

Agronomic, physiological and quality response of *romaine* and *red oak-leaf* lettuce to nitrogen input

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Abstract

Protecting the environment by improving the crop-system nitrogen (N) use efficiency (NUE) while maximising yield and quality is a primary challenge for modern agriculture, and understanding the processes that govern N fluxes in the plant-soil system is essential to improve NUE. An on-farm study was conducted over two fall-winter seasons to evaluate the NUE, agronomical and physiological response of *romaine* (var. *longifolia*, cv Manavert) and *red oak-leaf* (var. *crispa*, cv Aruba) lettuce (*Lactuca sativa* L.) to different N-rates (0, 60, 120, 180 kg ha⁻¹ of N). Nitrogen rate influenced all tested parameters, including plant fresh and dry weight, N accumulation, leaf NO₃⁻ and dry matter content, NUE indices, N nutrition index (NNI), soil residual N and the estimated N losses at the end of the crop season. Fresh yield, dry weight and N-accumulation response to N rate were influenced by lettuce genotype. Manavert had higher N recovery, NUE, and lower leaf NO₃⁻ concentration than Aruba. Analysing the NNI overtime, 120 kg ha⁻¹ of N assured an optimal N status in both Manavert and Aruba, while N deficiency and excess were observed at lower and higher N-rates, respectively. An empirical

relationship was observed between NNI and leaf NO₃⁻ concentration, suggesting that leaf NO₃⁻ concentration may be used to predict NNI and thus the crop N status. The relationship between NNI and leaf NO₃⁻ concentration may be used to define optimal leaf NO₃⁻ concentration ranges for the rapid and site-specific assessment of the crop N status, and the dynamic adjustment of N-fertilisation, contributing to improve crop NUE, minimise N-losses, and optimise yield and quality of lettuce crops.

Introduction

Matching temporal and spatial nitrogen (N) supply with N demand in vegetable crops, without incurring in excess or deficiency is still a challenging task for growers. Especially when dealing with crops like lettuce, characterised by shallow roots, high sensitivity to N deficiency (Broadley *et al.*, 2000), relatively low N uptake efficiency (Greenwood *et al.*, 1989), and high nitrate-accumulation capacity (Santamaria, 2006).

In spite of the relatively low N demand, during the early growth stage, lettuce crops require the maintenance of adequate levels of N in the root zone, because N shortage, even if temporary, can permanently compromise the final yield (Broadley *et al.*, 2000). Nitrogen demand in lettuce crops is particularly high in the last third of the growing cycle, and sub-optimal N supply may lead to N deficiency with consequent yield reduction, poor quality and maturity delay (Salomez and Hofman, 2009). On the other hand, over applications of N, exceeding crop N demand, may cause N luxury consumption with consequent nitrate accumulation (Di Gioia *et al.*, 2013), and increase the risks of N losses, causing environmental pollution and contamination of water bodies and drinking water.

Vegetables are considered one of the major sources of human nitrate intake (Santamaria, 2006; Di Gioia *et al.*, 2013). Despite there are increasing evidences that nitrate and nitrite may have a beneficial action against cardiovascular diseases (Hord *et al.*, 2009), nitrates are considered anti-nutrients. Lettuce commercialisation is in fact subject to limits of the nitrate concentration set by the EC Regulation No. 194/97 (European Commission, 1997), recently amended and integrated by the EC Regulation No. 1258/2011 (European Commission, 2011), or to limits imposed by importing Countries and food-chain stores (Di Gioia *et al.*, 2013).

Attempting to protect public health and environment, governments have implemented regulatory or volunteer measures and monitoring programmes aimed at reducing water pollution from agricultural sources. In Europe, with the Nitrates Directive (91/676/EEC), about 39.6% of the European Union (27 Member States) area has been designated as nitrate vulnerable zone (NVZ), and all Member States have defined good agricultural practices

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(GAPs) and have established action programmes in their NVZs (European Commission, 2010). Similarly, in the United States, with the definition of the *Federal Total Maximum Daily Load* mandate described in the *Federal Clean Water Act* (US Environmental Protection Agency, 2010), best management practices (BMPs) have been developed and implemented in each state, providing tools and important practices – including irrigation and fertilisation management practices aimed at reducing crop environmental impact while assuring optimal yields (Simonne *et al.*, 2010).

The increased awareness of the potential impact of agricultural activities on the environment and on public health, and the mandatory or volunteer adoption of GAPs and/or BMPs is leading farmers to a more attentive approach to crop fertiliser management. Protecting the environment by improving crops nitrogen use efficiency (NUE) has become one of the primary and most challenging goals for farmers (Hirel *et al.*, 2007).

Crop NUE, yield and quality are the result of a complex interaction between plant genotype, management practices and several environmental factors, and understanding the processes that govern N fluxes in the plant-soil system is of major importance in any attempt to improve NUE (Lemaire *et al.*, 2008). A better knowledge of the factors that determine plant N uptake, accumulation and use efficiency during the crop cycle may enable the development of more rational fertiliser management strategies and technologies, thereby minimising the risks of N losses into the environment (Good *et al.*, 2004).

The optimal N rate for a given crop is not constant, but can vary considerably from cultivar to cultivar, site to site, and season to season (Ozores-Hampton *et al.*, 2015). Therefore, practical on farm management tools are required to rationalise crop N fertilisation. An approach to optimise crop N fertiliser management consists in implementing modern *site-specific* management strategies, which may involve the use of diagnostic tools for the assessment of the crop or soil N status during the growing season (Peña-Fleitas *et al.*, 2015), or the use of models and decision support systems (Conversa *et al.*, 2015). Nevertheless, the implementation of site-specific N management strategies requires knowledge and basic information on N crop demand and accumulation, soil N mineralisation, potential N losses and residue, and crop NUE. Such basic information may be used to develop, calibrate and update predictive models, and to develop monitoring techniques and indicators of the soil-crop system N status, capable of detecting or predicting both N deficiency and excesses. Moreover, established indicators of the crop nutrient status, such as the N nutrition index (NNI) proposed by Lemaire *et al.* (1989) may be used to define optimal N rates, and test monitoring techniques and the reliability of diagnostic tools and indicators of the plant and soil N status.

Given the importance of lettuce crops, grown at global level on over a million of hectares every year (FAOSTAT, 2016), a field study was conducted to analyse and compare the agronomic, physiological and quality response of *romaine* (heading) and *red oak-leaf* (non-heading) lettuce to N inputs. Specific objectives of the study were: i) evaluate N rate effects on yield, N, nitrate and dry matter accumulation of both genotypes; ii) estimate their N recovery and utilisation efficiency, and potential soil N losses; iii) analyse *a posteriori* the crop N status and the optimal N rate for both genotypes using the NNI; and iv) evaluate the possibility to predict the NNI, and thus, the crop N status through the leaf NO_3^- concentration.

Materials and methods

Plant material, growing conditions and experimental design

Two field experiments were conducted on-farm at Conversano (40° 58' N, 17° 7' E; 140 m asl) and Polignano a Mare (40° 59' N, 17° 13' E; 40 m asl), in Southern Italy, during the fall-winter season of 2006-2007 (Exp-1) and 2007-2008 (Exp-2), respectively. In both sites the soil was a typical Mediterranean *Terra Rossa* (*red earth*) clay soil, classified as Alfisols according to the U.S. Department of Agriculture soil taxonomy (USDA, 2003), or as Luvisols according to the World Reference Base for soil resources (WRB, 2006). Soil chemical properties were as follows (for Exp-1 and Exp-2, respectively): organic matter 14.4 and 14.7 g kg⁻¹, total N 1.02 and 1.35 g kg⁻¹, organic carbon 8.4 and 8.5 g kg⁻¹, C/N 8.13 and 6.3, P₂O₅ 364.7 and 688.3 mg kg⁻¹, K₂O 130.6 and 196.9 mg kg⁻¹, cation exchange capacity 291.9 and 258.8 meq kg⁻¹, pH 7.8 and 7.0, electrical conductivity 0.5 and 1.1 dS m⁻¹.

