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Effect of different levels of nitrogen fertilizer on morphological and physiological parameters and nitrates accumulation of lettuce cultivars (*Lactuca sativa* L.)

M. M'hamdi*, I. Boughattas, H. Chikh-Rouhou, E. Souhli, T. Bettaieb

Higher Institute of Agronomic Sciences of Chott-Mariem. P.O. Box 47 4042 Chott-Mariem. Tunisia
* Corresponding Author email : mhamdimahmoud@yahoo.fr

The effect of nitrogen fertilizer level on growth and nitrate accumulation was studied in six lettuce cultivars (*Lactuca sativa* L.): 'Batavia rouge', 'Vitalia', 'Great Laks', 'Type Beurre', 'Romaine' and 'Romaine LO3'. Three nitrogen levels: 0, 120 and 240 Kg.ha^{-1} was applied. During plant growth, agronomical parameters (leaves weight, root weight, dry matter, head diameter) and physiological parameters (nitrate concentration, chlorophyll fluorescence and sugar content) were evaluated. Results showed significant differences between cultivars and nitrogen treatment for the most agronomical and physiological parameters. The nitrogen treatment affects head weight and nitrate concentration in all cultivars; the cvs 'Great Laks' and 'Type beurre' accumulated respectively the less and high nitrate concentration. The highest nitrate concentration was recorded in external leaves whereas the lowest concentration was recorded in the central leaves for all cultivars. Furthermore, nitrate distribution and chlorophyll fluorescence on the leaves are closely related. This study revealed also correlation between root nitrate concentration, chlorophyll fluorescence and sugar content for all cultivars. This correlation depended on nitrogen fertilization level and the cultivar used.

Key words: Fertilizer, Lettuce, Nitrate, Nitrogen.

Lettuce (*Lactuca sativa* L.) is an important leafy vegetable crop and it is considered as an excellent nutritive source of minerals and vitamins since it is consumed as fresh green salad (Abu-Rayyan et al., 2004). It is the most popular vegetable according to the highest consumption rate and economic importance through the world (Coelho et al., 2005). This vegetable requires a high rate of nitrogen which is the most important nutrients affecting plant growth and yield. Nitrate is often the major source of nitrogen available to higher plants (Marschner, 1995). However, high levels of nitrates in the nutrient solution can leads to an accumulation of nitrates in leaves which can be harmful for human health (Santamaria, 2006); because edible parts contain very high concentrations of nitrate that has been implicated in the occurrence of methaemoglobinemia and possibly in gastric cancer as well as other diseases (Walker, 1990; Du et al., 2007; Thomas and Chan, 2011). Furthermore, nitrate, not taken up by a crop, may potentially contribute to ground and surface water pollution through nitrate leaching and soil erosion (Gastal and Lemaire, 2002; Wang et al., 2002).

Nitrate accumulation in plants is a natural phenomenon resulting from uptake of the nitrate ion in excess of its reduction and subsequent assimilation (Maynard *et al.*, 1976). It has been shown to be affected by the form and level of nitrogen fertilizer (Maynard et al., 1976; Scaife et al., 1986; Hanafy et al., 2012.; Kostantopulou et al., 2012), the timing of nitrogen fertilizer release (Tesi and Lenzi, 1998), the light intensity and duration (Maynard et al., 1976; Chadjaa et al., 1999) and lettuce type and cultivar (Reinink and Eenink, 1988; Behr and Wiebe, 1992). In addition, nitrate concentration differs in plant parts (Maynard et al., 1976; Santamaria, 2006). In most types of lettuce, the highest concentration of nitrates is normally observed in the external leaves (Marsic and Osvald, 2002; Abu Rayyan et al., 2004; Kostantopulou et al., 2010).

The present study was conducted in order to determine the effect of nitrogen treatment on (i) some agronomical parameters such as fresh and dry matter, root weight, leaves size and head diameter (ii) physiological parameters: Chlorophyll fluorescence, sugar and nitrate content and its distribution in leaves.

Materiel and methods

Plant material and growth conditions

The experiment was carried out on 6 lettuce cultivars 'Vitalia', 'Batavia rouge', 'Great Laks', 'Romaine', 'Type Beurre' and 'Romaine LO3'. The seeds were sown on 24 January 2012 and were transplanted at three to five true-leaf stages.

The experimental design was a split plot fitted to randomized complete block. The treatments included three nitrogen rates (N0=0, N1=120, N2=240 kg N ha^{-1}) as the main plot and six lettuce cultivars as the sub-plot.

