Heliyon

Plant growth, phytochemical accumulation and antioxidant activity of substrate-grown spinach

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Abstract

Spinach production in soilless cultivation systems, mainly in substrate, transplanted with soil blocks and drip-irrigation is increasing worldwide. However, spinach establishment with soil blocks, with several seedlings per block compared with traditional planting methods, may affect light interception by plant canopy, wetting and salt patterns in root medium and therefore the plant growth and functional value of spinach. The effects of soil block number (plant density) and emitters spacing on plant growth, nitrate, proline and total phenols content and antioxidant activity were evaluated in spinach (*Spinacia oleracea* L). Five seedlings per soil block were transplanted to Styrofoam boxes filled with the substrate and grown during winter in an unheated greenhouse. Four treatments were carried out with two soil block numbers [8 (160 plants/m^2) and 14 (280 plants/m^2) soil blocks per Styrofoam box] and two emitter spacing [emitters spaced every 25 and 12.5 cm, respectively, with 4 and 8 emitters per Styrofoam box]. Neither plant density nor emitter spacing had any effect on shoot dry weight. Fresh yield increased as planting density and the number of emitters per Styrofoam box increased. The yield in Styrofoam boxes with 160 plants/m^2 and 8 emitters (3.85 kg m^{-2}) was $\approx$ 31 % lower than that obtained in treatment with

280 plants/m^2 and 8 emitters (5.09 kg m^{-2}). However, the leaf blade of the latter treatment presented a higher content of phenols and proline and greater antioxidant activity (FRAP and DPPH) as well as lower content of nitrate and lesser PDH activity. Decrease of space between emitters reduced the leaf blade nitrate concentration of spinach grown in a greenhouse during the winter. Leaf blade antioxidant activity (FRAP) decreased as planting density increased.

Keywords: Plant biology, Agriculture, Environmental science

1. Introduction

Spinach plays an important role in the Portuguese diet since is consumed in great quantities all year around. Moreover, it is low in calories and fat but is rich in minerals, fiber, vitamins, and phytochemicals (Alvino and Barbieri, 2016) and has high antioxidant activity (Ou et al., 2002; Ismail et al., 2004). Bioactive phytochemicals, ubiquitously distributed in nature, may constitute pigments that give a colorful appearance to foods, or secondary metabolism products that protect plant species from environmental attacks (Ismail et al., 2010) and increase their functional and nutritional value (Sucupira et al., 2012). However, phytochemical accumulation and antioxidant activity, are influenced by cultivation techniques (Ou et al., 2002), which in spinach production are changing. Spinach production in soilless cultivation systems, mainly in substrate, using soil blocks in plant establishment and drip-irrigation is increasing. The use of soil blocks, with several seedlings per block compared with traditional planting methods, greatly changes plant spacing which may affect photosynthetic photon flux density (PPFD) interception by plant canopy. Light is one of the primary factors that affect plant growth, yield, and the accumulation of phytochemicals such as phenols (Ghasemzadeh et al., 2010; Karimi et al., 2013; Bian et al., 2014; Chua et al., 2015; Kałużewicz et al., 2017; Jaganath and Zainal, 2016; Ilic et al., 2018). The concentration of NO$_3$ in plant tissues increases due to low-level light conditions (Colla et al., 2018; Marschner, 2012; Santamaria et al., 1999; Santamaria, 2006). High levels of light exposure could induce the synthesis of phenolic compounds and, consequently, antioxidant activity in a number of vegetables (Oh et al., 2009; Samuoliené et al., 2012; Pérez-Lopez et al., 2018). However, the use of soil blocks affects correct emitter spacing, since it may also influence substrate covering, wetting and salt patterns in the root medium and water and nutrient uptake by plants (Valdés et al., 2015) which may lead to abiotic stress such as water deficit and/or salinity. Abiotic stress may influence yield, phytochemical accumulation, and antioxidant activity. For example, salt stress was found to increase polyphenols content in spinach (Shimomachi et al., 2008). However, the response in peas (Miljus-Djukic et al., 2013) and beans (Telesiñski et al., 2008) was influenced by the level of osmotic stress. In beans, moderate salinity stress

decreased phenols content (Telesiński et al., 2008). Proline (Pro) accumulation is also related to abiotic stress (Wang et al., 2009, 2011; Per et al., 2017) such as salinity and water deficit (Hayat et al., 2012; Reddy et al., 2015; Ashraf et al., 2018). In tomato grown hydroponically, proline was found to be a reliable indicator of the environmental stress imposed on plants (Claussen, 2005). Pro degradation is suppressed during saline or drought stress (Verbruggen and Hermans, 2008) by a pathway which involves Pro dehydrogenase (PDH, EC 1.5.5.2) a key enzyme of Pro catabolism (Per et al., 2017).

Despite increasing in the use soil blocks, for plant establishment in substrate drip-irrigated, there is currently very little information available concerning the influence of soil block number and emitter spacing on plant growth, and the functional value of spinach grown in substrate. Therefore, the aim of this study was to evaluate the effect of soil block number and emitter spacing on plant growth, nitrate, total phenols and proline concentration, FRAP and DPPH antioxidant activity and PDH enzyme activity in spinach grown in a substrate in an unheated greenhouse during the winter.

2. Materials and methods

2.1. Growth conditions and substrates

The experiment was conducted between december 2015 and february 2016 in a greenhouse located at the "Herdade Experimental da Mitra" (38°31′52″N; 8°01′05″W), University of Évora, Portugal. The greenhouse was covered with polycarbonate and had no supplementary lighting. Air temperatures inside the greenhouse ranged from 6 to 27 °C, and solar radiation ranged from 21.9 to 151.1 W $m^{-2} \cdot d^{-1}$. The experiment comprised four treatments: two plant densities (PD), low plant density (LPD) (8 soil blocks per Styrofoam box - 160 plants/m^2) and high plant density (HPD) (14 soil blocks per Styrofoam box − 280 plants/m^2) and two emitter spacing (NE) [emitter spaced every 25.0 (NE4) and 12.5 cm (NE8), respectively 4 and 8 emitters per Styrofoam box].