On 3 November of 2006 and 30 October of 2007, *romaine* (var. *longifolia*, cv Manavert; Enza Zaden Italia s.r.l., Tarquinia, Italy) and *red oak-leaf* (var. *crispa*, cv Aruba; Enza Zaden Italia s.r.l.) lettuce (*Lactuca sativa* L.) were transplanted at the third true-leaf stage in rows 0.35 m apart, establishing a density of 9.5 plants m⁻². Both cultivars were harvested at 105 and 94 days after transplanting (DAT), in Exp-1 and Exp-2, respectively.

In both experiments, four N rates [0 (N₀), 60 (N₆₀), 120 (N₁₂₀), and 180 (N₁₈₀) kg ha⁻¹ of N] were applied through side-dressing, 50% as ammonium sulphate in pre-transplanting and 50% as ammonium nitrate in post transplanting at 48 and 40 DAT in Exp-1 and Exp-2, respectively. An unfertilised control was included to calculate the crop NUE, and estimate N soil mineralisation. Treatments were arranged according to a split plot experimental design with three replicates in both experiments. The cultivars (Manavert and Aruba) were arranged in the main plots, while N rates were randomised in subplots of 21 m² (3.5 m wide and 6 m long). Crop water requirements were satisfied by rainfall events (about 64.6 mm in Exp-1 and 144 mm in Exp-2) and supplemental irrigation (about 85 mm and 50 mm, respectively) applied using low-pressure mini-sprinkler irrigators, which represents the most common irrigation method used in the area for leafy vegetables. An integrated crop protection management approach was used to control weeds, pests, and main diseases. Meteorological data for both seasons were recorded and provided by the local public service, considering the closest weather station of the existing network. Mean daily air temperature was on average 10.3 and 9.1°C in Exp-1 and Exp-2, respectively. In Exp-1, daily minimum and maximum air temperature ranged from 0 to 9.1°C and from 12.3 to 22.4°C, respectively (Figure 1). In Exp-2, daily minimum and maximum air temperature ranged from -4.7 to 2.4°C and from 15.8 to 23.4°C, respectively. Temperatures below 0°C reduced plant growth but did not damage both lettuce genotypes. During the growing season, cumulative solar radiation was 978 and 503 MJ m⁻² in Exp-1 and Exp-2, respectively.

Plant growth analysis, tissue and soil laboratory analysis

Total aboveground fresh weight (FW) was measured on a sample of six plants per plot, at 20, 47, 61, 75 and 105 DAT in Exp-1, and 28, 49, 66 and 94 DAT in Exp-2. Plant samples were oven-dried at 65°C until constant weight to determine dry weight (DW) and dry matter (DM) content. Oven-dried plant samples were finely ground through a mill (IKA; Labortechnik, Staufen, Germany)

and used for quantitative chemical analyses. Total N was determined by Kjeldahl method (2300 Kjeltac Auto Analyser) with the addition of salicylic acid for the recovery of the $\text{NO}_3\text{-N}$ (Simonne *et al.*, 1997). Nitrates, previously extracted from dry matter samples of 0.5 g with 50 mL of sodium carbonate (3.5 mM) and sodium bicarbonate (1.0 mM) solution, were determined by ion chromatography (model Dionex DX500; Dionex Corp., Sunnyvale, CA, USA) as described by Boari *et al.* (2013).

Only for the cv Manavert, soil samples were collected before N treatment (N_0 , N_{60} , N_{120} and N_{180}) application and at harvest, 105 and 94 DAT for Exp-1 and Exp-2, respectively, to monitor the soil mineral N (NO_3^- and NH_4^+) content. Using an Eijkelkamp soil auger with a diameter of 5 cm, four borings per plot were taken at 30 cm depth, excluding border rows. Subsamples were combined in a single bulk sample per plot. Soil samples were stored in freezer (-18°C) pending analysis, then were defrosted at 4°C and extraction was conducted on two subsamples within 24 hours with a 1:4 ratio (w/w) soil: 0.025 M KCl solution. The filtered solution was then analysed to determine $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ content by ion chromatography (model QIC; Dionex Corp.). Values of $\text{NH}_4\text{-N}$ were negligible or below detection limit during both seasons (data not shown).

Soil core subsamples collected in pre-transplanting were air-dried, sieved to 2 mm, and used for soil physical and chemical characterisation.

Nitrogen use efficiency indices and nitrogen nutrition index calculation

Total N accumulation (N_{acc} ; $\text{kg}\cdot\text{ha}^{-1}$) was calculated multiplying the aboveground DW by the total N concentration. In correspondence of each sampling date, the critical nitrogen (N_{crit}) content, which is defined as the minimum N concentration in the aboveground biomass required for maximum growth, was calculated as proposed by Justes *et al.* (1994) according to the equation parametrised by Tei *et al.* (2003) for lettuce:

$$\text{N}_{\text{crit}} = 4.56 \times \text{DW}^{-0.357} \quad (\text{Eq. 1})$$

The NNI was calculated as proposed by Lemaire *et al.* (1989), dividing the measured N concentration of the aboveground biomass ($\%N_{\text{DW}}$) by the predicted $\%N_{\text{crit}}$:

$$\text{NNI} = \%N_{\text{DW}} / \text{N}_{\text{crit}} \quad (\text{Eq. 2})$$

At final harvest NUE indices were calculated for each N rate according to Elia and Conversa (2012) as follows.

First, apparent N fertiliser recovery efficiency (REC_N) by the crop, which represents the kg increase in N_{acc} per kg of applied N:

$$\text{REC}_N = (\text{A}_F - \text{A}_0) / \text{N}_F \quad (\text{Eq. 3})$$

Second, partial factor productivity of applied N (PFP_N), which represents the kg of product harvested per kg of applied N:

$$\text{PFP}_N = Y_F / \text{N}_F \quad (\text{Eq. 4})$$

Third, agronomic efficiency of applied N (AE_N), which represents the kg of yield increase per kg of applied N:

$$\text{AE}_N = (Y_F - Y_0) / \text{N}_F \quad (\text{Eq. 5})$$

Fourth, physiological efficiency of applied N (PE_N), which repre-

sents the kg of yield increase per kg increase in N_{acc} from fertiliser:

$$\text{PE}_N = Y_F - N_0 / A_F - A_0 \quad (\text{Eq. 6})$$

where: Y_F is the crop yield ($\text{kg}\cdot\text{ha}^{-1}$) obtained with the application of a determinate N-fertiliser (N_F) rate ($\text{kg}\cdot\text{ha}^{-1}$); Y_0 is the crop yield obtained in the unfertilised control; A_F is the total plant N_{acc} ($\text{kg}\cdot\text{ha}^{-1}$) in aboveground biomass at maturity when an amount of N_F is applied, and A_0 is the corresponding total plant N_{acc} ($\text{kg}\cdot\text{ha}^{-1}$) in aboveground biomass at maturity when no N-fertiliser is applied. Nitrogen use efficiency indices were calculated both on fresh and dry weight basis.