Analytical methods

Just after harvest, fresh weight and diameters of heads were determined. Five external leaves from each head were removed and their chlorophyll fluorescence, nitrate and sugar content were determined. Roots were also washed and its fresh weight and nitrate content were measured.

Nitrate measurement was taken by Twin NO3- Meter. Each leaf is crushed in a mortar and the juice collected is put into the device and then the value is displayed on the screen. Chlorophyll florescence was determined by CM-1000 chlorophyll Meter. Total soluble sugar was determined with the method of Dubois et al. (1956). Dry matter was determined after drying in an oven at 80°C.

Statistical analysis

Data were subjected to multivariate analysis using General Linear Model (GLM) procedure from SPSS 17.0 including two fixed factors (cultivar and nitrogen level). Correlation analysis between nitrate concentration of roots and leaves and the agronomic and physiological parameter were determined.

Results

Agronomic parameters

Fresh weight of lettuce head

The effect of nitrogen fertilizer level on leaves fresh weight was significant only for 'Vitalia' and 'Batavia rouge' cvs. However, the cultivars effect was highly significant. The lowest leaf fresh weight (405 g) was obtained for 'Type beurre' at N2 level and the greatest value (1833 g) for 'Great Laks' at N1 level. Increasing nitrogen fertilizer up to 240 Kg ha^{-1} decreased fresh weight of all cultivars except 'Vitalia' (Figure 1).

Diameter of lettuce head

Nitrogen fertilizer level significantly affected lettuce diameter. Increasing the nitrogen level from 0 to 120 Kg ha^{-1}, increased the 'Romaine LO3' and 'Vitalia' diameters, contrary to the other cultivars whose diameter decreased. With N2 treatment, all cultivars diameters decreased. Also, with NO treatment 'Batavia rouge', 'Great laks' and 'Romaine' showed the greatest diameter compared to the other nitrogen level (Figure 2).

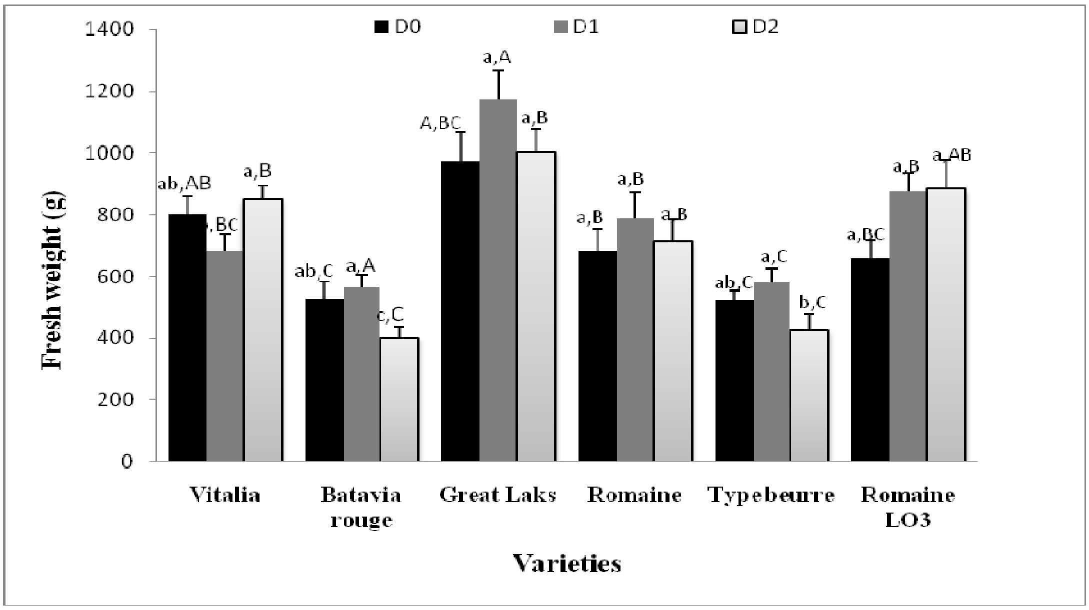

Fig.1. Effect of nitrogen level on lettuce fresh weight. Means followed by the same letters are not significantly different (P<0.05) according to Duncan test. The first letter indicates the effects of nitrogen level within the same variety and the second varietal differences within the same dose.

