statement that blue light exerts an inhibitory effect on microbes' growth and development. However, it cannot be excluded that the lower carbon allocation to root, observed in RB plants, might indicate also an efficient nutrient and water transport *via* fungi (Kothari *et al.* 1990). In this case, the blue light might have promoted a higher nitrate reductase activity improving the nitrate assimilation in these plants, as found by other authors (Agarwal *et al.* 2018).

Conclusions: Light quality influences the phenotypic plasticity of spinach plants, inducing changes in morphology and physiology. The green wavelength promotes the plant carbon gain enhancing the photosynthetic rates and reducing the limitation to CO_2 diffusion. The exposure to pure red increases the photosynthesis promoting the light harvesting and improving the CO_2 diffusion to carboxylation sites that reduce significantly the photorespiration. Light modulation also affects the secondary metabolites synthesis and the antioxidant capacity. The interaction between light quality and microorganism-based biofertilizer alters the spinach phenotypic plasticity affecting the plant responses to microbes. In particular, the growth under pure red light promotes the root colonisation by microorganisms raising the costs of symbiosis. Under this condition, the interaction of plant–microorganisms becomes unfavourable.

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