Soil nitrogen mineralisation and losses estimate

Nitrogen derived from the mineralisation of the soil organic matter ($\text{N}_{\text{organic}}$) in each growing season was estimated by subtracting the initial soil inorganic N (N_{min}) content of the top 30-cm soil layer in unfertilised control ($0\text{ kg}\cdot\text{ha}^{-1}$ of N) from the sum of aboveground N_{acc} and residual soil N_{min} at harvest in the same soil layer according to Cui *et al.* (2010):

$$\text{N}_{\text{organic}} = (\text{soilN}_{\text{min}}(\text{residual}) + \text{cropN}_{\text{acc}}) - \text{soilN}_{\text{min}}(\text{initial}) \quad (\text{Eq. 7})$$

Nitrogen losses were estimated as difference between N inputs (initial soil N_{min} , apparent N mineralisation, applied N_F), and outputs (aboveground N_{acc} , residual soil N_{min}):

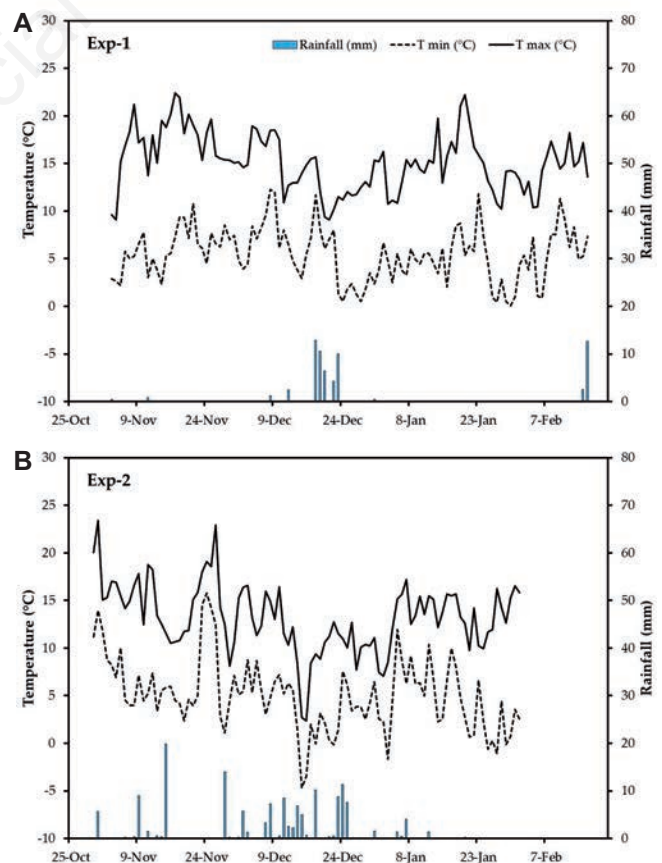


Figure 1. Daily minimum and maximum temperature and rainfall recorded at Conversano and Polignano a Mare (Southern Italy), during the fall-winter season of 2006-2007 (Exp-1; A) and 2007-2008 (Exp-2; B), respectively.

$$N_{losses} = (\text{soil } N_{\min(\text{initial})} + N_{\text{organic}} + N_F) - (\text{soil } N_{\min(\text{residual})} + \text{crop } N_{\text{acc}}) \quad (\text{Eq. 8})$$

where: soil $N_{\min(\text{initial})}$ and $N_{\min(\text{residual})}$ were the inorganic N content of the top 30-cm soil layer, before pre-planting fertiliser application and at harvest, respectively.

Statistical analysis

Statistical analysis was performed separately for each experiment. Analysis of variance (ANOVA) was performed using the GLM procedure of SAS software package (SAS Institute Inc., Cary, NC, USA). Linear and quadratic regression analyses were performed using PROC REG of SAS to estimate the parameters of the relationships between N rate and FW, DW, and N_{acc} at harvest. All means were compared by the least significant difference (LSD) test, at 5% level of probability.

Results

Effect of nitrogen supply on fresh yield, dry biomass and nitrogen accumulation

Nitrogen rate and lettuce genotype influenced plant FW, DW and N_{acc} (Figure 2). In both experiments, at harvest (105 and 94 DAT in Exp-1 and Exp-2, respectively) FW, DW and N_{acc} were on average 2.2, 1.8 and 1.5 times higher in Manavert than in Aruba (Figure 2), respectively.

Except for the N_{acc} response to N rate of the cv Manavert in Exp-2 (Figure 2C), a second order polynomial function showed always a better fit than the linear function, as demonstrated by the higher adjusted R^2 ($\text{Adj}R^2$) coefficient (Table 1). Nevertheless, the quadratic coefficients were very low, especially in the case of the relationship between N rate and DW (Table 1).

Based on the estimated quadratic functions the optimal N rates to achieve the maximum fresh yield were 182 and 148 kg ha^{-1} for Manavert, and 160 and 169 kg ha^{-1} of N for Aruba, in Exp-1 and Exp-2, respectively (Figure 2A). Considering the DW, estimated optimal N rates ranged between 145 and 160 kg ha^{-1} of N for both cultivars and experiments (Figure 2B).

Effect of nitrogen supply on nitrate and dry matter plant content

Leaf NO_3^- concentration was higher in Aruba than in Manavert in both experiments (Figure 3A and B). In both experiments, Aruba increased the leaf NO_3^- concentration with increasing N rate, whereas Manavert increased the leaf NO_3^- concentration only when grown with N_{120} and N_{180} . Maximum leaf NO_3^- concentration values achieved with N_{180} were on average 1534 and 1881 mg kg^{-1} of FW for Aruba, and 1042 and 685 mg kg^{-1} of FW for Manavert, in Exp-1 and Exp-2, respectively (Figure 3A and B). In Exp-1, the DM percentage was not different between the two cultivars at N_{60} . When non-fertilised (N_0) Manavert had a higher DM content compared to Aruba, while when fertilised with the highest N rates (N_{120} and N_{180}) Manavert DM content was lower than in Aruba. Instead, in Exp-2 the DM content was consistently higher ($P=0.01$) in Aruba than in Manavert regardless the N rate (Figures 3C and D). The DM content was higher in unfertilised plants than in fertilised ones, in both experiments, except for Aruba grown with N_{60} that in Exp-2 had DM values similar to the unfertilised plants. In Exp-1 only for Manavert, DM content was higher with N_{60} than in plants grown at higher N rate (Figure 3C).

An inverse relationship was observed between DM and leaf NO_3^- concentration: with increasing the N fertiliser rate the leaf NO_3^- concentration increased, while the DM content decreased in both cultivars and experiments (Figure 4).

Nitrogen use efficiency and its components

In Exp-1, REC_N was lower ($P=0.02$) in Aruba than in Manavert (32% vs 42%, respectively), while in Exp-2 it was on average 22% and was not different between cultivars (Figure 5). In both experiments the REC_N was not significantly influenced by N rate in Manavert, while it decreased with the highest N rate in Aruba

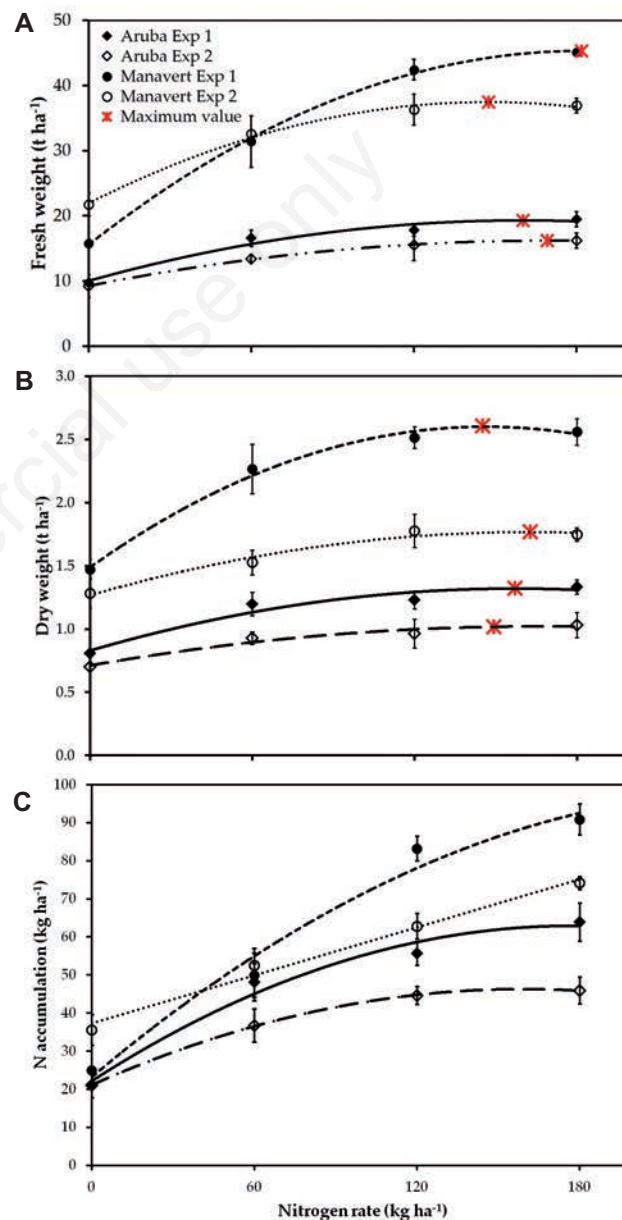


Figure 2. Final aboveground fresh weight (A), dry weight (B), and N accumulation (C) as functions of nitrogen fertilizer rates in *romaine* (cv Manavert) and *red oak-leaf* (cv Aruba) lettuce grown at Conversano and Polignano a Mare (Southern Italy), during the fall-winter season of 2006-2007 (Exp-1) and 2007-2008 (Exp-2), respectively. Vertical bars represent \pm standard error ($n=3$).