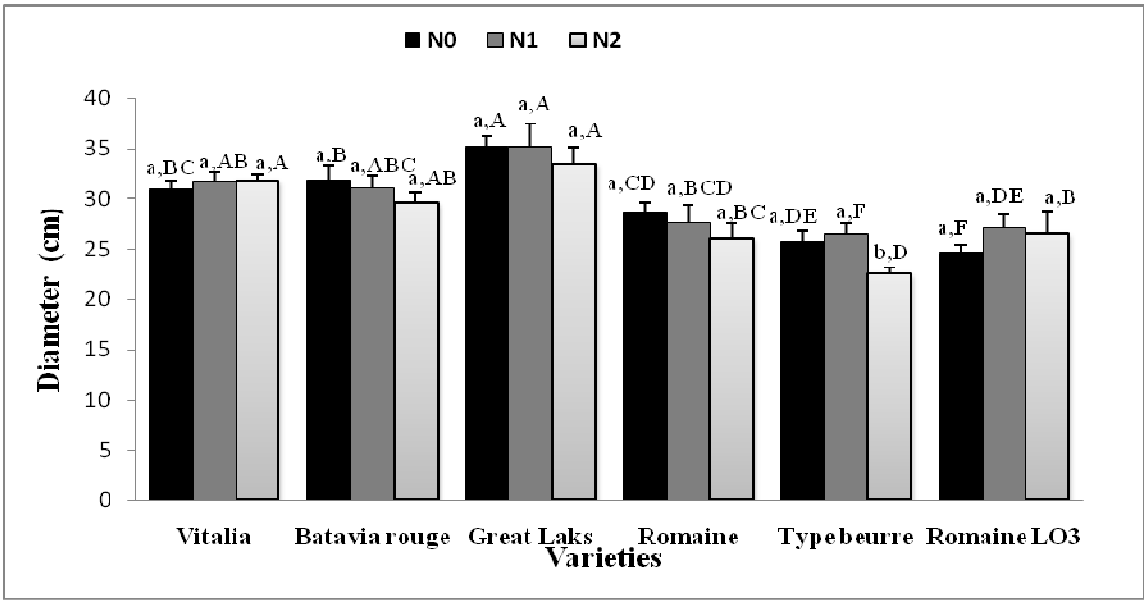

Fig.2. Effect of nitrogen level on lettuce diameter. Means followed by the same letters are not significantly different (P<0.05) according to Duncan test. The first letter indicates the effects of nitrogen level within the same variety and the second varietal differences within the same dose.

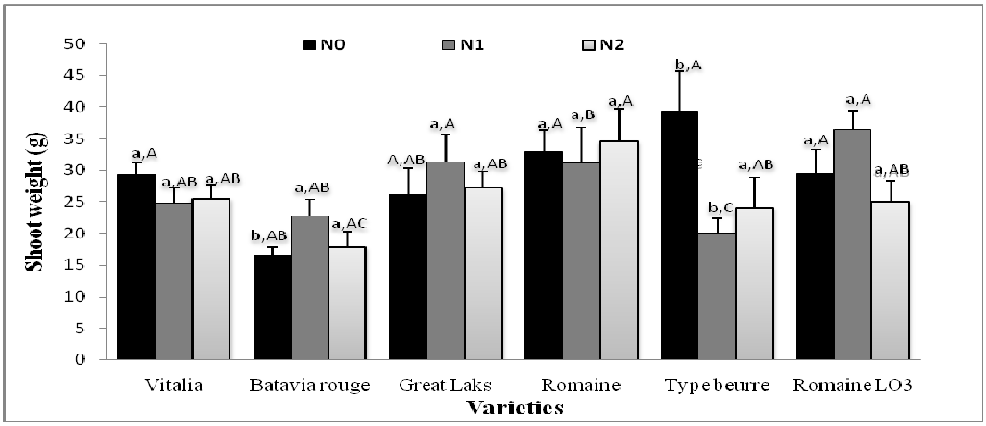

Fig .3. Effect of different nitrogen rate on root weight of lettuce varieties. Means followed by the same letters are not significantly different (P<0.05) according to Duncan test. The first letter indicates the effects of nitrogen level within the same variety and the second varietal differences within the same dose.

