

Modelling plant yield and quality response of fresh-market spinach (*Spinacia oleracea* L.) to mineral nitrogen availability in the root zone

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Abstract

Spinach is one of the most important green-leafy vegetables, consumed worldwide, and its intake is beneficial for human beings. In this crop, produce yield and quality are closely related to plant nitrogen (N) nutrition. A precise supply of N is also essential for high environmental and economic sustainability. Main aims of the work were: i) to establish relationships between produce yield or quality and mineral N availability in the root zone; and ii) to define an optimal mineral N level to be maintained in the root zone for spinach. Eight experiments were carried out during a four-year-long period under typical Mediterranean climate conditions. Different amounts of N fertilisers were supplied leading to twenty different levels of mineral N in the root zone. Experimental measurements included climate parameters, plant growth, tissue and soil analyses, produce yield and quality indicators. A segmented linear model significantly represented the relationship between crop yield (1.7 to 21.7 t ha⁻¹) and soil mineral N concentration (7.6 to 41.0 mg kg⁻¹). Basing on this model, an optimal mineral N

threshold was fixed at 23.4 mg kg⁻¹. Above this threshold, crop yield did not show any significant variations as well as tissue characteristics and produce quality. Plants grown under suboptimal N levels showed reduction in growth, tissue mineral (nutrients) content, and SPAD index. The proposed models could be implemented in fertilisation protocols for the optimization of N supply and the estimation of spinach growth and yield.

Introduction

In the Mediterranean basin, savoy spinach (*Spinacia oleracea* L.) represents an important typical produce exported in many countries of northern Europe. For fresh market, savoy spinach is harvested at early growth stage. The most important quality attributes of this leafy vegetable are related to the leaf greenness and morphology (*i.e.*, wrinkledness), to the content of beneficial mineral elements, and to the low content of toxic compounds such as oxalic acid and nitrates (Santamaria *et al.*, 1999; Cavaiuolo and Ferrante, 2014).

Well-balanced nitrogen (N) supply is crucial for high yield and market quality of spinach. Many authors reported a positive relationship between crop yield and increasing N fertiliser rate in spinach cultivated in open field or under greenhouse (Biamond *et al.*, 1996; Wang and Li, 2004; Gülser, 2005; Lefsrud *et al.*, 2007; Stagnari *et al.*, 2007; Rodriguez-Hidalgo *et al.*, 2010). The spinach content in mineral elements and antioxidants, such as lutein and β -carotene, are both positively related to N availability (Lefsrud *et al.*, 2007; Stagnari *et al.*, 2007).

On the other hand, N excess may lead to large leaf accumulation of oxalic acid and nitrates in leaf tissues, especially when N is supplied in the nitric form (Chen *et al.*, 2004; Wang and Li, 2004; Zhang *et al.*, 2005; Stagnari *et al.*, 2007). Similarly to nitrates, oxalic acid and related compounds are harmful molecules for human beings and their continuous intake can induce blood diseases, especially in infants, nutritional disorders, and other health disturbs in human body (Noonan, 1999; Bryan and Loscalzo, 2011; Agnoli *et al.*, 2017). In the European Union (EU) specific limits have been laid down for the nitrate content of some leafy vegetables (The Council of the European Community, 2006; EFSA, 2008). In spinach, these limits are 2000 and 3500 mg NO₃⁻ kg⁻¹ (fresh weight basis) for frozen and fresh products, respectively.

Excessive N supply also results in increased water content of leaf tissues (Lefsrud *et al.*, 2007), which may negatively influence plant resistance to pathogens (Dordas, 2008) and its shelf life as well (Lombardo *et al.*, 2016). A correct N fertiliser management is essential for reducing the crop environmental impact associated with nitrate leaching (Robertson and Vitousek, 2009; Zhou and

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Butterbach-Bahl, 2014), which easily occurs in sandy soils where spinach is typically cultivated. In the EU, the Nitrates Directive (The Council of the European Communities, 1991) was issued to preserve the quality of ground and surface water bodies from the pollution of nitrates produced by agricultural activity, and to promote the adoption of good agricultural practices. According to the Nitrates Directive, growers must follow mandatory rules to tackle nitrate loss from their crops; for example, in the area of Val di Cornia (Tuscany, Italy), where the experiment took place, a maximum N dose of 120 kg ha⁻¹ and well defined (limited) periods of distribution have been ruled for spinach by the local authorities.

Moreover, N waste has negative economic impact on the production costs of field crops (Robertson and Vitousek, 2009). Some authors have related crop yield and N supply to economic parameters, to calculate the N fertiliser rate that maximizes grower's incomes (Wang and Li, 2004; Milne *et al.*, 2012).

Literature on leafy vegetables mostly focuses on the effects of N on yield and quality (Chen *et al.*, 2004; Wang and Li, 2004; Zhang *et al.*, 2005; Lefsrud *et al.*, 2007; Stagnari *et al.*, 2007) while less attention is paid to effective N management (Canali *et al.*, 2014).

However, most works attempt to describe growth and yield response curves as a function of fertiliser doses instead of the actual N availability in the root zone. For optimal N management different authors have therefore introduced the concept of *minimum optimal concentration* (Heckman *et al.*, 2002; Cui *et al.*, 2008; Bottoms *et al.*, 2012); this would represent the reference value to be maintained in the root zone to minimize crop environmental impact and to support high yield and quality (Incrocci *et al.*, 2017). To the best of our knowledge, no previous study addresses the above issue for spinach, thus an N optimal concentration for this crop has not been yet determined.

The paper reports experimental data collected in eight different experiments carried out during a four-year-long period. Main aims of the work are: i) to assess the effects of soil N concentration on spinach yield and quality throughout a medium-long observation

period under different climate conditions; ii) to define an optimal soil mineral N concentration for effective N management; and iii) to test an optical sensor for the quick monitoring of N nutritional status in fresh-market spinach grown under open-field Mediterranean climate conditions.

Materials and methods

Growing conditions and treatments

Experimental data were collected in eight different experiments (E), on spinach (*Spinacia oleracea* L.) crops, during a four-year-long period (from September 2007 to April 2011). Experimental fields were located in Val di Cornia (Tuscany, Italy), a coastal area with sandy-loam soils. The area is intensively cultivated with vegetables under typical Mediterranean climate conditions (Figure 1) with mild winters and 650 mm annual rainfall (ten-year average).

The physico-chemical characteristics of the soil (Table 1) were determined in the root zone of spinach (5-40 cm) prior to sowing. The quantity of fertilisers containing P, K, Ca, Mg and micronutrients was then calculated through a soil nutrient balance aimed to: i) replace the nutrients taken up by a crop grown under optimal conditions; and ii) restore the initial soil fertility if necessary. Only N was supplied in a variety of different doses (Table 2) based on the following criteria. During the first two experiments (E1 and E2), N was supplied at the fixed rate of 0, 80, 120 or 160 kg ha⁻¹. In the other experiments (E3-E8), N was supplied following growers' fertilisation practice. This is based on the standard fertilisation rate of 120 kg N ha⁻¹, according to the blueprint laid down for spinach production in the Val di Cornia area. The above quantity is usually increased by growers up to 175 kg ha⁻¹ in relation to the rainfalls occurred in the growing period, which may increase the risk for N shortage due to nitrate leaching. Each experiment always

Table 1. Chemical and physical characteristics in the 0-40 cm depth layer of the different experimental fields (n=8) used for spinach cultivation.