(Figure 5). The PFN_N decreased ($P < 0.001$) as the N rate increased in both cultivars and experiments (Figure 6A). In both experiments the PFN_N was higher ($P < 0.001$) in Manavert than in Aruba, however, PFN_N values were lower in Exp-2 than in Exp-1. The AE_N was lower ($P < 0.001$) in Aruba (on average 4.2 kg of DW kg^{-1} of N) than in Manavert (on average 9.3 kg of DW kg^{-1} of N) in Exp-1, whereas no differences were found in Exp-2, AE_N was on average

2.8 and 3.6 kg of DW kg^{-1} of N in Aruba and Manavert, respectively (Figure 6C). The PE_N ranged on average between 13 and 14 kg of DW per kg of increased N_{acc} , in both cultivars and experiments regardless the level of N supply, except for the cv Manavert that in Exp-1 had higher ($P < 0.001$) PE_N values with N_{60} (32.5 kg of DW kg^{-1} N) than with N_{120} (17.8 kg of DW kg^{-1} N) and N_{180} (16.4 kg of DW kg^{-1} N).

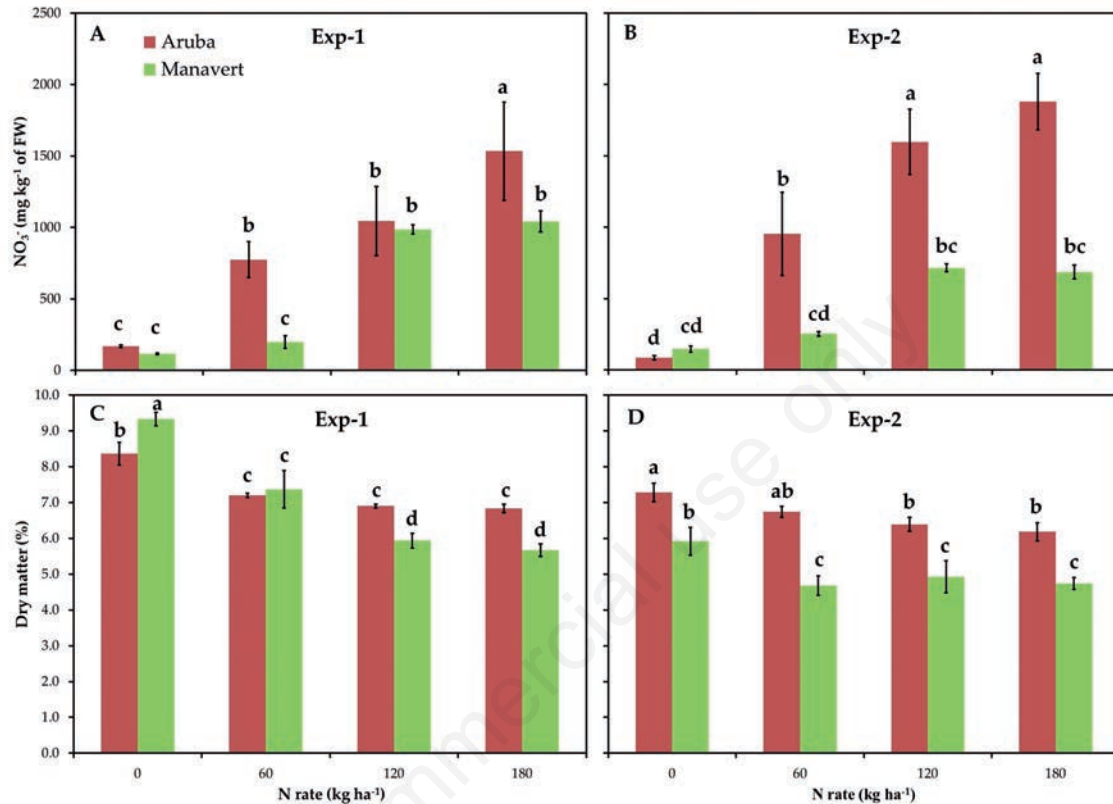


Figure 3. Effect of nitrogen fertiliser rate on nitrate accumulation (A and B) and dry matter content (C and D) observed at final harvest in cv Aruba (red oak-leaf) and cv Manavert (romaine), grown at Conversano and Polignano a Mare, during the fall-winter season of 2006-2007 (Exp-1) and 2007-2008 (Exp-2), respectively. Vertical bars represent \pm standard error ($n=3$). Different letters indicate significant differences at $P=0.05$.

Table 1. Parameter estimates, their significance, and adjusted R^2 for the function representing the fresh weight-, dry weight- and nitrogen uptake-nitrogen rate relationships in lettuce cv Aruba and Manavert grown with different levels of nitrogen supply in two Mediterranean locations in 2006-2007 and 2007-2008 fall-winter cropping seasons.

Experiment	Cultivar	Intercept ^o		Slope ^o		Quadratic coefficient ^o		Adjusted R^2 quadratic function	Adjusted R^2 linear function	
		Estimate	P	Estimate	P	Estimate	P			
FW-N rate relationship	Exp-1	Aruba	10.02	0.01	0.12	0.01	-0.00036	0.03	0.81	0.71
		Manavert	15.56	0.01	0.33	0.01	-0.00090	0.01	0.92	0.85
	Exp-2	Aruba	9.25	0.01	0.08	0.01	-0.00024	0.12	0.71	0.65
		Manavert	21.93	0.01	0.21	0.01	-0.00071	0.01	0.85	0.69
DW-N rate relationship	Exp-1	Aruba	0.83	0.01	0.006	0.01	-0.000020	0.07	0.72	0.63
		Manavert	1.49	0.01	0.015	0.01	-0.000053	0.01	0.83	0.66
	Exp-2	Aruba	0.67	0.01	0.005	0.04	-0.000015	0.17	0.51	0.44
		Manavert	1.27	0.01	0.006	0.04	-0.000019	0.21	0.55	0.51
N uptake-N rate relationship	Exp-1	Aruba	22.15	0.01	0.46	0.01	-0.00130	0.04	0.85	0.78
		Manavert	23.20	0.01	0.60	0.01	-0.00121	0.08	0.92	0.90
	Exp-2	Aruba	21.13	0.01	0.32	0.01	-0.00100	0.05	0.76	0.67
		Manavert	35.92	0.01	0.28	0.01	-0.00038	0.44	0.85	0.86

FW, fresh weight; N, nitrogen; DW, dry weight; Exp-1, field experiment 1 conducted on-farm at Conversano (40° 58' N, 17° 7' E; 140 m asl) during the fall-winter season of 2006-2007; Exp-2, field experiment 2 conducted on-farm at Polignano a Mare (40° 59' N, 17° 13' E; 40 m asl) during the fall-winter season of 2007-2008. ^oIntercepts, slopes and quadratic coefficients in italics are not significantly different from zero at the 5% level.

Calculated on FW basis PFN_N , AE_N and PE_N assumed a different trend as compared to the same indices calculated on DW basis: PFN_N , AE_N and PE_N values were consistently higher in Manavert than in Aruba in both experiments (Figure 6B, D, F).