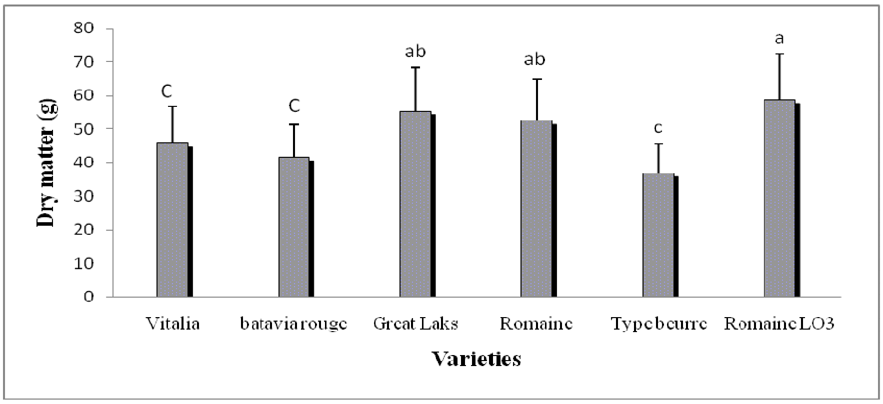

Fig.4. Dry matter content of six lettuce cultivars. Means followed by the same letters are not significantly different (P<0.05) according to Duncan test.

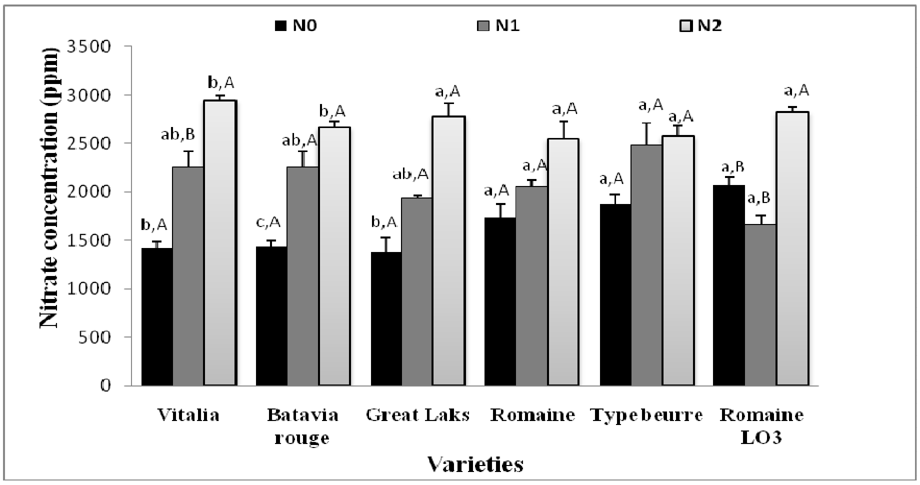

Fig.5. Effect of different nitrogen levels on nitrate accumulation in 6 lettuce cultivar. Means followed by the same letters are not significantly different (P<0.05) according to Duncan test. The first letter indicates the effects of nitrogen level within the same variety and the second varietal differences within the same dose.