Substrate [composted forest residues, husks, and white peat (2:1:1 v/v)], physico-chemical properties were the following: organic matter = 63.0 % (w/w), pH 5.5−6.5, total nitrogen = 200−400 mg L^{-1}, potassium = 150−300 mg L^{-1}, phosphorus = 100−200 mg L^{-1}, EC = 1−3 mS cm^{-1}, C/N = <20, bulk density = 0.27 g cm^{-3}, mass wetness = 2.4 g water per g substrate, moisture content = 71 %. Soil-blocked spinach (*Spinacia oleracea* L. cv. Tapir) seedlings (five seedlings per block) were transplanted (on 22 december) at 18 days after emergence in Styrofoam planting boxes (100-cm long × 25 cm wide × 10-cm high) filled with 14 L of substrate. Soil blocks with five spinach seedlings were spaced at 25-cm intervals in 160 plants/m^2 treatment (8 soil blocks per Styrofoam box) and 14.5-cm intervals in 280 plants/m^2 treatment (14 soil blocks per Styrofoam box), planted in two rows per box,

10 cm apart. Treatments were arranged in a randomized complete block design with five replicate boxes per treatment.

Each planting box was irrigated using $4 \, L \, h^{-1}$ pressure-compensating and anti-drain emitters, attached to 4 fine tubes (70-cm in length and with a diameter of 5 mm), inserted into substrate through a straight plastic arrow, 25.0 cm and 12.5 cm apart, along the center the line of the Styrofoam box, in the treatments with 4 and 8 emitters per Styrofoam box, respectively. Irrigation was scheduled three to seven times per day using a timer and averaged 20–30% drainage (leaching fraction) at Styrofoam box control (NE4 HPD) for each application. The duration of each irrigation event in the treatments with 8 emitters was half that of the treatments with 4 emitters.

Nutrient solution was applied daily by fertigation, from two days after transplanting to the day before harvesting. The solution was made from fresh tap water [electrical conductivity (EC) of 0.4–0.48 dS m^{-1}; pH 7–7.4; 0.10–0.30 mmol L^{-1} NO_3, 1 mmol L^{-1} Ca, 1 mmol L^{-1} Mg, 2.1 mmol L^{-1} Cl^{-}, 0.7 mmol L^{-1} Na, 0.53 μ mol·L^{-1} Fe and 0.16 μmol L^{-1} Mn] and initially contained 9.84 mmol L^{-1} NO_3, 3.14 mmol L^{-1} NH_4, 0.76 mmol L^{-1} P, 4.24 mmol L^{-1} K, 3.19 mmol L^{-1} Ca, 1.08 mmol L^{-1} Mg, 1.25 mmol L^{-1} S, 46 μmol L^{-1} B; 3 μmol L^{-1} Cu, 16 μmol L^{-1} Fe, 19 μmol L^{-1} Mn, 1 μmol L^{-1} Mo' and 2 μmol L^{-1} Zn and 2.1 Cl and 0.7 mmol L^{-1} Na. The concentration was adjusted (N-nitrate concentration in the nutrient solution was reduced in order to avoid high nitrate accumulation in spinach tissues) at 29 days after transplanting (DAT) to 4.24 mmol L^{-1} NO_3, 4.10 mmol L^{-1} NH_4, 0.62 mmol L^{-1} P, 3.00 mmol L^{-1} K, 2.12 mmol L^{-1} Ca, 0.88 mmol L^{-1} Mg, 0.87 mmol L^{-1} S, 46 μmol L^{-1} B, 7.86 μmol L^{-1} Cu, 8.95 μmol L^{-1} Fe, 18.3 μmol L^{-1} Mn, 1 μmol L^{-1} Mo, 1 μmol L^{-1} Zn, 2.1 mmol L^{-1} Cl and 0.7 mmol L^{-1} Na. The final pH of both solutions ranged from 5.9 to 6.1.

2.2. Measurements

The EC and the concentration of NO_3 in the drainage water from each box was measured twice a week using a conductivity meter (LF 330 WTW, Weilhein, Germany), and an ion-specific electrode and meter (Crison Instruments, Barcelona, Spain), respectively, following the procedures outlined by Prazeres (2005).

For yield evaluation, the plants of 4 soil blocks per box were harvested at 44 DAT. The plants (shoots) were cut off at 1 cm above the leaf base. Two representative plants (shoots) were also harvested at 30, 36 and 44 DAT from each box, were washed, oven-dried at 70 °C for 2–3 days, and weighed.

Additional leaf samples were stored at −80 °C for NO_3 determination (Lastra, 2003). The samples were oven-dried at 65 °C for 48 h, weighed (0.1000 g), macerated in a mortar, homogenized in a test tube with 10 mL of distilled water, agitated in a vortex, and incubated for 1 h at 45 °C in a shaking water bath. Filtrated extract was then

mixed with salicylic acid in 5% sulfuric acid (1:4), incubated for 20 min at room temperature, and mixed with 9.5 mL of 2 M sodium hydroxide. The concentration of NO_3 in the solution was then determined reading the absorbance at 338 and 440 nm, using a calibration curve (NO_3, n = 6 concentrations between 0 and 500 mg/L).

For the determination of total phenols, free proline content and antioxidant activity (FRAP, DPPH) 1,000 g of leaf blade and petiole sample was homogenized in 8 mL of methanol:water solution (80:20,v/v) for 1 min and then centrifuged at 5 °C at 6,440 *g* for 5 min.