Soil nitrate residual and nitrogen losses estimate

In Exp-1, at harvest, residual soil NO_3-N content in the top 30-cm of soil in plots of Manavert was not influenced by N rates and was on average 26.8 kg ha^{-1} (Figure 7A). In Exp-2, residual soil NO_3-N content was on average 15.0 kg ha^{-1} , ranging from 6.6 kg ha^{-1} in treatment N_{60} to 29.0 kg ha^{-1} in treatment N_{180} . Residual soil NO_3-N was higher ($P=0.003$) with N_{180} than with lower N rates (Figure 7A). In both experiments, estimated N losses from the top 30-cm of soil in plots of Manavert increased ($P<0.001$) with increasing the N rate (Figure 7B). Estimated N losses for treatments N_{60} , N_{120} and N_{180} were 32, 55 and 111 kg ha^{-1} in Exp-1, and 44, 83 and 125 kg ha^{-1} in Exp-2, respectively (Figure 7B).

Nitrogen nutrition index and relationship with nitrate content

Analysing the NNI time course during the crop cycle, for each level of N supply, it was observed that in both genotypes and experiments, the N status of plant grown at different N rates started to diverge at 60 DAT. After 60 DAT, the NNI was always lower than 1.0 (optimal N status) in unfertilised plants or in those grown at lower N rate (N_{60}), proving the occurrence of N deficiency. In plants grown at higher N rates (N_{120} and N_{180}) the NNI remained close to 1.0 during the entire crop cycle, and only at harvest, plants fertilised with N_{180} had NNI values above 1.0, revealing the occurrence of N luxury consumption (Figure 8). Based on the NNI time-course, in both cultivars and experiments, only the application of 120 kg ha^{-1} of N assured the maintenance of an optimal crop N status during the entire crop cycle, while N deficiency or luxury consumption were observed at lower and higher N rates, respectively.

Analysing the variation of the leaf NO_3-N ($\text{g } 100 \text{ g}^{-1}$ of DW) to total-N ($\text{g } 100 \text{ g}^{-1}$ of DW) ratio in relation to the NNI, in both cultivars and experiments, it was observed that the fraction of NO_3-

N in the leaves markedly increases with increasing the NNI (Figure 9A). Considering as optimal NNI values of one, with a tolerance of $\pm 15\%$, the fraction of NO_3-N ranged from 0.1 to 20% when the NNI was lower than 0.85 (N deficiency), from 14 up to 72% when the NNI was close to 1 (optimal N status) and was detected only one point at 55% of NO_3-N when the NNI was higher than 1.15 (N excess).

Examining the relationship between NNI and the leaf NO_3^- concentration (mg kg^{-1} of FW) of both cultivars (Figure 9B), it was observed that the leaf NO_3^- concentration ranged from 0 to 520 mg kg^{-1} of FW when the NNI was lower than 0.85, from 230 up to 2170 mg kg^{-1} of FW when the NNI was within the optimal interval ($0.85 < \text{NNI} < 1.15$), and the leaf NO_3^- concentration was about 1870 mg kg^{-1} of FW in the only point with value of NNI above 1.15.

Discussion

Agronomic and physiological response of lettuce to nitrogen input

The variability of the crop response to N supply, observed in this study (Figure 2), confirms the existence of a complex interaction between plant genotype and several environmental factors (Ozores-Hampton *et al.*, 2015; Di Gioia *et al.*, 2017), which makes the prediction of crop N fertiliser requirement a difficult task.

The most common method used to define the optimal crop N requirements is based on the estimate of the maximum yield, or the maximum economical return (Ozores-Hampton *et al.*, 2012), using response curves to increasing N rates as shown in Figure 2A and B. However, data collected from N rate studies can be fitted to several statistical models, and the choice of the most appropriate model in each particular cropping situation is not obvious (Cerrato and Blackmer, 1990; Bélanger *et al.*, 2000). In this study the quadratic terms were very low and in some cases not significantly different from zero. When in doubt, the adjusted coefficient of

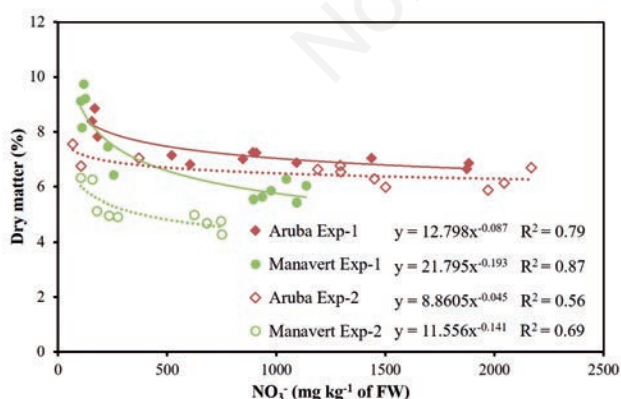


Figure 4. Variation of the dry matter content (%) in function of nitrate concentration in cv Manavert (*romaine*) and cv Aruba (*red oak-leaf*), grown at Conversano and Polignano a Mare, during the fall-winter season of 2006-2007 (Exp-1) and 2007-2008 (Exp-2), respectively. Regression lines are negative power functions representing the specific relationships between the two variables in both cultivars and experiments ($n=3$).

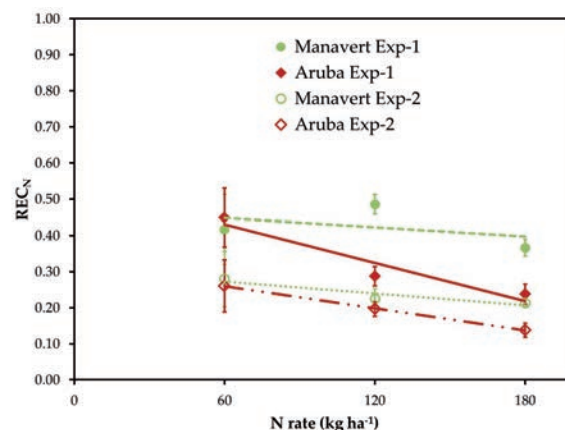


Figure 5. Effect of nitrogen fertiliser rate on apparent recovery of applied nitrogen at final harvest for cv Manavert (*romaine*) and cv Aruba (*red oak-leaf*), grown at Conversano and Polignano a Mare, during the fall-winter season of 2006-2007 (Exp-1) and 2007-2008 (Exp-2), respectively. Vertical bars represent \pm standard error ($n=3$).

determination ($AdjR^2$) was used as discriminant between different models. Nevertheless, different models with similar R^2 may give different optimal fertiliser rates (Cerrato and Blackmer, 1990). Moreover, response curves are usually quite flat around the maximum point (Burns, 2006), and quadratic models tend to overestimate the response, if the maximum point on the curve is taken as optimal fertiliser rate (Cerrato and Blackmer, 1990). Although simple, as observed in this study, the major limit of this method is the variability of the estimated optimal N fertiliser rate between years, seasons and locations. Such variability may be explained in part by the variability of the amount of N available in the soil, deriving from different sources, other than the fertiliser applications. Available N in the soil is in fact the result of a complex balance, in which inputs (N deriving from mineralisation) and outputs (leach-

ing, volatilisation) of N in the root-zone are determined by several variable and interacting factors (soil type, soil organic matter content, soil temperature, climatic conditions, microbiological fertility). Finally, comparing the N response of two different lettuce types, it was observed that despite the higher fresh yield and dry biomass produced by Manavert as compared to Aruba (Figure 2A and B), the optimal N rates estimated for the two cultivars using the yield curve-response method were similar. Moreover, in Exp-2 the estimated optimal N rate was higher in Aruba than in Manavert. These results further support the hypothesis that the quadratic model overestimates the optimal N rate (Cerrato and Blackmer, 1990). The lower response to N fertilisation of the aboveground DW biomass compared to the fresh biomass (Figure 2A and B) may be explained by the lower DM content observed in plants