Parameter	Maximum value	Minimum value	Average	SD	CV
Sand (%)	76.1	58.6	69.1	6.4	0.1
Silt (%)	19.8	10.8	15.4	3.1	0.2
Clay (%)	26.0	7.8	15.5	5.6	0.4
Bulk density (t m ⁻³)	1.48	1.40	1.45	0.3	0.3
Field capacity (% v/v)	26.7	13.2	18.4	4.5	0.2
Wilting point (% v/v)	16.7	5.7	10.3	3.5	0.3
Organic matter (%)	2.1	1.0	1.4	0.4	0.1
Total N (mg kg ⁻¹)	800.0	700.0	757.1	53.5	0.4
N-NO ₃ ⁻ (mg kg ⁻¹)	19.7	3.6	13.2	5.0	0.4
N-NH ₄ ⁺ (mg kg ⁻¹)	11.3	3.6	7.3	2.9	0.4
P ₂ O ₅ (mg kg ⁻¹)	109.0	37.0	73.4	27.1	0.3
K ₂ O (mg kg ⁻¹)	235.0	110.0	187.2	48.2	0.4
CaO (mg kg ⁻¹)	3190.0	1451.0	1946.0	711.3	0.3
MgO (mg kg ⁻¹)	230.0	113.0	174.0	49.6	0.3
CEC (meq 100g ⁻¹)	18.1	9.2	12.4	4.0	0.1
pH (H ₂ O)	7.8	6.8	7.4	0.5	0.3
EC (dS m ⁻¹)	0.7	0.4	0.5	0.1	0.1

included a dose of N equal to 0 as untreated control (Table 2). In all treatments, the total amount of N was split over three periods: i) 40% blended with the soil, before sowing (4-6 days), using a mixed organic-mineral fertiliser (1% organic, 5% ammonia and 30% ureic); ii) 30% distributed as top-dressing fertilisation, at 1/3 of the cultivation cycle (roughly 4-5 true leaves), using ammonium nitrate; iii) 30% distributed as top-dressing fertilisation, at 2/3 of the cultivation cycle (roughly 10-11 true leaves), using calcium nitrate. In the case of rainy periods (*i.e.*, when total rainfall in the 8-14 true-leaf phase was 50% higher than the average of the previous ten years), with high N leaching, a third top-dressing fertilisation (40-50 kg N ha⁻¹) was applied when spinach had 14-15 true leaves (E3, E4 and E8).

Soil preparation of seedbed included ploughing, harrowing and levelling for bringing the soil into the better tilt for water drainage. Spinach (*cv.* Spitfire, Seminis®, Monsanto Company, USA) was sown in order to have a plant density of 30 plants m⁻² taking into account the percentage of emergency. Each treatment was applied in a completely randomized experimental design on an area of 800-1000 m².

Irrigation was applied rarely (only once at germination in E1 and E3), using traveling sprinklers, to restore the field capacity when rainfall events were not sufficient to preserve the quantity of easily available water (50-60% of the available water) in the root zone (Table 1). Soil moisture was monitored by using a tensiometer (Delta-T SWT 4, Delta-T Device Ltd, Cambridge UK) with the ceramic cup positioned within 20-30 cm depth in the driest area of the field. Crop protection was accomplished following the standard protocol used by local growers that includes treatment against insects, fungi and weeds.

Climate parameters were monitored hourly using a meteorological station (Pessl Instruments GmbH, Weiz, Austria) located in the experimental area. Air and soil temperature, radiation, wind speed, rainfall and air humidity data were collected and summarized in Figure 1 and Table 2.

Plant and soil analysis

Plant samples were collected at different crop stages, *i.e.*, at: i) 4-5 true leaves; ii) 10-11 true leaves; and iii) harvest time; for each treatment, three replicates were collected in the first and second sampling, and four replicates at harvest. Each replication unit con-

sisted of an area of 3.5 m² that corresponded roughly to 100 plants. Sample units were collected randomly in each treatment. Plants were harvested by hand, stored in plastic bags to limit water loss, and moved rapidly to the laboratory for growth and tissue analyses. The growth analysis consisted in the measurement of fresh (FW) and dry weight (DW, obtained in a forced-air oven at 80°C for 96 h), number of true leaves, and leaf area, determined by a planimeter (Delta-T Device, Cambridge, UK) for the calculation of leaf area index. Leaf chlorophyll was assessed through SPAD index (SPAD-502, Konica Minolta Optics, 2970 Ishikawa-machi, Hachioji, Tokyo, Japan). Plant dry matter was analysed for its mineral nutrient content. In more detail, total N was determined as the sum of reduced N (by the Kjeldhal method) and N-NO₃; the latter was determined in the aqueous extract of dry matter (1:300, w/w) using a colorimetric method (Cataldo *et al.*, 1975). After nitric-perchloric acid digestion of dried samples (90 min at 150°C), K, Ca, and Mg were determined by atomic absorption spectroscopy (Spectra-AA240 FS, Varian, Australia), while P was measured through a colorimetric method (Olsen and Sommers, 1982).

In occasion of plant destructive analyses, soil samples were also collected in each replication unit and analysed for mineral N content (N_{min}). Nitrate was measured in the aqueous extract of dry soil (soil-water ratio 1:2 w/w) using the Cataldo's method (1975). Ammonium was extracted from soil using 1 M KCl (soil-KCl ratio 1:2 w/w) and quantified spectrophotometrically through the indophenol method (Kempers and Kok, 1989).

Data analysis and modelling

Most of the variables analysed in the work refer to the averaged concentration of total (N-NH₄⁺ plus N-NO₃⁻) mineral N (N_{min}) in the root zone. Nitrogen concentration in this work is mostly expressed as mg of element per kg of dry soil. The conversion from mg kg⁻¹ N to N expressed as kg per hectare can be computed using the following equation:

$$N_{\min} (\text{kg ha}^{-1}) = N_{\min} (\text{mg kg}^{-1}) \cdot \text{BD} \cdot \text{RD} \cdot 10 \quad (1)$$

where BD is the soil bulk density (1.45 t m⁻³, on average; Table 1), RD is the root depth (0.40 m), and 10 is a multiplicative factor for unit conversion (from mg kg⁻¹ to kg ha⁻¹); then in our growing

Table 2. Period of cultivation (dates), experiment duration (days after sowing, DAS), and nominal doses of N supplied in each experiment (E) are reported in the table as single values. Mean air temperature (Ta), growing degree days (GDD), mean and cumulative daily global radiation (Rad and Cum. Rad, respectively), photothermal units (PTU) and cumulative rainfall (Rain) recorded in each experiment (E).

Experiment	Sowing	Harvest	DAS	Dose of N applied (kg ha ⁻¹)	Ta (°C)	GDD (°C)	Rad (MJ m ⁻² day ⁻¹)	Cum. Rad (MJ m ⁻²)	PTU (°C MJ m ⁻²)	Rain (mm)
E1	01/10/2007	07/01/2008	98	0-80-120-160	11.5	831.3	6.3	622.1	3383.9	256.0
E2	24/10/2007	25/02/2008	124	0-80-120-160	9.2	769.0	5.2	643.2	2575.8	369.4
E3	29/09/2008	29/12/2008	91	0-160	12.9	907.3	6.4	583.5	3553.7	649.0
E4	20/10/2008	19/02/2009	122	0-155	9.9	862.2	4.9	596.3	2666.0	829.8
E5	06/10/2009	29/12/2009	84	0-130	12.3	783.7	7.4	624.3	2620.1	271.4
E6	18/11/2009	30/03/2010	132	0-120	9.2	815.3	8.5	1117.0	5550.3	485.6
E7	08/10/2010	10/01/2011	94	0-145	11.2	772.0	5.4	511.4	2297.7	344.1
E8	16/12/2010	04/04/2011	109	0-175	9.0	652.6	8.4	911.7	4588.3	219.4
Average	-	-	-	-	10.6	799.2	6.6	701.2	3404.5	428.1
SD	-	-	-	-	1.5	75.9	1.4	204.8	1140.2	214.4
CV	-	-	-	-	0.1	0.1	0.2	0.3	0.3	0.5

conditions the product of BD, RD and 10 was 5.8.