Total phenols content was determined using Folin—Ciocalteau's phenol reagent as described earlier (Bouayed et al., 2011), by spectrophotometry UV/Vis, reading the absorbance at 760 nm. The total phenols content expressed as milligrams of gallic acid equivalents per 100 g of fresh weight (FW) was calculated using a calibration curve (gallic acid, n = 6 concentrations from 0 to 50 mg/L).

Free proline levels were determined using the acid ninhydrin reaction in accordance with the method of Bates et al. (1973), reading the absorbance of formed formazone at 546 nm. Proline concentration was calculated using a calibration curve (L-proline, n = 6 concentrations from 0 to 20 mg/L).

Ferric-reducing antioxidant activity (FRAP) was determined in accordance with the method of Bouayed et al. (2011). Briefly, the FRAP reagent was prepared freshly by mixing 300 mM acetate buffer pH 3.6, 10 mM TPTZ solution in 40 mM HCl and 20 mM iron(III) chloride solution (10:1:1, v/v/v) and warmed to 37 °C before use. Then 0.050 mL of the sample (suitably diluted extracts or standard) was mixed with 0.950 mL of FRAP reagent. Absorbance was determined at 593 nm after the incubation of the mixture during 4 min. Antioxidant activity reported as milligrams of Trolox equivalents per 100 g of FW was calculated using a calibration curve (Trolox solution, n = 8 concentrations from 0 to 1120 mg/L).

Scavenging antioxidant activity (DPPH) was determined by measuring the ability of plant extracts to capture the stable organic radical DPPH• (2, 2-diphenyl-1-picryl-hydrazyl). Aliquots of an extemporaneous metanolic solution of 0.03 g/L DPPH•, kept in the dark, were added to a known volume of sample or standard solution. The reduction of DPPH• to DPPH-H (diphenyl-picryl hydrazine) was followed by reading the absorbance at 515 nm, at 25 °C, for 180 s. Antioxidant activity reported as milligrams of gallic acid equivalents per 100 g of FW was calculated using a calibration curve (gallic acid solution, n = 8 concentrations from 0 to 200 mg/L) (Brand-Williams et al., 1995).

Samples of 0.25 g of spinach leaf blade or petiole were macerated in the presence of liquid N_2 and homogenized in 50 mM phosphate buffer pH 7.0 for the extract preparation used in the determination of proline dehydrogenase (PDH) enzyme activity. The supernatant obtained by means of the centrifugation of this extract for 15 min at

15,000 g at 4 °C, was collected and stored in aliquots at −20 °C for further use in determining protein content and PDH activity.

PDH activity was measured as described by Costilow and Cooper (1978). Enzyme activity was assayed following the reduction of NAD^+ at 340 nm at 30 °C during 180 s in a double-beam Hitachi-U2001 spectrophotometer with the control of temperature. The 2 cm^3 assay mixture contained 100 mM Na_2CO_3-$NaHCO_3$ buffer pH 10.3, 10 mM NAD^+, 2 mM L-proline and the enzyme extract, in which proline was used to initiate the reaction. The control cuvette contained all the solutions except NAD^+. Enzyme activity was calculated on the basis of the reaction curve slope, using the extinction coefficient of $\varepsilon = 6.22$ mM^{-1} cm^{-1}. PDH activity was expressed in nmol min^{-1}/mg protein.

The protein content of the extract was determined in accordance with the Lowry method (Lowry et al., 1951), using a calibration curve (bovine serum albumin, BSA; n = 6 concentrations from 0 to 200 μg/mL).

A Thermo Scientific, Genesys 10S spectrophotometer (Waltham, Massachusetts, USA) was used in all content determination for spectrophotometric analysis.

2.3. Data analysis

Data were processed by means of the analysis of variance (Sokal and Rohlf, 1997), using SPSS Statistics 24 software (Chicago, Illinois, USA), licensed to University of Évora. Means were separated at the 5% level using Duncan's new multiple range test. Pearson correlation coefficients (r) are from SPSS.