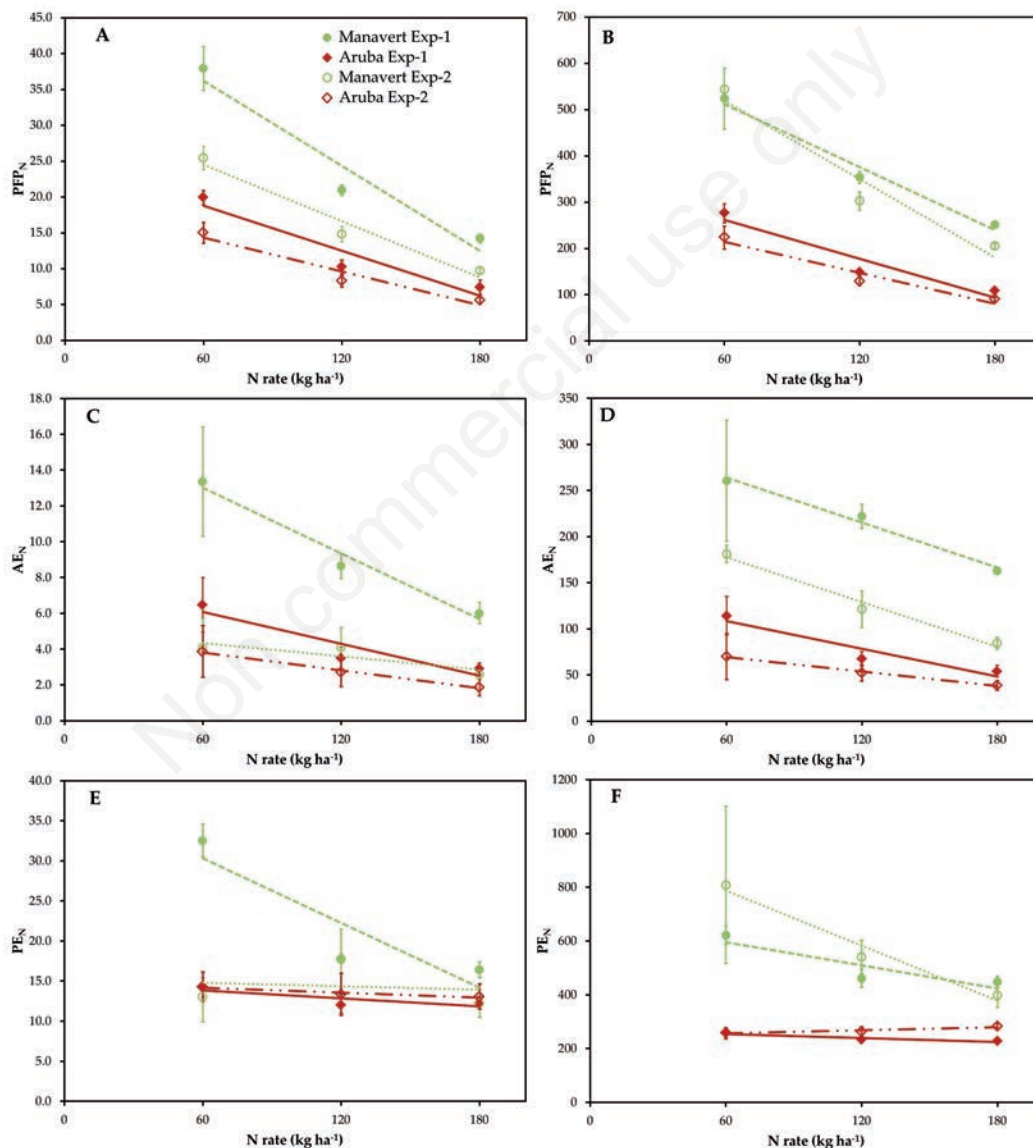


Figure 6. Effect of nitrogen fertiliser rate on partial factor productivity (A, B), agronomic efficiency (C, D), and physiological efficiency (E, F) of applied nitrogen, calculated on dry (A, C, E) and fresh (B, D, F) weight basis, at final harvest for cv Manavert (*romaine*) and cv Aruba (*red oak-leaf*), grown at Conversano and Polignano a Mare, during the fall-winter season of 2006-2007 (Exp-1) and 2007-2008 (Exp-2), respectively. Vertical bars represent \pm standard error ($n=3$).

grown at higher N rate (Figure 3C and D). Likely, the lower DM content of plants grown at higher N rate was determined by the higher nitrate accumulation. Nitrates, exerting an osmotic effect, increase the capacity of the plant to retain water, and cause a decrease of DM content, which is consistent with the negative relationship observed between nitrates and DM content, in both cultivars and experiments (Figure 4), as well as in other studies (Reinink *et al.*, 1987; Cárdenas-Navarro *et al.*, 1999). At the same time, the osmotic effect of nitrate can explain the positive relationship observed between nitrate content and fresh yield (data not shown).

As hypothesised by Tei *et al.* (2000), the nitrate accumulation in leafy vegetables is not useful to plant growth and dry biomass accumulation, and therefore it is considered the result of N luxury consumption. However, the nitrate accumulation may cause an increase in water content, and thus, in fresh biomass, which may be not important in cereal and pulse crops, harvested with a low water content, but it assumes a significant role in leafy vegetables like lettuce, which are harvested fresh. The results of this study were in agreement with the findings of other authors (Tei *et al.*, 2000; Dapigny *et al.*, 1996), who observed a relationship between plant N content and relative growth rate, only when both water contribution (fresh weight) in biomass and reduced-N (total-N minus nitrate-N) were taken into account.

These results should be duly considered in both, empirical and mechanistic crop models, currently proposed to predict crop growth, crop N uptake and crop water requirement (Cárdenas-Navarro *et al.*, 1999). The inverse relationship between nitrate and DM content was in part considered by Seginer *et al.* (2004) who modified the NICOLET model, used to predict the crop N uptake, on the assumption that because of the high nitrate-N content of lettuce, N uptake depends on plants water content.

From a quality perspective, although at harvest, leaf NO_3^- content significantly increased with increasing N rate, the levels of NO_3^- accumulated in the edible portion were always lower than maximum limits set by the EC Regulation No. 1258/2011 for lettuce grown in open field and harvested from October 1st to the end of March (4000 mg kg^{-1} of FW). The relatively low nitrate accumulation observed in both cultivars and experiments may be due to the high levels of solar radiation that characterise the Mediterranean area, even during the fall-winter season. In fact, high levels of solar radiation can substantially reduce the crop nitrate accumulation by enhancing the plant nitrate-reductase activity (Weightman *et al.*, 2012; Di Gioia *et al.*, 2013).

The higher leaf NO_3^- accumulation capacity consistently observed in Aruba (non-heading) as compared to Manavert (heading lettuce), for two years, despite N input, highlights the importance of the genotype in determining the level of leaf NO_3^- accumulation, and the possibility to select lettuce genotypes that accumulate less nitrates (Burns *et al.*, 2011).

Nitrogen recovery and utilisation efficiency of lettuce crops

The NUE of a crop may be considered as the product of two components: i) the N uptake efficiency (NupE), that expresses the ability of a genotype to acquire nitrogen from the soil; and ii) the N utilisation efficiency (NutE), that expresses the ability of a genotype to use N taken up to produce plant biomass. In this study, the N uptake efficiency expressed as REC_N was on average 37% and 22% in Exp-1 and Exp-2, respectively (Figure 5). According to Greenwood *et al.* (1989), REC_N should remain constant with increasing the N rate, until N supply exceeds the crop N demand,

after which REC_N should start decreasing with increasing the N rate. In both experiments, Manavert REC_N values remained constant with increasing the N rate from 60 to 180 kg ha^{-1} , while Aruba REC_N values decreased with increasing the N rate, suggesting that the two genotypes had different N requirements. The decline of REC_N observed in Aruba N_{180} compared to Aruba N_{60} in both experiments, indicates that the higher N rate tested exceeded the crop N demand. Therefore, in the case of Aruba N rate should not exceed 120 kg ha^{-1} . The same N rates did not exceed the N demand of Manavert, suggesting that applications of N within the range 120-180 kg ha^{-1} may be adequate for Manavert.

Values of REC_N observed in these experiments were similar to those observed on lettuce in previous studies (Greenwood *et al.*, 1989; Tei *et al.*, 1999, 2000), however, were lower than those observed by Karam *et al.* (2002), confirming that lettuce crops are characterised by a low efficiency in recovering applied N.

Manavert was more efficient than Aruba also in terms of NutE, as expressed by the indices PPFN , AE_N and PEN , calculated both on dry (Figure 6A, C, E) and fresh weight basis (Figure 6B, D, F).