To compare the yield (Y) of different experiments carried out under different temperature and radiation levels (Figure 1 and Table 1) the photothermal use efficiency of each single treatment (Y_{PTU} ; $\text{mg MJ}^{-1} \text{ } ^\circ\text{C}^{-1}$) was calculated according to Thornley and Johnson (1990). For each experiment, Y was divided by the photothermal units (PTU; $\text{MJ m}^{-2} \text{ } ^\circ\text{C}$) accumulated during in the growing period from the appearance of the first true leaf to harvest, (Eq. 2):

$$Y_{PTU} = \frac{Y}{\sum_{i=1}^n \text{Rad}_i \cdot (\text{Ta}_i - \text{Tb})} \quad (2)$$

where Rad_i and Ta_i are the radiation and the mean air temperature measured at the i^{th} day, respectively, and Tb represents the base temperature below which plant development does not occur. The base temperature was estimated by empirical methods, as suggested in other works (Wolfe *et al.*, 1989; Jenni *et al.*, 1996). Specifically, Tb was calculated as the value (ranging between -4°C and 8°C , with steps of 1°C) that maximized the determination coefficient of the linear regression (Eq. 3) between the number of true leaves (n_{Leaves}) and growing degree ($\sum_{i=1}^n (\text{Ta}_i - \text{Tb})$; GDD):

$$n_{\text{Leaves}} = a + b \sum_{i=1}^n (\text{Ta}_i - \text{Tb}) \quad (3)$$

In our case, the best fit was obtained with the following equation, using $\text{Tb}=3^\circ\text{C}$: $n_{\text{Leaves}} = -6.47 + 0.030 \cdot \text{GDD}$, ($n=260$; $R^2=0.81$, $P<0.001$).

For each experiment, normalized values of Y_{PTU} (Y^*_{PTU}) were obtained as the ratio between Y_{PTU} and its maximum value (Y^{max}_{PTU}):

$$Y^*_{PTU} = \frac{Y_{PTU}}{Y^{\text{max}}_{PTU}} \quad (4)$$

Following the approach proposed by Magán *et al.* (2008), (Y^{max}_{PTU}) was represented by the average yield of those treatments that did not differ statistically (*i.e.*, following ANOVA results) from the maximum yield obtained among all treatments.

A segmented linear-plateau model was adopted to fit normalized data of dry and fresh Y_{PTU} (Y^*_{DW} and Y^*_{FW} , respectively). With this model, biomass production is assumed to be zero if N_{min} is less than or equal to a minimum threshold value (N_0); afterward, Y starts to increase linearly with N_{min} up to the optimal N concentration (N_{opt}) that represents the level of N_{min} above which Y reaches its maximum value (Eq. 5):

$$Y^*_{PTU} = \begin{cases} 0 & \text{if } N_{\text{min}} \leq N_0 \\ a + b \cdot N_{\text{min}} & \text{if } N_0 < N_{\text{min}} < N_{\text{opt}} \\ 1 & \text{if } N_{\text{min}} \geq N_{\text{opt}} \end{cases} \quad (5)$$

where Y^*_{PTU} is the ratio $Y_{PTU}/(Y^{\text{max}}_{PTU})$. Following the approach proposed by Magán *et al.*, (2008), the coefficients a and b were determined by consecutive linear regression analyses run fitting the complete group of treatments and step by step subtracting the treatments at the right of a possible N_{opt} threshold till achieving the highest coefficient of determination (R^2). Finally, N_{opt} and N_0 were obtained solving the equation $Y^*_{PTU} = a + b \cdot N_{\text{min}}$ when Y^*_{PTU} was equal to 1 and 0, respectively.

A linear model was used to describe the relationship between the N nutrition index (NNI) and the SPAD index of spinach. Both parameters were calculated by the average of data collected during the entire cultivation cycle in each treatment. The NNI was calculated as the ratio between the tissue total N concentration for each treatment and the tissue average N concentration of those treatments with optimal N_{min} levels in the root zone (*i.e.*, $N_{\text{min}} \geq N_{\text{opt}}$).

Other data (*i.e.*, crop Y and N uptake at harvest, and tissue nutrient concentrations) were analysed through one-way ANOVA and Tukey test (HSD) for the separation of the means.

The programs Statgraphics Centurion XV (Statpoint Technologies, Inc., Warrenton, Virginia, USA) and Prism 5 (GraphPad Software, Inc., La Jolla, California, USA) were used for data analysis.

Results

Growing conditions

Figure 1 and Table 2 report the main climate variables monitored during the whole experimental period. The average values of Ta did not differ much among the different trials, showing the lowest CV as compared with other climate variables (Table 2). Mean daily Ta was used to calculate GDD (see Eq. 2 for details), which averaged 799.2°C among all trials, with the minimum and maximum values recorded for E8 (652.6°C) and E3 (907.3°C), respectively (Table 2).

Mean daily Rad varied more than Ta during the experiment (Table 2) with the minimum daily-averaged value recorded for E5 and E6 ($0.44 \text{ MJ m}^{-2} \text{ day}^{-1}$) in December 2009 (Figure 1), and the maximum value recorded for E6 in March 2010 ($25.0 \text{ MJ m}^{-2} \text{ day}^{-1}$). The combination of Ta and Rad in Eq. 2 resulted in different PTU values that ranged between $2297.7^\circ\text{C MJ m}^{-2}$ for E7 and $5550.3^\circ\text{C MJ m}^{-2}$ for E6 with an average of $3404.5^\circ\text{C MJ m}^{-2}$ (Table 2).

The accumulated rainfall, among the other climate variables (Table 2), showed the highest variability with the minimum and maximum value recorded for E8 (219.4 mm) and E4 (829.8 mm), respectively. In general, rainfall events were regularly distributed during the growing seasons, excluding some exceptional precipitations above 60 mm day^{-1} recorded for E3, E4 and E5 (Figure 1).

Due to diverse climate conditions, the duration of different crop cycles varied over the entire experimental period (Figure 1 and Table 2). The shortest culture was recorded in 2009, when E5 lasted 84 days, while the longest one (E6) was registered in 2009-2010 and lasted 132 days (Table 2).

The different N treatments (Table 2) and growing conditions produced a variety of N_{min} levels in the root zone as summarized in Figure 2. Data reported in Figure 2A represent the averages of soil samples collected during the experimentation in each treatment (5 samplings). To evaluate the time-dependent variability of collected data, CV and SD were also calculated and averaged at each sampling time. The coefficient of variation averaged 30% and the maximum values were generally recorded for those treatments with no supply of N fertiliser (N_0). High CV values in these treatments were therefore due to high variability in N_{min} that generally tended to decrease with time. This effect could mainly be related to N depletion in the root zone, which occurred because of the absence of N fertilisers supply. On the contrary, the lowest variability for N_{min} concentration was observed in those treatments that underwent N fertilisations and scarce rainfall with limited N-NO_3^- leaching. However, the different experiments differed significantly

(ANOVA, $P < 0.001$) for the actual mean values of N-NO_3^- , N-NH_4^+ and as a consequence N_{\min} concentration in the root zone (Figure 2A); the latter ranged from 7.6 to 41.0 mg kg^{-1} that corresponded to 44.3 and 237.6 kg ha^{-1} N_{\min} available in the soil profile explored by the root system (see Eq. 1 for conversion coefficients). Mineral

N was strongly correlated to the concentration of N-NO_3^- ($R=1.00$, $n=300$, $P < 0.001$), which accounted for roughly 81% N_{\min} on the average of all treatments. A significant albeit weak correlation was also found between N-NH_4^+ and N_{\min} ($R=0.49$, $n=300$, $P=0.02$).