3. Results and discussion

3.1. Drainage water

The concentration of NO_3 and EC in drainage water was not significantly affected by interaction between plant density and emitter spacing. The concentration of NO_3 in the drainage water collected after 30 DAT was greater in LPD than in HPD (Fig. 1). The concentration of NO_3 in drainage water on the first six dates of measurement (Fig. 1) was slightly lower than the concentration applied in the nutrient solution (610 mg NO_3 L^{-1}) ranging from 488 to 587 mg L^{-1} (Fig. 1). The EC in drainage water after 30 DAT was lower in HPD than in LPD (Fig. 1). The NO_3 concentration and the EC in the drainage water on the last four dates, as compared with the NO_3 and the EC in the nutrient solution, decreased significantly (Fig. 1), due to the reduction of nitrate applied in the nutrient solution (262 mg NO_3 L^{-1}) and due to high nutrient uptake by spinach plants, as ≈ 85 % of total shoot dry weight was accumulated during this period (Fig. 3).
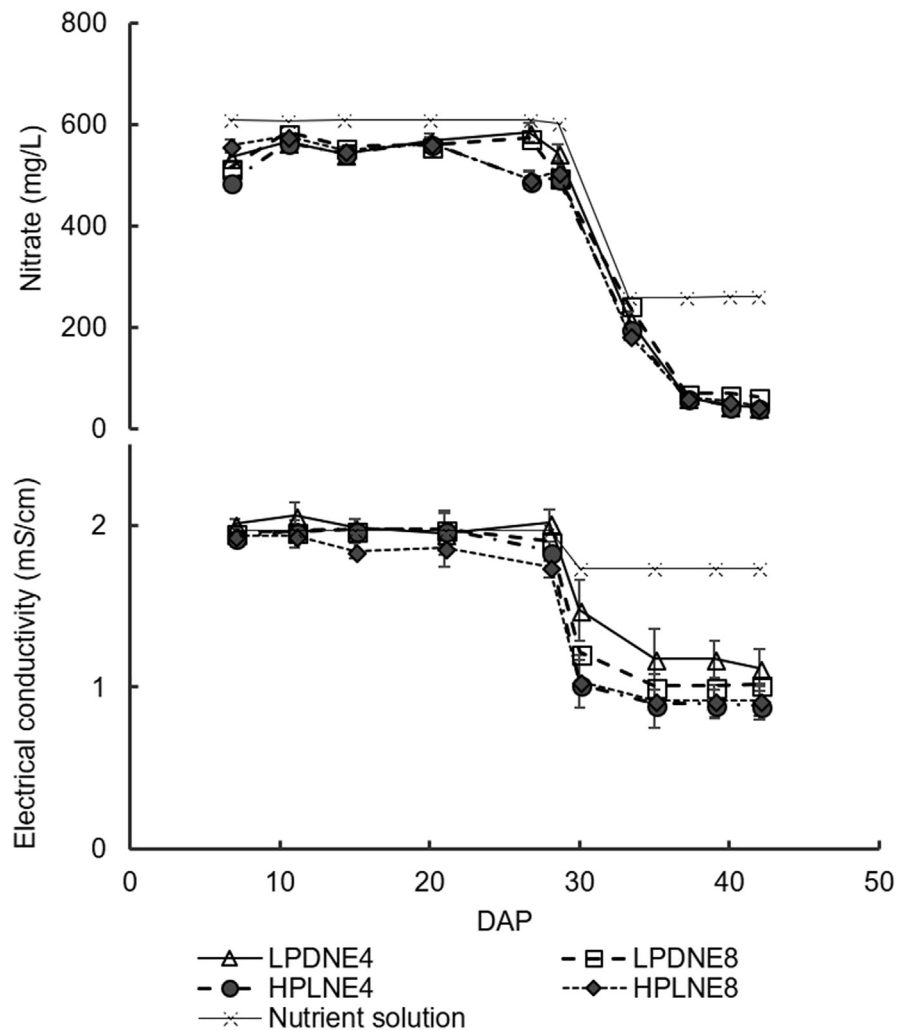
Fig. 1. Effects of plant density (LPD — 160 plants/m^2; HPD — 280 plants/m^2) and emitter spacing (NE4 — emitters 25.0 cm apart; NE8 — emitters 12.5 cm apart) on the concentration of nitrate (NO$_3$) and electrical conductivity (EC) of the drainage water. Each symbol represents the mean of five replicates, and the error bars represent ±1 SE.

3.2. Plant growth and yield

Shoot dry weight per plant was affected neither by planting density nor emitter spacing (Fig. 2) and it increased exponentially during the crop cycle ($\approx 85\%$ of total shoot dry weight was accumulated from 30 to 44 DAT) (Fig. 3). Plant growth in the first 30 DAT was minimal, thus, the influence of plant density on light interception in the first 30 days may have been low.

Leaf area per plant decreased as plant density increased (Fig. 2). However, in high planting density treatment shading may be increased, since close planting usually causes mutual shading among individuals.
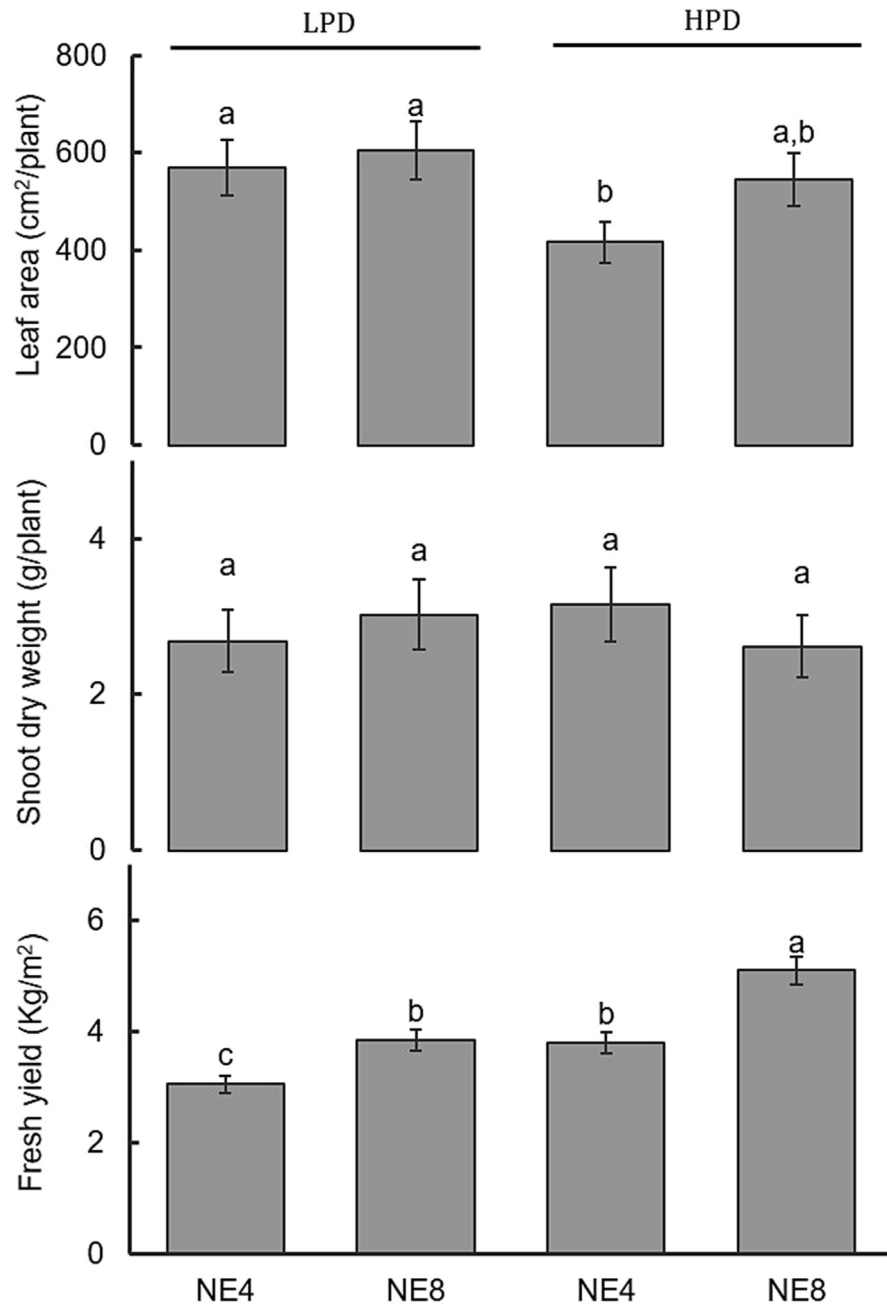
Fig. 2. Effects of plant density (LPD − 160 plants/m^2; HPD − 280 plants/m^2) and emitter spacing (NE4 − emitters 25.0 cm apart; NE8 − emitters 12.5 cm apart) on leaf area, shoot dry weight and fresh yield of spinach. Means with different letters are significantly different at $p < 0.05$. Each bar represents the mean of five replicates, and the error bars represent ±1 SE.