Proposed primarily to evaluate the efficiency of grain and pulse crops characterised by low water content, NutE indices are usually calculated on DW basis, also to enable the comparison between different species and environments. Nevertheless, the

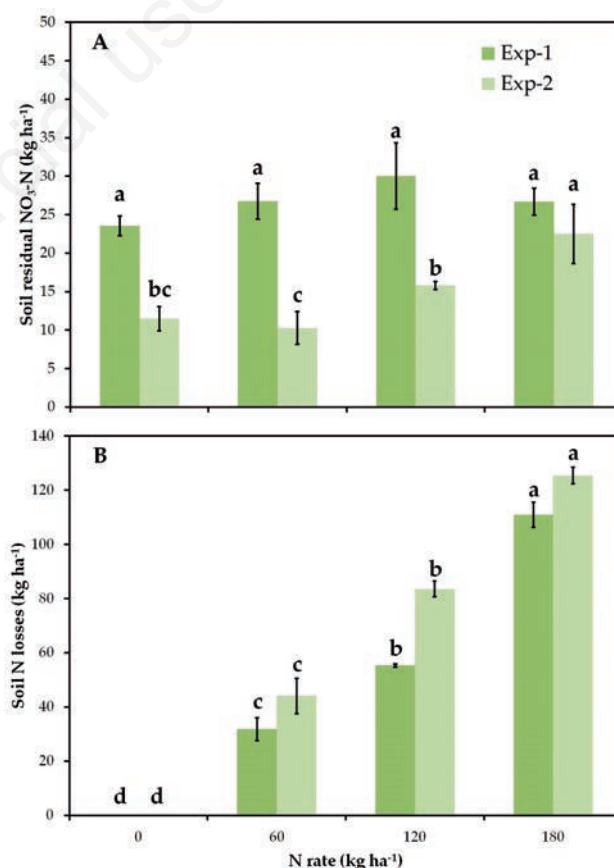


Figure 7. Effect of nitrogen rate on residual soil nitrate-nitrogen (A) (in the top 30 cm) and apparent soil nitrogen losses (B) determined at harvest in plots of the cv Manavert (*romaine*), grown at Conversano and Polignano a Mare during the fall-winter season of 2006-2007 (Exp-1) and 2007-2008 (Exp-2), respectively. Vertical bars represent standard error (n=3). Different letters within each experiment indicate significant differences at P=0.05.

results of this study suggest that for lettuce crops, characterised by relatively high water content, considering that the N rate may affect the water content, it may be meaningful to express the NutE indices also on FW basis.

As expected, PPF_N , which represents the yield per unit of N fertiliser applied, significantly decreased with increasing N fertiliser rate, and was higher in Manavert than in Aruba. Observed PPF_N values were comparable to those reported by Conversa *et al.* (2004) on hydroponically-grown butterhead lettuce. The scarce influence of N rate on AE_N in both cultivars and experiments, was mainly due to the high variability observed between replications at N_{60} (Figure 6C), which may be explained by the fact that at lower N rates, crop yield performances are more susceptible to variations depending on the amount of N available in the soil, deriving from alternative sources, other than the fertiliser. The PE_N which represents the ability of a plant to transform N acquired from fertiliser into yield, and depends mostly on the genotype characteristics and only in part on environmental factors (Dobermann, 2005), was not influenced by N supply, either when expressed in terms of DW and FW basis, except in the case of Manavert N_{60} in Exp-1. The higher PE_N value observed in Exp-1 in plants of Manavert grown at lower N rate (N_{60}) in comparison to those grown at higher N rates, was due to environmental factors rather than to genotype traits, as demonstrated by the variability observed between replications for

both DW and N_{acc} in treatment N_{60} .

Values of NutE estimated in this study were lower than those observed from other authors in cereal crops (Cassman *et al.*, 2002; Dobermann, 2005), while were comparable to those observed by López-Bellido *et al.* (2005) on rainfed wheat under Mediterranean environment.

Soil nitrogen losses estimate

At the end of the crop cycle, although some residual NO_3-N was found in the top 30-cm of soil in both experiments, estimated N loss from the same soil layer were high, and in treatment N_{180} reached 111 and 125 $kg\ ha^{-1}$ of N in Exp-1 and Exp-2, respectively, further proving the low N recovery efficiency of lettuce crops (Figure 7). Moreover, in absence of a catch crop, also soil residual NO_3-N may be subject to environmental losses.

The variability of climatic conditions, soil texture, biological and chemical properties makes it difficult any kind of comparison with other situations; however, these results may be considered normal for lettuce crops, and suggest that under the specific conditions, the mineralisation of the soil organic matter may provide substantial amounts of N for the crop. Therefore, to improve crop NUE and minimise the risks of N losses, the amount of N potentially deriving from mineralisation process should be accounted in the fertilisation programme.

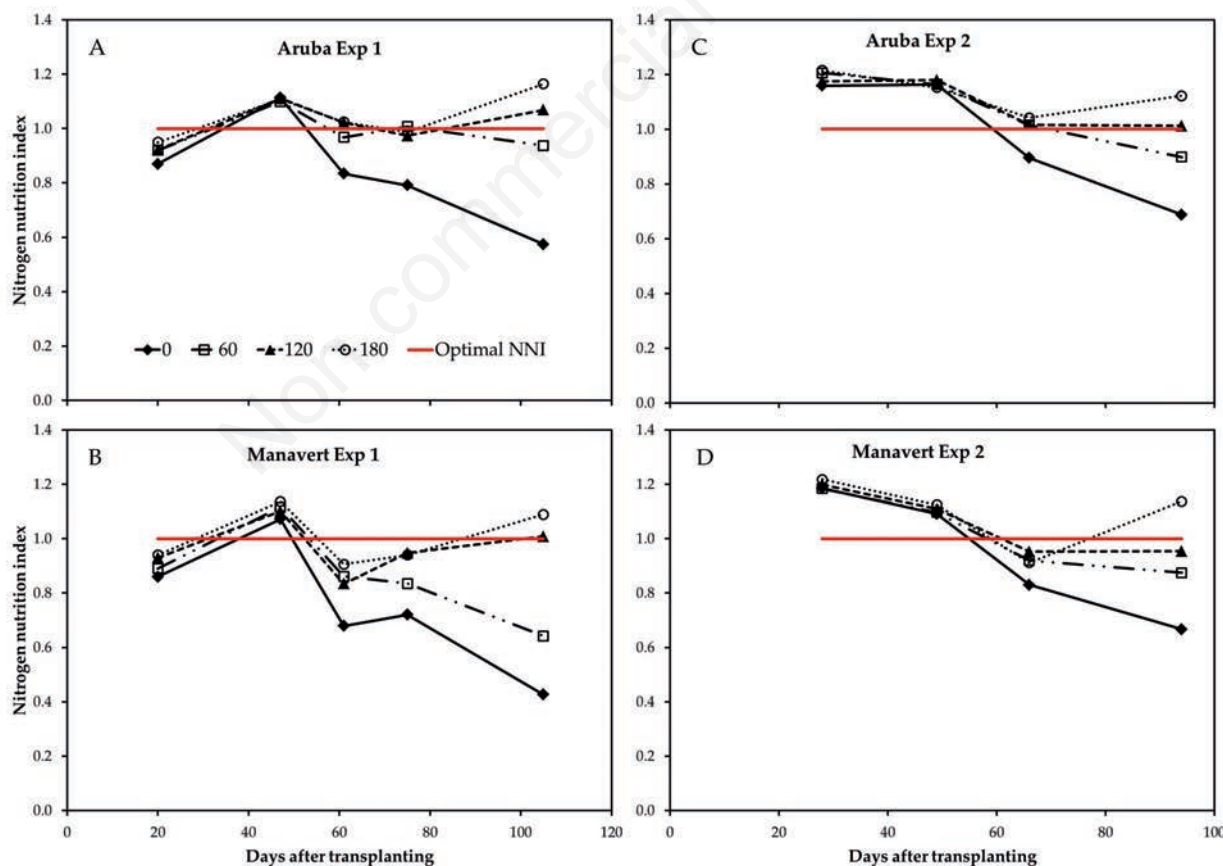


Figure 8. Effect of nitrogen rate on the nitrogen nutrition index time course in cv Aruba (A, C) and cv Manavert (B, D) grown at Conversano and Polignano a Mare during the fall-winter season of 2006-2007 (Exp-1) and 2007-2008 (Exp-2), respectively. Nitrogen nutrition index was calculated according to Lemaire *et al.* (1989) using the critical nitrogen dilution curve proposed by Tei *et al.* (2003) for lettuce. Standard error bars were smaller than the symbols used in the graphs ($n=3$).