On the other hand, it appears crucial to highlight that a very

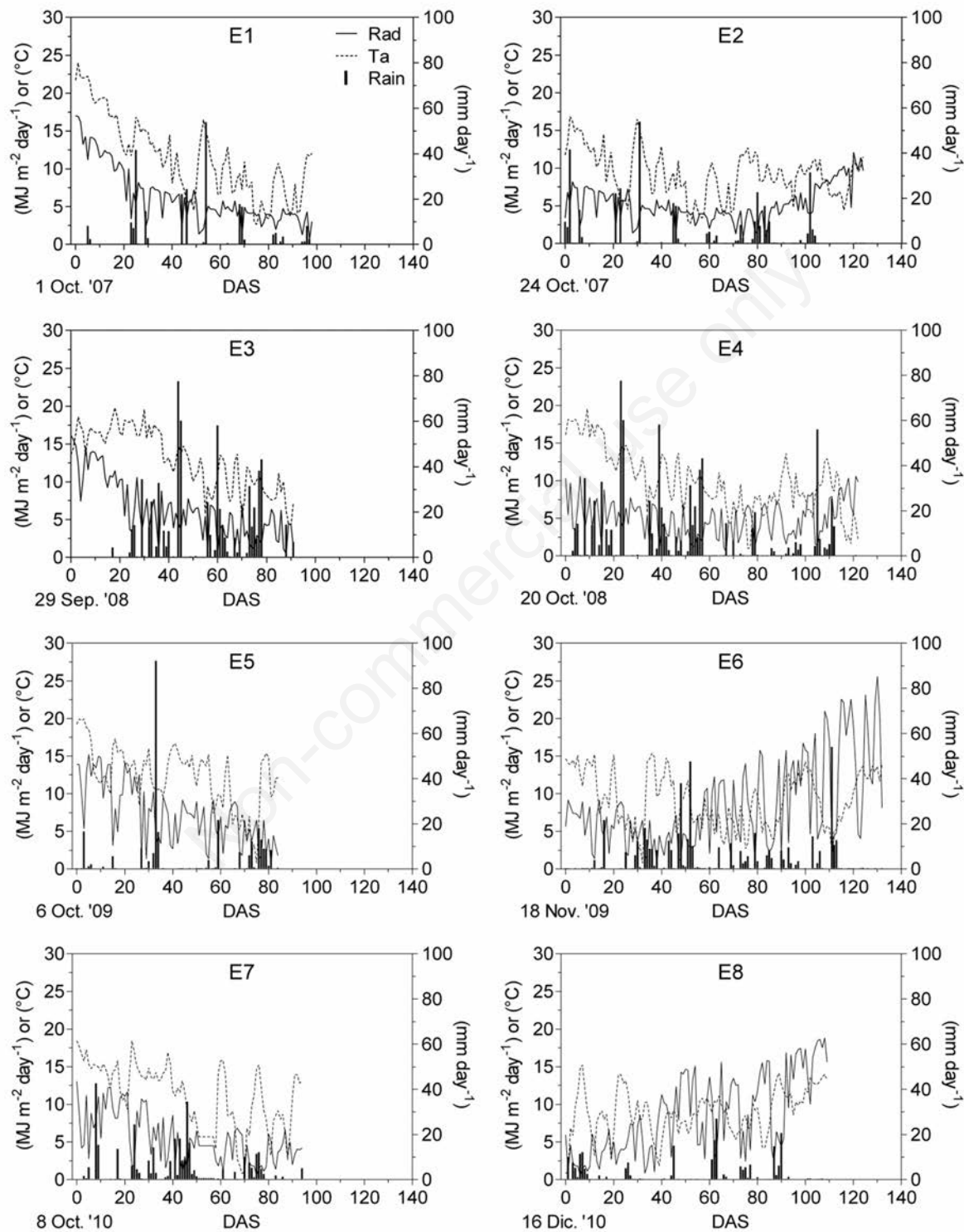


Figure 1. Daily global radiation (Rad, $\text{MJ m}^{-2} \text{ day}^{-1}$), mean air temperature (Ta, $^{\circ}\text{C}$) and rainfall (Rain, mm) plotted versus days after sowing (DAS) in the different experiments (E, see Table 2 for details). The date reported below 0 represents the sowing date.

poor relationship was instead found between the actual N_{min} and the nominal N dose supplied by fertilisers (Figure 2B).

Biomass accumulation and biometric parameters

At harvest, plant DW and FW significantly varied among treatments (Table 3). The dry weight accumulated in the aboveground biomass ranged between 0.25 (E6-0) and 2.43 (E1-160) t ha⁻¹ while FW varied from 1.72 (E6-0) to 21.69 (E1-120) t ha⁻¹.

Leaf area index ranged between 0.3 and 2.3 and was significantly correlated to both the accumulated DW ($R=0.75$, $n=80$, $P<0.001$) and FW ($R=0.71$, $n=80$, $P<0.001$). Similar results were also found for the total N uptake calculated on a DW basis, which was significantly correlated to plant biomass accumulation at harvest ($R=0.95$, $n=80$, $P<0.001$); N uptake increased from 6.28 (E6-0) to 87.37 kg ha⁻¹ (E1-160) depending on treatments (Table 3).

Significant differences among N treatments were also observed for the specific leaf area that increased with N_{min} availability. With respect to the thermal time (GDD), the specific leaf area significantly changed during the crop cycle, decreasing linearly from emergence to harvest (157.8 to 88.1 cm² g⁻¹ DW, calculated as the average of all treatments).

Modelling crop response to N_{min}

Figure 3A clearly shows that N nominal doses, supplied by fertilisers, and plant biomass accumulation were poorly correlated. This was consistent with: i) the poor correlation observed between N nominal dose and actual N_{min} in the root zone (Figure 2B); and ii) the high variability of Y in relation to the different growing conditions (*i.e.*, Ta and Rad, Table 2 and Figure 1). Yield values were therefore divided by the PTU accumulated in each experiment, thus obtaining Y_{PTU} , and then plotted *versus* N_{min} (Eq. 2 for details).

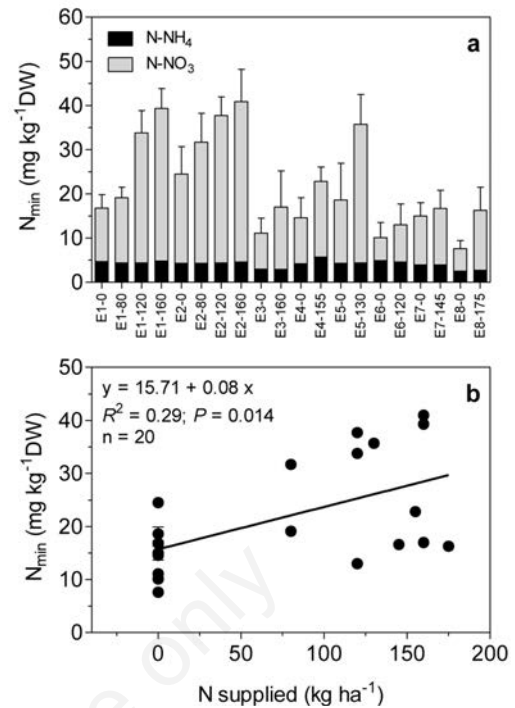


Figure 2. A) Ammonia (N-NH₄), nitric (N-NO₃) and total mineral N concentration (N_{min}) in the root zone (0-40 cm depth) averaged over the cultivation in the different experiments; each column represents the average of the replicates \pm SD. B) Relationship between actual N_{min} and the nominal doses of N supplied with fertilisers during the cultivation period; each point represents the average of replicates \pm SE.

Table 3. Effect of different N supplies on dry weight and fresh weight accumulated at harvest. Each value represents the average of replicates \pm SD.