Yield (fresh weight) increased significantly as planting density and as the number of the emitters per Styrofoam box increased (Fig. 2). As shoot biomass was not affected by treatments, fresh yield increase due to an increase in emitter numbers, was probably due to an increase in leaf water content. This indicates that an increase in emitter
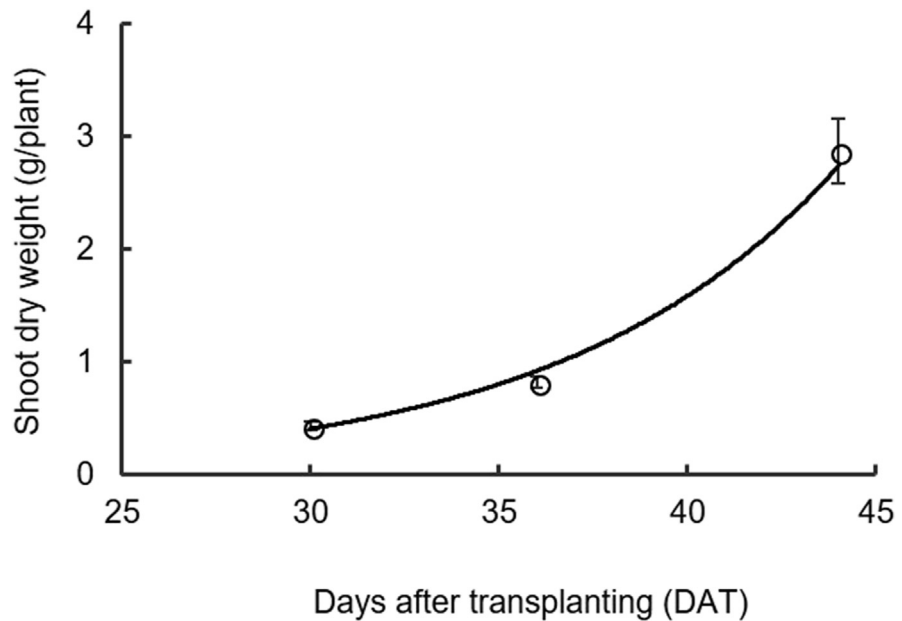
Fig. 3. Relationship between shoot dry weight (g/plant) and days after transplanting (DAT). [Shoot dry weight (g/plant) $= 0.0063e^{0.1365(DAT)}$, $R^2 = 0.99$, $p < 0.001$]. Each symbol represents the mean of five replicates averaged over four treatments, and the error bars represent ± 1 SE.

numbers increased the rate of water uptake from the substrate. This may have a dilution effect on phytochemical content, which is highest in HPDNE8.

The yield in Styrofoam boxes with 280 plants/m^2 and 8 emitters reached a maximum of 5.09 kg m^{-2} (≈ 31 % higher than in LPDNE8 and HPDNE4) (Fig. 2), and it was also higher than obtained when spinach was grown both in a floating system (Conesa et al., 2009) and in a substrate (Barcelos et al., 2016).

3.3. Phytochemical accumulation

3.3.1. Nitrate

Leaf blade NO_3 concentration was not significantly affected either by the interaction between treatments or by soil block number (Fig. 4). Therefore, an increase of leaf blade nitrate concentration due to higher leaf shading in treatments with 280 plants/m^2 was not observed. Leaf blade nitrate concentration in baby spinach grown in a hydroponic system increased as plant density increased (Machuca et al., 2015), but planting densities (862, 1296 and 1728 plants/m^2) were much higher than those in present study.