Nitrogen nutrition index and nitrate content as indicators of the crop nitrogen status

The NNI represents an indicator of the crop N status and may be used as a guide to optimise the N fertilisation programme in terms of timing and N application rate, enabling the adjustment of N fertiliser applications to N demand and corresponding target yield, in any moment of the crop growth cycle (Lemaire *et al.*, 2008).

Analysing *a posteriori* the NNI time course of a crop is possible to define when N-deficiency and/or N-excess occurred, and thus, when and how much N fertiliser should be applied to the crop. In this study, the NNI analysis revealed that in both cultivars and experiments, only the application of 120 kg ha⁻¹ of N ensured the maintenance of an optimal crop N status during the entire crop cycle, while N deficiency or excess were observed at lower and at higher N rates, respectively. These results further confirm that yield N response quadratic models overestimate the optimal N rates. Examining the NNI pattern, it was observed that at lower N rates, N deficiency occurred only after 60 DAT in correspondence of the maximum crop growth rate (Figure 9); while, N excess occurred at the highest N rate, either at the beginning of the crop cycle in Exp-2, or at the end of the crop cycle. These results highlight the importance of splitting N fertiliser applications to avoid both N excess and deficiency.

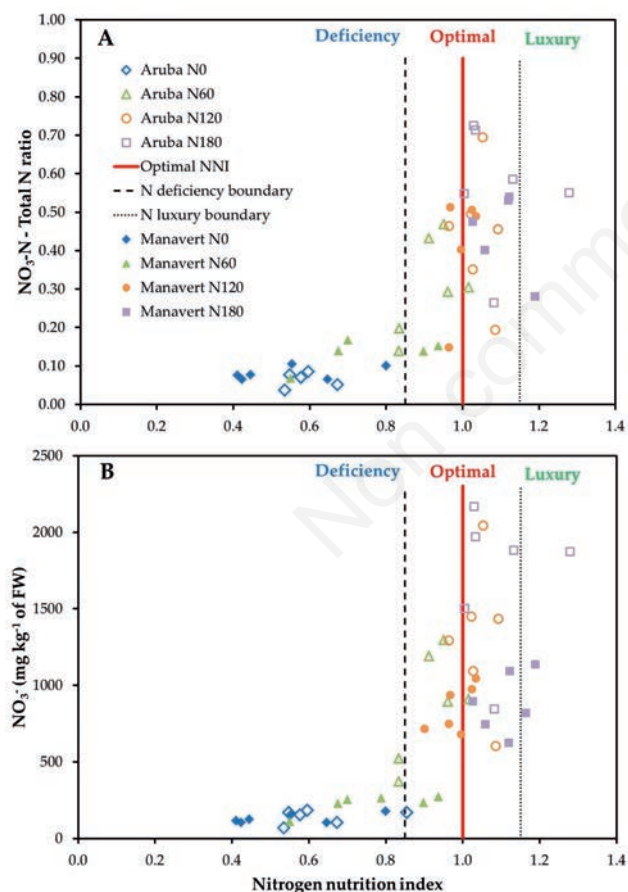


Figure 9. Relationship between nitrogen nutrition index and plant nitrate-nitrogen to total-nitrogen ratio (A) and between nitrogen nutrition index and nitrate concentration (B) in cv Aruba and Manavert, grown with different nitrogen rates, at Conversano and Polignano a Mare during the fall-winter season of 2006-2007 (Exp-1) and 2007-2008 (Exp-2), respectively.

Although the NNI is an established and reliable indicator of the crop N status, its determination is time consuming and requires laboratory equipment and expertise that farmers usually do not have. For this reason, it is necessary to develop methods to indirectly estimate the NNI (Lemaire *et al.*, 2008). Pooling the data of the two experiments and lettuce genotypes, an empirical relationship was found between leaf NO₃-N to total-N ratio and NNI (Figure 9A), and an equivalent relationship was observed between NNI and leaf NO₃⁻ concentration expressed on FW basis (mg kg⁻¹ of FW; Figure 9B). Although the equivalence of the two relationships may appear obvious, it is of fundamental importance for the practical application of NNI as a diagnostic tool of the crop N status. In fact, while it is difficult to analyse the fraction of NO₃-N on the total-N, the leaf NO₃⁻ concentration, may be easily determined on-farm, directly by growers, using ion selective electrodes or other rapid methods (Di Gioia *et al.*, 2010; Peña-Fleitas *et al.*, 2015).

Despite the great variability of the NO₃-N to total-N ratio and leaf NO₃⁻ concentration for a given N level, as observed also by Justes *et al.* (1994), examining the variation of the leaf NO₃⁻ concentration in relation to the NNI, it was possible to associate N deficiency or N excess crop status to specific leaf NO₃⁻ concentration levels for both cultivars. In both cultivars and experiments, leaf NO₃⁻ values below 500 mg kg⁻¹ of FW were consistently associated to N deficiency, suggesting that to assure an optimal N status the leaf NO₃⁻ content should be above this threshold. Moreover, considering as optimal the status of plants grown at the rate of 120 kg ha⁻¹ of N, to assure an optimal N status with a tolerance of ±15%, the leaf NO₃⁻ concentration should range between 600 and 1000 mg kg⁻¹ of FW in Manavert, and between 600 and 1500 mg kg⁻¹ of FW in Aruba, which showed a tendency to accumulate more NO₃⁻.

These results suggest that, although empirical, the relationship between leaf NO₃⁻ content and NNI may be used to define the leaf NO₃⁻ range associated to an optimal N status, or threshold limits to avoid N deficiency and/or luxury consumption for specific crops and growth stages. Developing these specific leaf NO₃⁻ concentration thresholds for different crops and using rapid and low cost methods such as NO₃⁻ ion-selective electrodes, growers may be able to monitor directly the nutritional status of their crops and dynamically adjust the N fertilisation programme according to the actual crop demand.

Conclusions

Fresh and dry biomass of *romaine* (cv Manavert) and *red oak-leaf* (cv Aruba) lettuce varied in response to N inputs according to a quadratic model in both fall-winter seasons. Nevertheless, the response varied from year to year and was different for the two lettuce genotypes. Manavert had higher N recovery and utilisation efficiency as compared to Aruba, suggesting that heading lettuce genotypes are more efficient than non-heading lettuce types. Therefore, NUE may be substantially improved by selecting more efficient lettuce genotypes.

Plants grown at higher N rates accumulated more NO₃⁻ in the leaves and a negative relationship was observed between leaf NO₃⁻ and DM content. Such interesting relationship characterise lettuce from other crops harvested at relatively higher DM content, and should be properly considered in the N fertilisation management.

The analysis of the NNI time course of the two genotypes revealed that only the application of 120 kg ha⁻¹ of N assured an

optimal crop N status, while N deficiency and excess were observed at lower and higher N rates, respectively. An empirical relationship was found between leaf NO_3^- concentration and NNI, which may be used to define optimal leaf NO_3^- ranges for specific lettuce crops and growth stages. Using the NNI- NO_3^- relationship growers may be able to predict and indirectly monitor the NNI, and the crop N status, on *site-specific* basis, by measuring the leaf NO_3^- concentration with simple rapid on-farm tests.

Combining the selection of more efficient lettuce genotypes with the dynamic and site-specific adjustment of the N fertilisation programme based on the actual crop N status may contribute to improve the crop NUE, minimise the risks of N losses and the crop environmental impact, while maintaining high yields and quality standards.

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