Treatment*	Dry weight (t ha ⁻¹)	Fresh weight (t ha ⁻¹)	Total N uptake (kg ha ⁻¹)
E1-0	1.56 \pm 0.12 cde	17.69 \pm 1.32 ab	57.12 \pm 4.18 c_f
E1-80	1.88 \pm 0.30 abc	18.70 \pm 2.65 ab	64.91 \pm 10.39 a_e
E1-120	2.31 \pm 0.15 ab	21.69 \pm 1.95 a	78.41 \pm 5.18 abc
E1-160	2.43 \pm 0.19 a	21.60 \pm 1.30 a	87.37 \pm 6.66 a
E2-0	1.86 \pm 0.10 bc	17.46 \pm 1.31 abc	68.13 \pm 3.57 a_e
E2-80	1.97 \pm 0.07 abc	18.16 \pm 0.62 ab	78.16 \pm 4.07 abc
E2-120	1.94 \pm 0.12 abc	18.43 \pm 1.01 ab	76.14 \pm 4.35 a_d
E2-160	1.97 \pm 0.26 abc	17.66 \pm 2.44 ab	73.72 \pm 10.35 a_d
E3-0	0.97 \pm 0.27 efg	8.15 \pm 2.31 efg	33.01 \pm 4.92 gh
E3-160	1.18 \pm 0.30 def	10.66 \pm 2.78 def	45.65 \pm 9.34 efg
E4-0	1.50 \pm 0.21 c_f	12.65 \pm 1.93 cde	52.38 \pm 4.75 d_g
E4-155	1.99 \pm 0.11 abc	17.71 \pm 1.34 ab	81.62 \pm 4.38 ab
E5-0	1.46 \pm 0.02 c_f	14.75 \pm 0.60 bcd	57.82 \pm 6.51 b_f
E5-130	1.88 \pm 0.17 abc	17.83 \pm 1.72 ab	82.40 \pm 12.12 a
E6-0	0.25 \pm 0.04 h	1.72 \pm 0.26 h	6.28 \pm 1.27 i
E6-120	1.13 \pm 0.58 def	6.87 \pm 3.51 fgh	27.29 \pm 5.15 ghi
E7-0	0.44 \pm 0.19 fgh	4.20 \pm 1.77 gh	14.48 \pm 3.22 hi
E7-145	0.95 \pm 0.11 e_h	10.40 \pm 0.81 def	38.55 \pm 7.81 fgh
E8-0	0.31 \pm 0.10 gh	2.17 \pm 0.92 h	6.55 \pm 2.42 i
E8-175	1.62 \pm 0.25 cd	15.81 \pm 1.47 bcd	53.93 \pm 8.24 c_g
Significance	P<0.001	P<0.001	P<0.001

*The abbreviations represent Experiment number-Dose of N supplied with fertilisers (kg ha⁻¹, see Table 2); P-value for one-way ANOVA is reported. Different letters in each column represent significant differences according to Tukey's (HSD) test ($P\leq 0.05$).

This expedient allowed the standardization of Y in terms of different growing seasons with significant improvement in data analysis (Figure 3B). Y_{PTU} response to increasing N_{min} was zero below a minimum threshold; then, it began to increase linearly with N_{min} up to a maximum Y_{PTU} (Y_{PTU}^{max}), after which no significant variation was

observed. Data analysis produced (Y_{PTU}^{max}) values of 73.0 and 675.5 $mg\ MJ^{-1}\ ^\circ C^{-1}$, respectively for DW (Figure 3B) and FW (data not shown). These quantities were finally used for the normalization of the biomass datasets. The normalized Y_{PTU} (Y^*_{PTU}), which represents Y_{PTU} as a proportion of (Y_{PTU}^{max}), was fitted using a segmented

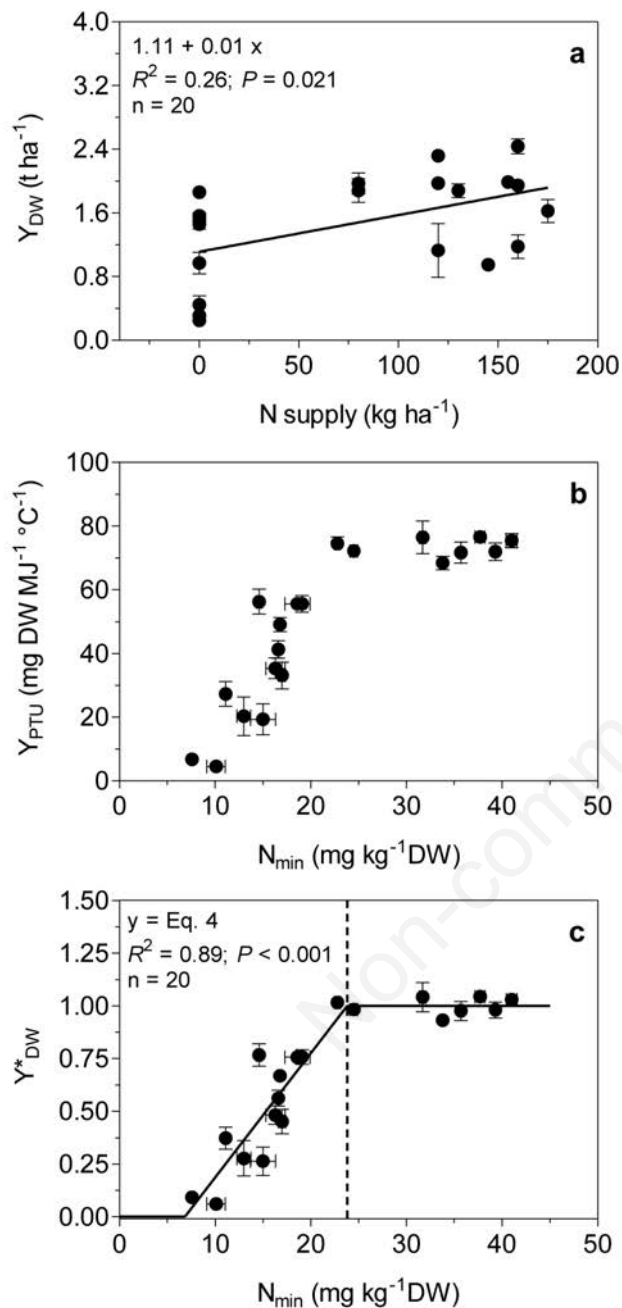


Figure 3. Relationship between: A) the nominal doses of N supplied with fertilisers during the cultivation period and the dry weight accumulated at harvest (Y_{DW}); B) the actual mineral nitrogen concentration in the root zone (N_{min}) and crop photothermal use efficiency (Y_{PTU}); or C) its normalized values (Y^*_{PTU}), both calculated on the basis of dry weight accumulated at harvest. Each point represents the mean of replicates ($\pm SE$). Continuous lines in panel C represent the model fitting the experimental data by Eq. 5; dotted lines represent the optimal value of N_{min} ($N_{opt} = 23.44\ N-NO_3\ mg\ kg^{-1}$) for fresh-market spinach.