Leaf blade NO_3 concentration in HPD treatment decreased in plants irrigated with 8 emitters (Fig. 4), which could be related to higher leaf water content due to high water availability in the root medium. In leafy vegetables, Qiu et al. (2014) observed that nitrate accumulation decreased with increasing soil water content.
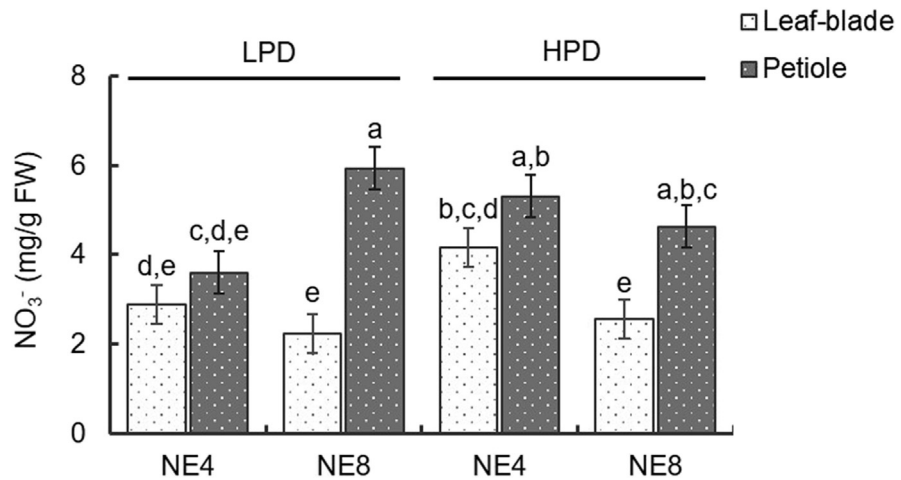
Fig. 4. Effects of plant density (LPD — 160 plants/m^2; HPD — 280 plants/m^2) and emitter spacing (NE4 — emitters 25.0 cm apart; NE8 — emitters 12.5 cm apart) on leaf blade and petiole nitrate concentration. Means with different letters are significantly different at $p < 0.05$. Each bar represents the mean of five replicates, and the error bars represent ± 1 SE.

In plants irrigated with 8 emitters leaf blade NO_3 concentration values ranged from 2.23 to 2.56 mg g^{-1} fresh weight (FW). These values were lower than those allowed by European Commission Regulation no. 1258/2011 for fresh spinach (3.5 mg g^{-1} fresh weight). Therefore, the use of spacing emitters could provide a way to reduce nitrate concentration in the fresh weight of leaf blade of spinach grown in a greenhouse during the winter.

Petiole NO_3 concentration was affected by the interaction between the factors ($p < 0.001$) and it was greater in the treatment with 160 plants/m^2 and 8 emitters per Styrofoam box (5.9 mg·NO_3 g^{-1} FW). Petiole NO_3 concentrations were higher than those in the leaf blade, as reported by others authors (Marschner, 2012), and higher than the value allowed by the European Commission (3.5 mg g^{-1} FW) (Fig. 4).

3.3.2. Total phenols content

The interaction between plant density and the number of emitters per Styrofoam box on leaf blade total phenols content (TPC) was significant ($p < 0.001$), which indicates that the response of leaf blade TPC to plant density differed as a function of the number of emitters per Styrofoam box (Fig. 5).

Leaf blade TPC was highest in the LPDNE8 treatment, reaching a maximum of 86.5 mg GAE/100 g FW. Leaf blade TPC in the HPD treatment was not affected by spacing emitters, although fresh yield in NE8 was 30 % higher than in NE4 (Fig. 5), therefore the high leaf water content of the leaves of the plants grown in HPDN8 did not lead to a decrease in TPC concentration in leaves. As leaf shading may have increased when plant density increased (Fig. 2), a light-dependent
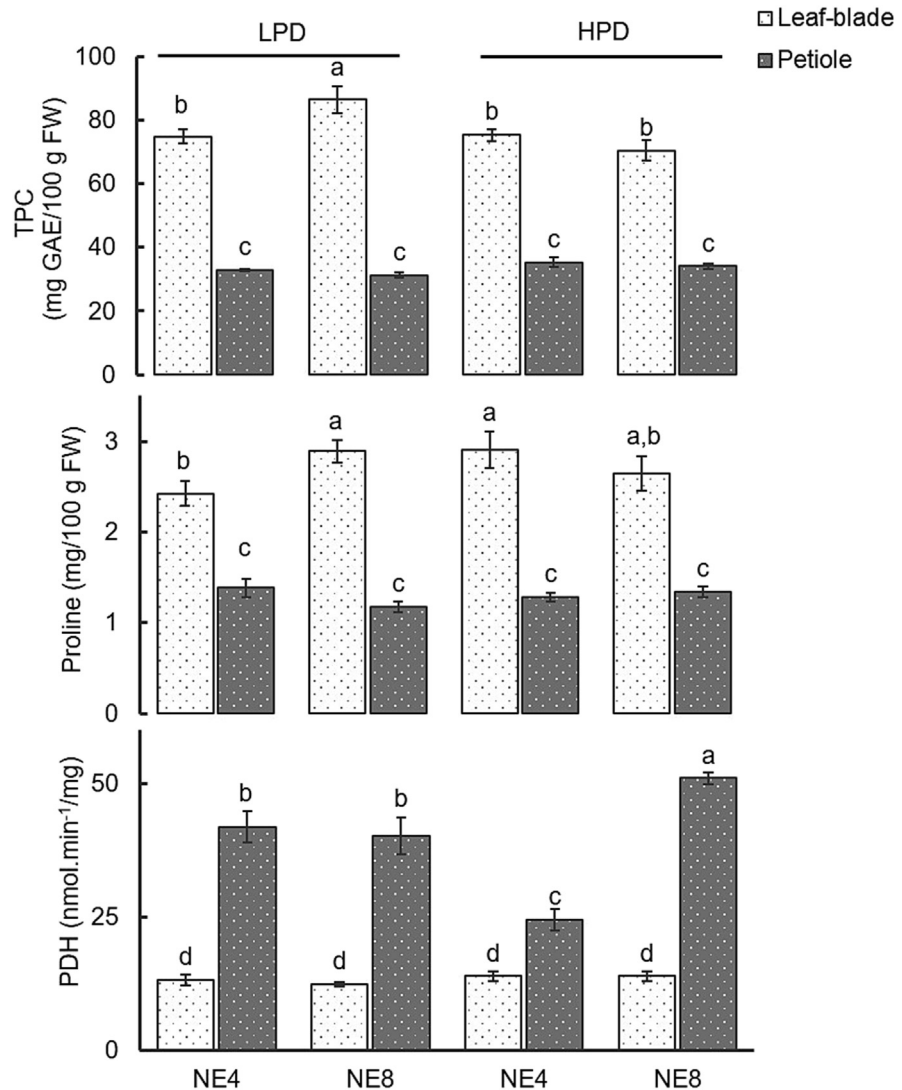
Fig. 5. Effects of plant density (LPD − 160 plants/m^2; HPD − 280 plants/m^2) and emitter spacing (NE4 − emitters 25.0 cm apart; NE8 − emitters 12.5 cm apart) on total phenols content, proline content and PDH enzyme activity in the leaf blade and petiole. Means with different letters are significantly different at $p < 0.05$, FW-fresh weight. Each bar represents the mean of five replicates, and the error bars represent ±1 SE.

induction of phenylpropanoid biosynthesis may have occurred in spinach of LPDNE8 treatment, accounting in part, for the highest value of TPC (Pérez-Lopez et al., 2013, 2015, 2018; Crozier et al., 2007; Leyva et al., 1995).

Leaf blade TPCs ranged from an average 79−86.5 mg GAE/100 g FW. These values were similar to those measured in spinach by Proteggente et al. (2002) (72.1 mg GAE/ 100 g FW) and Chu et al. (2002) (90 mg GAE/100 g FW) and lower than the levels reported by Machado et al. (unpublished results) (106−130 mg GAE/100 g FW), Bayili et al. (2011) (182.5 mg GAE/100 g FW) and Mazzucotelli et al. (2017)

(104.7 mg GAE/100 g FW). The variation in TPC values may be due to many factors, such as spinach variety, maturity at harvest and growth conditions (Howard et al., 2002). In general, total phenols content increases as abiotic stress (light conditions, salinity, etc.) increases (D'Souza and Devaraj, 2010; Karimi et al., 2013; Miljus-Djukic et al., 2013; Bian et al., 2014; Klados and Tzortzakis, 2014; Alam et al., 2015; Kyriacou and Rouphael, 2018) though in beans, moderate salinity stress led to a decrease in phenols content (Telesiñski et al., 2008).

Petiole TPCs were not influenced by treatments, while they were $\approx$ 2.3 times lower than in leaf blade ($p < 0.05$) (Fig. 5). Different content of total phenols in different plant parts was also determined in spinach by Machado et al., (unpublished results), and by Kollia et al. (2017) in artichoke.

3.3.3. Proline

Leaf blade proline content was lower in plants in the LPD treatment irrigated with four emitters (2.42 mg/100 g FW) (Fig. 5). Leaf blade proline content ranged from an average 2.42−2.91 mg/100 g FW. These values are lower than those obtained by Yoon et al. (2017) (148.8 mg/100 g DW) and Aslan et al. (2016) (5.4−7.2 mg/100 g FW) in spinach grown in the greenhouse and in the open air, respectively. The lower values of Pro found in the present study may indicate that spinach plants were not subject to high levels of abiotic stress, since its accumulation is closely related to salinity and water deficit (Claussen, 2005; Pérez-López et al., 2018).

Petiole proline content was not affected by the treatments ($p < 0.05$) (Fig. 5) and ranged from an average 1.26−1.34 mg/100 g FW. These values were lower than those found in the leaf blade.

3.3.4. PDH enzyme activity

Leaf blade PDH enzyme activity was not significantly affected by treatments and ranged from 12.36 to 13.94 nmol min^{-1}/mg prot, these values being higher than those reported in maize by Li et al. (2013) (14.5 nmol min^{-1}/g FW), but lower than those described by Tian et al. (2016) in millet cultivar seeds (35 U min^{-1}/mg prot).

Petiole PDH enzyme activity was affected by treatments ($p < 0.01$). The highest value for this enzyme activity was found in plants in the HPD treatment irrigated with 8 emitters. However, proline petiole values were not affected (Fig. 5).

PDH activity found in petioles was higher than that found in the leaf blade, which may explain the low levels of proline found in the petiole (Fig. 5), because, as previously mentioned, PDH is one of the enzymes involved in Pro catabolism

(Kiyosue et al., 1996; Per et al., 2017). Leaf blade and petiole PDH enzyme activity did not correlate with the respective proline levels. However, in mulberry, proline dehydrogenase activity was found to decreases progressively as proline increased owing to water stress (Chaitanya et al., 2009).

3.3.5. Antioxidant activities

Leaf blade FRAP decreased as plant density increased (Fig. 6) ($p < 0.01$). This could be related to an increase in leaf shading caused by plant number in the HPD treatment. Light conditions, as previously mentioned, influence phytochemical accumulation, and antioxidant activity (Bian et al., 2014; Karimi et al., 2013; Kyriacou and Rouphael, 2018). Leaf blade FRAP antioxidant activity was not affected by spacing emitters. Thus the high leaf water content of the leaves irrigated with 8 emitters did not lead to a decrease in FRAP.