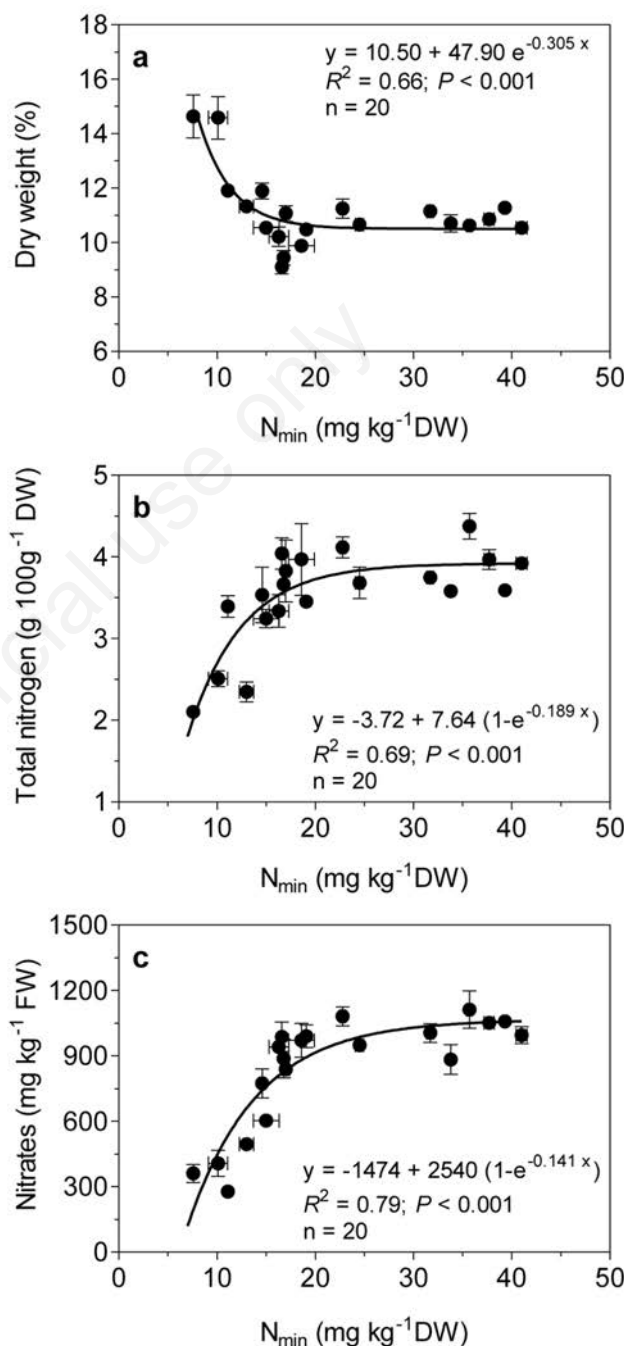


Figure 4. Relationship between the mineral nitrogen concentration in the root zone (N_{min}) and A) dry matter percentage; B) total nitrogen concentration; and C) leaf nitrate content of spinach tissues (shoot) as determined on samples collected at harvest. Each point represents the mean of replicates ($\pm SE$). Continuous lines represent the non-linear equation proposed for data fitting.

linear model (Figure 3C). Equation 5 significantly fitted Y^*_{PTU} , calculated for both Y_{DW} (Figure 3C) and Y_{FW} (data not shown), explaining 89 and 91% of the experimental variability, respectively ($P < 0.001$). Model parameterization produced the following coefficients: i) an intercept (a ; in Eq. 5) equal to -0.40 for Y^*_{DW} and -0.47 for Y^*_{FW} ; ii) a slope (b ; in Eq. 5), which represents the relative yield increase per N_{min} unit, equal to 0.06 for both Y^*_{DW} and Y^*_{FW} ; iii) N_0 equal to 6.85 mg kg^{-1} for Y^*_{DW} and 7.36 mg kg^{-1} for Y^*_{FW} ; iv) N_{opt} equal to 23.80 mg kg^{-1} for Y^*_{DW} and 23.08 mg kg^{-1} for Y^*_{FW} . Since no significant difference was found for the parameterisation of Eq. 5 using the two datasets, it could be concluded that the averaged values of N_0 (i.e., 7.11 mg kg^{-1}) and N_{opt} (i.e., 23.44 mg kg^{-1}) were representative for spinach crops. Basing on Eq. 1, the quantity of N_{min} per surface unit corresponded to $N_{opt} = 140.0 \text{ kg ha}^{-1}$ and $N_0 = 41.2 \text{ kg ha}^{-1}$.

Plant tissue analyses and N uptake

N_{min} in the root zone significantly affected dry matter percentage in plant tissues. A one-phase exponential decay equation was fitted to experimental data, explaining 62% and 66% of the measurement variability for data averaged over the whole crop cycle (data not shown) or only at harvest (Figure 4A), respectively. At harvest, DW percentages were 9.1 and 14.6%, for treatments with N_{min} above and below N_{opt} , respectively. Data averaged during the whole crop cycle showed a more restricted range, from 9.6 to 12.9%.

An opposite pattern was observed for tissue total N concentration. In this case, a one-phase exponential growth equation was fitted to experimental data explaining 63% and 69% of the measurement variability for data averaged over the whole crop cycle (data not shown) or only at harvest (Figure 4B).

At harvest, a positive relationship was found between $N\text{-NO}_3^-$ accumulation in plant tissues and N_{min} in the root zone. However, in all treatments, $N\text{-NO}_3^-$ concentration did not exceed the limits suggested by the European Food Safety Authority (EFSA) for this crop (Figure 4C). A one-phase exponential equation fitted the experimental data, showing a plateau value at 1030 g kg^{-1} FW (Figure 4C).

Plant DW and tissue N content (Figure 4C) were combined to calculate the crop total N uptake at harvest (Table 3) and over the

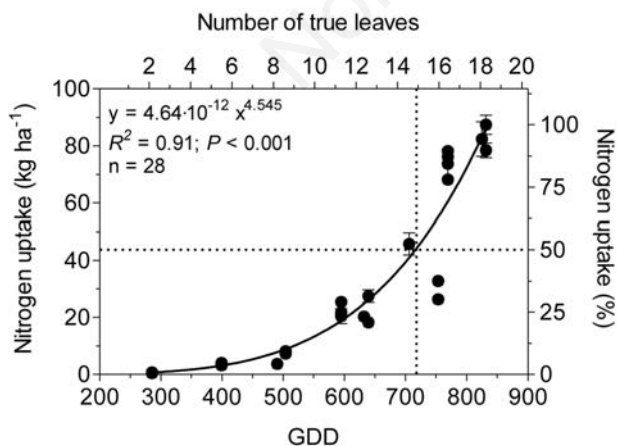


Figure 5. Relationship between crop nitrogen uptake and growing degree days (GDD) or number of true leaves. Dotted lines correspond to the growing phases at which 50% of the total N uptake occurs. Each point represents the mean of replicates (\pm SE) of only the treatments (seven) grown under optimal N levels (see Figure 3C).

cultivation cycle for each treatment. The latter data for spinach grown under optimal N conditions (i.e., excluding the treatments with N_{min} below N_{opt} in Figure 3C) are reported in Figure 5 as a function of the GDD accumulated from sowing. The number of true leaves, which is also reported in Figure 5, is a further parameter that could be used to estimate the N uptake rate for spinach.

Data on leaf mineral content (Figure 6) and SPAD index (Figure 7) were pooled into two groups corresponding to N_{min} values above N_{opt} (N^+_{opt}) or below N_{opt} (N^-_{opt}). The concentrations of N, P and Mg in plant tissues analysed at harvest were significantly

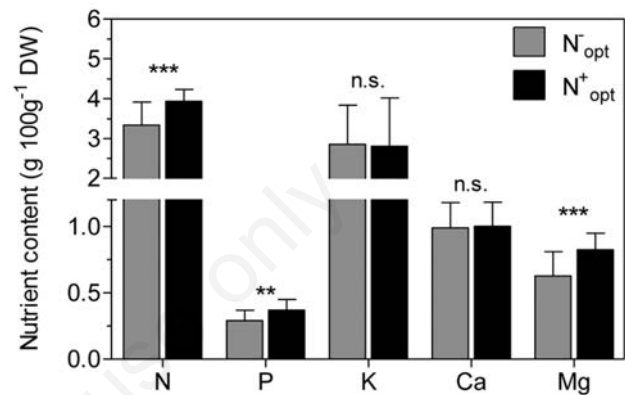


Figure 6. Concentration of total nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg), determined at harvest, in plant tissues (shoot) of spinach grown under suboptimal (N^-_{opt}) or optimal (N^+_{opt}) concentration of N_{min} in the root zone. Columns represents the average (\pm SD) of treatments below (N^-_{opt}) or above (N^+_{opt}) the optimal threshold (N_{opt}) established for fresh-market spinach. Not significant (n.s.) or significant differences are also reported for $P \leq 0.05$ (*), 0.01 (**) and 0.001 (***) according to one-way ANOVA.