Leaf blade FRAP values ranged from an average of 464.5−507 Trolox mg/100 g FW and from 374 to 401 Trolox mg/100 g FW in the LPD and HPD treatments, respectively. These values were higher than those reported for spinach by
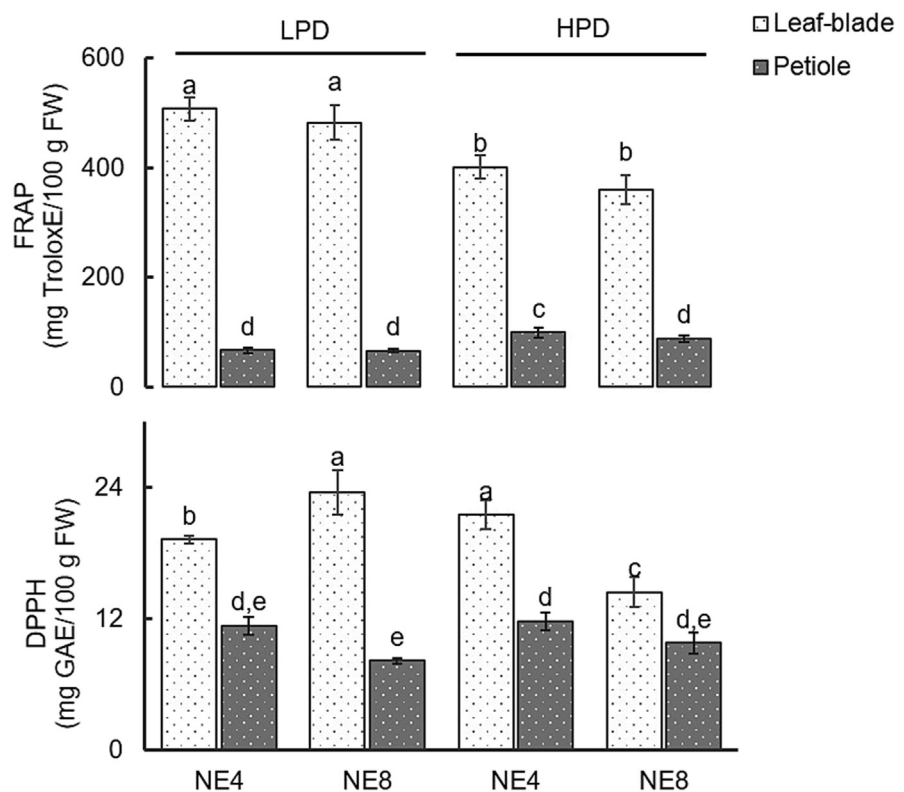

Fig. 6. Effects of plant density (LPD − 160 plants/m^2; HPD − 280 plants/m^2) and emitter spacing (NE4 − emitters 25.0 cm apart; NE8 − emitters 12.5 cm apart) on FRAP and DPPH in the leaf blade and petiole. Means with different letters are significantly different at $p < 0.05$, FW- fresh weight. Each bar represents the mean of five replicates, and the error bars represent ±1 SE.

Apak et al. (2007) (267 Trolox mg/100 g FW) but much lower than the values observed by Sreeramulu et al. (2013) (1380.6 Trolox mg/100 g FW) and Machado et al. (unpublished results) (700−800 Trolox mg/100 g FW). The variations between the findings of different studies may be related to methodological differences such as extraction technique, and the factors that affected antioxidant activity in vegetables such as genotype, location, harvest season, cultural techniques (Ou et al., 2002) and abiotic stress (Per et al., 2017).

Petiole FRAP values in the treatments ranged from an average 65.78 to 99.08 Trolox mg/100 g FW. These values were similar to those observed in spinach grown in co-copeat (Machado et al., unpublished results). The FRAP of the leaf blade was 4.5−7.0 times higher than in petioles. This behavior was also reported by Yosefi et al. (2010) in spinach and by Petropoulos et al. (2017) in Greek artichoke.

There was no overall effect of either number emitters or plant density on leaf blade ROS scavenging antioxidant activity (DPPH) because the interaction between the factors was significant ($p < 0.001$). Leaf blade DPPH in the LPD treatment with 8 emitters was 1.6 times greater than in the HPD treatment with 8 emitters (Fig. 6).

Petiole DPPH decreased as the number of emitters increased (Fig. 6). Petioles DPPH ranged from an average 8.1−11.8 mg/100 FW, and these values were 1.47−2.88 times lower than in leaf blade.

Leaf blade total phenols content was positively and linearly correlated with leaf blade DPPH antioxidant activity ($r = 0.862$, $p < 0.01$), but not with FRAP. The same correlation was also observed in some other crops while it was not observed in others (Stratil et al., 2006; Salandanan et al., 2009). These differing results can be explained by the fact that antioxidant activity depends not only on total phenols but also on other antioxidants (Salandanan et al., 2009), which are influenced by a large range of factors (Alvino and Barbieri, 2016).

Leaf blade DPPH and FRAP values were not related linearly with fresh yield. The lowest value of the DPPH was observed in the treatment with highest fresh yield (Figs. 2 and 6). Leaf blade FRAP values in the HPD treatment were similar, despite the fresh yield in the plants irrigated with 8 emitters having increased by thirty per cent.

4. Conclusions

Plant density and emitter number per Styrofoam had no effect on shoot dry weight but led to an increased in fresh yield. The decrease in the spacing of emitters led to a decrease in leaf blade NO_3 concentration. Leaf blade ferric reduction (FRAP) activity declined as plant density decreased. The interaction between plant density and the number of emitters affected proline, total phenols, and antioxidant scavenging

activity (DPPH). The leaf blade of spinach grown in low plant density treatment (8 soil blocks) with 8 emitters per Styrofoam box presented the highest levels of phenols content, proline content and antioxidant activity (FRAP and DPPH) and the lowest levels of PDH activity and nitrate content. Plant density and emitter spacing had no influence on the accumulation of proline, total phenols or the antioxidant activity of petiole.

Declarations

Author contribution statement

Rui Machado: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Isabel Alves-Pereira, Rui Fereira; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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