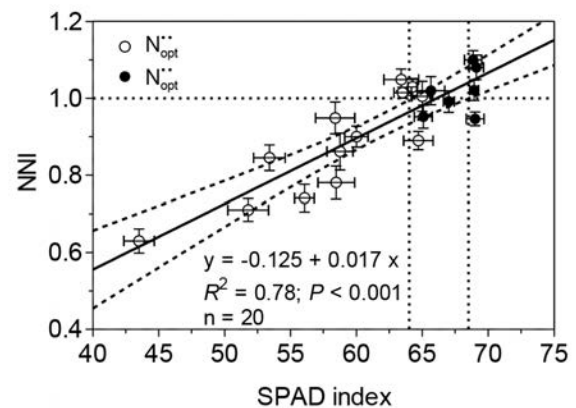


Figure 7. Linear regression between normalized nitrogen index (NNI) and SPAD index estimated by the averaged values of samples collected during the whole cultivation cycle in each treatment. Full and empty symbols represent values corresponding to plants grown under optimal (N^+_{opt}) and suboptimal concentrations (N^-_{opt}) of N_{min} in the root zone, respectively. The horizontal dotted line represents $NNI = 1$ for spinach; vertical dotted lines represent the values of SPAD index within which NNI is optimal as calculated by the 95% confidence interval of the linear regression (dashed lines). Each point represents the mean of three replicates (\pm SE).

affected by suboptimal N_{\min} availability in the root zone (Figure 6). Nitrogen was reduced by 15% from 3.9 to 3.3 g 100 g⁻¹ DW while P and Mg were more severely affected with a reduction of 21 and 23%, respectively. Conversely, no significant difference was observed at harvest for K and Ca tissue concentrations (Figure 6).

Suboptimal N_{\min} levels also affected the SPAD index that provides an indirect estimation of chlorophyll content. For this parameter, no significant difference was found between data averaged at harvest or during the whole crop cycle (ANOVA, $P > 0.05$) while a significant reduction was observed in N_{opt}^- treatments compared with N_{opt}^+ treatments. Figure 7 shows the relationship between NNI and SPAD index. The linear regression model significantly fitted the measured data, explaining 78% of the experimental variability. The collected data suggest that values of SPAD index between 64.0 and 68.5 would be optimal for savoy spinach to avoid N deficiency or excess. This range was calculated on the basis of the confidence interval resulting from the linear regression analysis (Figure 7).

Discussion

Yield response curve to N_{\min} concentration in the root zone

Very poor correlations between the nominal dose of N, applied through mineral fertilisers, and the actual N_{\min} concentration in the root zone or the harvested biomass were found in this work. In some cases (*i.e.*, treatments E1-0 and E2-0; Table 3) high Y occurred without any N supply, since a sufficient (optimal) level of N_{\min} was already present in the root zone before sowing, which supported adequately plant N nutrition throughout the whole cultivation cycle. Similar results have been obtained in previous studies with spinach (Gülser, 2005; Stagnari *et al.*, 2007). Defining the relationship between crop Y and N_{\min} appears therefore of fundamental importance for a balanced N supply whereby both economic and environmental sustainability of the crop can be maximized (Schroder *et al.*, 2000; Cui *et al.*, 2008; Bai *et al.*, 2013).

The harvest time varied widely among the different experiments. Spinach was harvested when the plants achieved 16 to 22 leaves, according to the market requirement and the weather (for example, rainfalls can delay the harvest). Furthermore, the quite different climate conditions (mainly Ta and Rad) during the years of observation caused a large variability in terms of plant growth. Crop Y was therefore standardized by PTU thus obtaining comparable data (*i.e.*, Y_{PTU}) collected over different growing seasons. The use of Y_{PTU} was successfully applied by other authors to assess differences in the theoretical Y of several crops grown in different regions (Hou *et al.*, 2012).

For modelling crop response to soil nutrient concentration, several authors fitted crop Y using linear-plateau models (Reid, 2002; Cui *et al.*, 2008; Bai *et al.*, 2013; Magán *et al.*, 2008) as was done in the present work by Eq. 5. On the other hand, many other authors described the response of different crops to N_{\min} or N fertiliser dose using non-linear equations (Thornley and Johnson, 1990; Van Noordwijk and Wadman, 1992; Reid, 2002; Milne *et al.*, 2012). Therefore, the preliminary data analysis in the present work included non-linear models consisting in quadratic and hyperbolic functions as suggested by Thornley and Johnson (1990). However, the resulting determination coefficients (in the range of 0.83-0.86), or other error performance indices, were close to the ones achieved with the proposed linear model or even worse. The same was

obtained for the other observed statistical parameters.

With respect to non-linear models, Eq. 5 has the advantage of producing an unequivocal value for N_{opt} ; in contrast, the use of non-linear equations may lead to higher uncertainty in the identification of N_{opt} . The N_{opt} value found in the present study (23.44 mg kg⁻¹) for spinach was quite similar to others reported in literature for different crops. In fact, optimal N_{\min} in the root zone have been found in the range of 16-30 mg kg⁻¹ dry soil for corn (Cui *et al.*, 2008; Peng *et al.*, 2013), 20-21 mg kg⁻¹ for wheat (Bundy and Andraski, 2004), 20-30 mg kg⁻¹ for potato (Doll *et al.*, 1971), 24 mg kg⁻¹ for cabbage (Heckman *et al.*, 2002), and 20-24 mg kg⁻¹ for celery and lettuce (Hartz *et al.*, 2000; Bottoms *et al.*, 2012).

Crop quality response to N_{\min} concentration in the root zone

Nitrate accumulation in spinach leaves is due to many factors depending on both environmental growth conditions (*e.g.*, temperature, radiation, N fertilisation management) and plant-specific characteristics (*e.g.*, nitrate reductase activity, leaf age) (Lasa *et al.*, 2001; Chen *et al.*, 2004; Gülser, 2005; Stagnari *et al.*, 2007). High availability of N_{\min} in the root zone indeed promotes nitrates and total N accumulation in leaves. Similar results have been observed in other leafy vegetables, such as romaine and red-oak leaf lettuce (Di Gioia *et al.*, 2017). However, the tissue nitrate content observed in this work was within the limits suggested by EFSA (2008) for spinach. It was likely due to the quite low plant density and to the N level in the soil that was not too much exceeding N_{opt} (maximum N-NO₃ value was 36 mg kg⁻¹, recorded in the treatment E2-160).

Tissue content of other plant mineral nutrients responded differently to N_{\min} treatments depending on the nutrient element. Literature on spinach is quite heterogeneous with regard to the relationship between tissue content of plant mineral nutrients and N_{\min} in the root zone. In several studies, positive correlations between N, Ca, or Mg and N_{\min} have been reported (Lefsrud *et al.*, 2007; Stagnari *et al.*, 2007). However, contrasting findings have been reported for P or K that were found to increase (Stagnari *et al.*, 2007), to be constant (Lefsrud *et al.*, 2007, only K) or even to decrease (Gülser, 2005, only P) by increasing N_{\min} in the root zone. A significant positive correlation between P or Mg and N_{\min} in the root zone ($P < 0.001$, $R = 0.70$ or 0.71 , respectively) was observed in this work, as previously reported by other authors (Lefsrud *et al.*, 2007; Stagnari *et al.*, 2007). On the other hand, no significant correlation was found between K or Ca tissue content and N treatments, in agreement with Gülser (2005).

Increasing N_{\min} in the root zone led to an enhanced blade colour. This is a relevant reference extrinsic characteristic, much appreciated by consumers of fresh-market spinach. The SPAD index was significantly lower in N_{opt}^- treatments, in which plants were grown under suboptimal nutritional conditions. SPAD index is significantly correlated to leaf N and leaf chlorophyll concentration (Schepers *et al.*, 1992), on which spinach colour depends mostly. Leaf chlorophyll concentration has been found positively correlated to N_{\min} in the root zone in spinach (Lefsrud *et al.*, 2007), and to leaf N and Mg concentration in many species (Shaahan *et al.*, 1999) as also observed in this work.

The use of chlorophyll meters, such as the SPAD, could be not reliable to estimate on-time crop N requirement because of possible delays between the nutrient status of the root zone and its effects on plants (Westerveld *et al.*, 2003; Wu *et al.*, 2007). Nevertheless, the information provided by these tools and other optical sensors may be valuable if coupled with data on soil com-

position obtained through either laboratory analyses or easy-to-use and rapid methods available at farm level (Hartz *et al.*, 2000; Maggini *et al.*, 2010; Thompson *et al.*, 2017).

Implementation of the results for improving spinach N fertilisation management

Growers usually adopt very simple N fertilisation plans for fresh-market spinach. In Tuscany (Italy), where the experiment took place, they usually apply from 120 to 175 kg ha⁻¹ N for each growing cycle, often as fixed reference dosages without pre-sowing soil analyses. This fertilisation approach is quite robust and effective from the growers' point-of-view. In common practice, the application of 120 kg ha⁻¹ N to a crop like savoy spinach, which takes up from roughly 60 to 75% of the above quantity, consists in a sort of *insurance* against possible low soil N concentration and rainy periods that could hinder N top dressing applications. Obviously, this approach contributes to N loss phenomena related to excess N in the root zone and may reduce produce quality. In the above scenario, the risk for high crop environmental impact and production costs increases drastically (Massa *et al.*, 2013).

According to our results, the presence of roughly 23 mg kg⁻¹ of N_{min} in the soil is enough for a regular spinach growth and development without any yield and/or quality reduction. An ideal management of N fertilisation would consist in the supply of small fertiliser amounts at high frequency to keep N_{min} concentration close to N_{opt}, as in fertirrigated crops. Nevertheless, a similar fertiliser application plan would not be sustainable for winter spinach under open-field operative conditions from both the agronomic and the economic point of view. Based on the crop N uptake curve reported in Figure 5, 50% of the total N absorbed by spinach is concentrated in the last part of the growing cycle (the last three to five weeks between the 13-15 true-leaf phase and harvest time). Similar results have been reported for other leafy vegetables such as lettuce (Bottoms *et al.*, 2012). Therefore, when N fertilisers have to be applied in advance, data reported in Figure 5 must be carefully taken into account in terms of doses and distribution frequency to avoid excess N supply when plants are not ready to take this element up.

The results obtained in this work could be implemented for advanced N fertilisation strategies, which are based on a pre-sowing N_{min} soil analysis followed by N_{min} monitoring in the root zone (Thompson *et al.*, 2017). At the pre-sowing stage, if the soil N_{min} is lower than N_{opt}, a base fertilisation is necessary. After sowing, top-dressing fertilisations will then be necessary only if the level of N_{min} in the root zone drops below N_{opt}. Nowadays, soil N_{min} can be easily monitored at farm level by using quick tests that are faster, simpler, and cheaper than the conventional laboratory analyses (Hartz *et al.*, 2000; Maggini *et al.*, 2010; Incrocci *et al.*, 2017). For example, this approach has been validated for the fertilisation plan of lettuce and celery in California (Hartz *et al.*, 2000), and for cabbage in North America (Heckman *et al.*, 2002) or in The Netherlands (Everaarts and de Moel, 1998).

Furthermore, the model calibrated in the present work (Eq. 5) can be implemented in decision support systems for the precise nutrient management of spinach. In addition to the estimation of N_{opt}, the implementation of Eq. 5 in decision support systems can be useful to simulate spinach growth, as a function of N_{min} in the root zone, and eventually calculate the dose and distribution fre-

quency of N fertilisers based on real plant needs.

The relationship between NNI and SPAD index provides additional information for the precise management of N nutrition in spinach using optical sensors, as previously reported for other vegetable crops (Padilla *et al.*, 2015; Thompson *et al.*, 2017; Incrocci *et al.*, 2017). Spinach for fresh market is harvested at early growth stage. We observed that, within this growing period, N tissue concentration did not vary significantly; it was therefore possible to find a significant correlation between SPAD index and NNI using data averaged over the whole cultivation cycle. On the other hand, much higher variability was observed for other vegetable crops in different growing periods (Padilla *et al.*, 2014, 2015).

As observed in the present work, SPAD values lower than 64.0 would imply N deficiency with reduced Y for fresh-market spinach. Lower values were observed for open-field processing (Canali *et al.*, 2014) and greenhouse-grown spinach (Liu *et al.*, 2006; Muchecheti *et al.*, 2016). However, the threshold values reported in Figure 7 (in the range of 64.0-68.5) were assessed in this work for the first time for NNI-based quick N monitoring in fresh-market spinach grown under open-field Mediterranean climate conditions. The combination of soil analyses and optical leaf sensors, like the SPAD used in this study, indeed appears of great interest for the optimized management of N fertilisation in vegetable crops (Incrocci *et al.*, 2017).

Conclusions and remarks

Meaningful relationships between crop yield or quality and mineral nitrogen availability in the root zone are reported in this work for fresh-market spinach. A linear-plateau model significantly represented the yield response to N concentration in the root zone. The adopted model was successful in determining an optimal threshold value (*i.e.*, N_{opt}) of 23.44 mg kg⁻¹ DW (140.0 kg ha⁻¹ in our experimental conditions) to be maintained in the root zone for efficient N fertilisation plans of this crop.

Plant tissue analyses supported the hypothesis that there is no reason to exceed N_{opt} in the root zone since above this threshold most of plant tissue characteristics tend to be unaffected. In contrast, plants grown under suboptimal levels of N in the root zone (*i.e.*, below N_{opt}) show a reduced yield and tissue content of mineral nutrients and SPAD index, which imply that N_{opt} must be maintained in the root zone to obtain high yield and quality. Maintaining the suggested conditions in the root zone may therefore significantly improve the environmental and economic sustainability of the crop.

The use of optical sensors (*e.g.*, the SPAD by Minolta used in the present work) can be helpful for growers to check quickly the N nutritional status of the crop; SPAD index higher than 64.0 would ensure optimal N nutrition in fresh-market spinach. The combined monitoring of the root zone and crop canopy therefore appears a strategy worth exploring for the correct management of N fertilisers in this crop. The results reported in this paper can be implemented in protocols, algorithms and decision support systems for the optimized N fertilisation of fresh-market spinach.

Abbreviations

Symbol	Description	Units
CV	Coefficient of variation	
DW	Dry weight	t ha ⁻¹
E	Experiment	
FW	Fresh weight	t ha ⁻¹
GDD	Growing degree days or thermal time	°C
LAI	Leaf area index	
N ₀	Soil mineral nitrogen concentration below which yield is zero mg kg ⁻¹ , kg ha ⁻¹	
n _{Leaves}	Number of true leaves	n
N ⁻ _{opt}	Pool of treatments below N _{opt}	
N ⁺ _{opt}	Pool of treatments above N _{opt}	
N _{min}	Total mineral nitrogen in the root zone	mg kg ⁻¹ DW or kg ha ⁻¹
N _{opt}	Optimal nitrogen concentration to be maintained in the root zone for spinach	mg kg ⁻¹ , kg ha ⁻¹
NNI	Nitrogen nutrition index	
PTU	Photothermal units	MJ m ⁻² °C ⁻¹
Rad	Global radiation	MJ m ² day ⁻¹
SD	Standard deviation	
SE	Standard error	
T _a	Air temperature	°C
T _b	Base air temperature	°C
Y	Crop yield	t ha ⁻¹
Y _{PTU}	Photothermal use efficiency or potential yield	t MJ ⁻¹ m ⁻² °C ⁻¹
Y _{PTU} ^{max}	Maximum photothermal use efficiency or potential yield	t MJ ⁻¹ m ⁻² °C ⁻¹
Y* _{PTU}	Y _{PTU} /Y _{PTU} ^{max}	

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