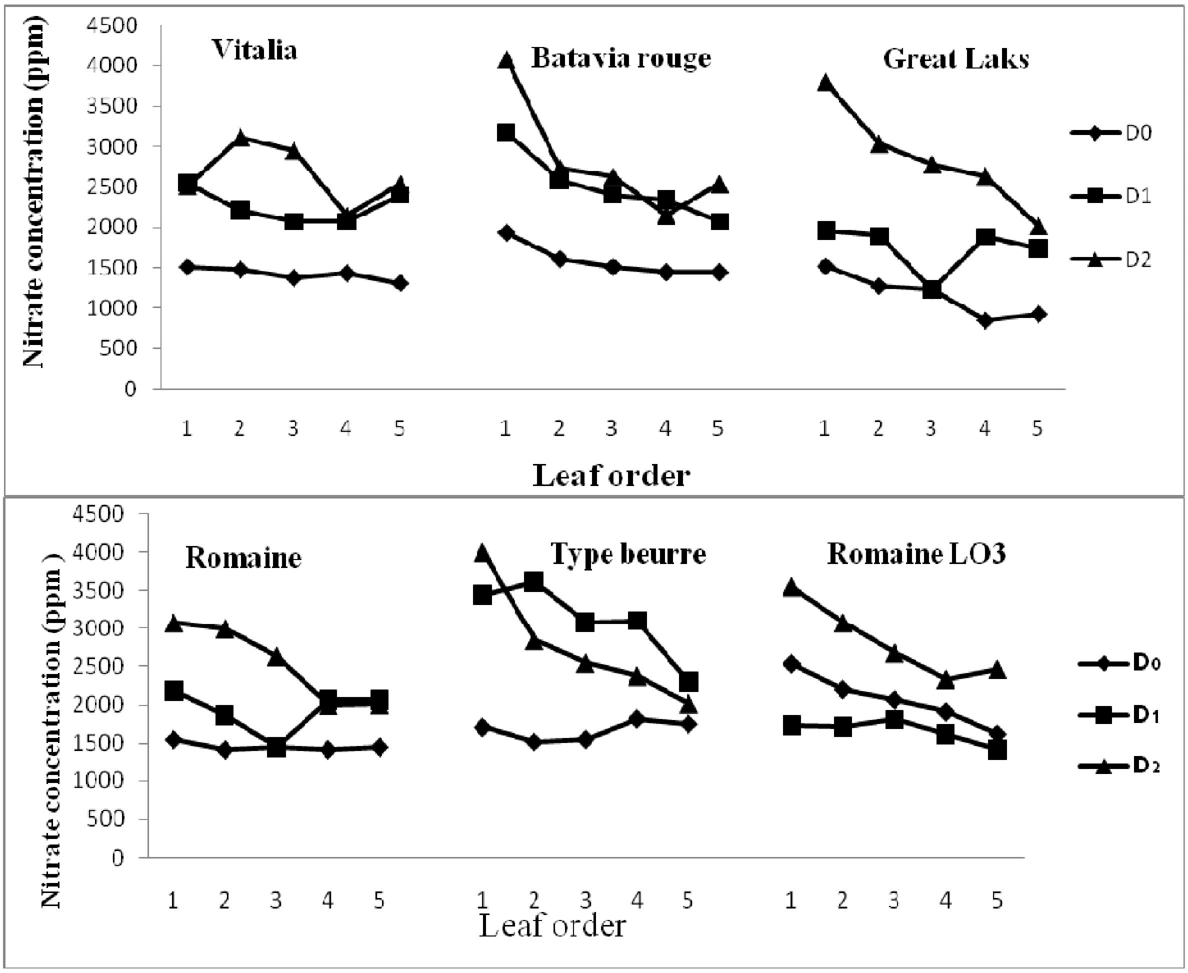

Fig.6. Nitrate distribution in the first five leaves in six lettuce varieties.

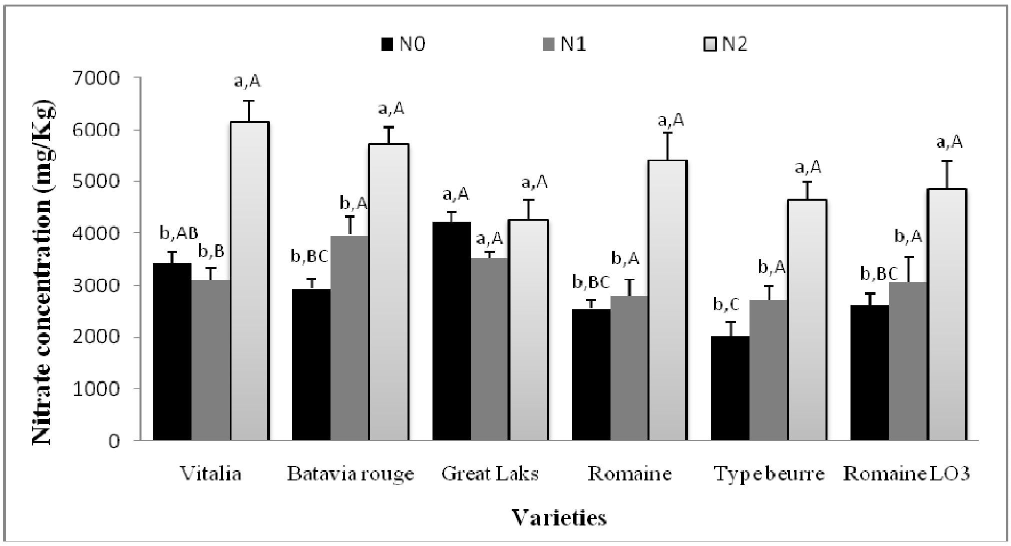

Fig .7. Effect of nitrogen level on nitrate concentration of roots. Means followed by the same letters are not significantly different (P<0.05) according to Duncan test. The first letter indicates the effects of nitrogen level within the same variety and the second varietal differences within the same dose.

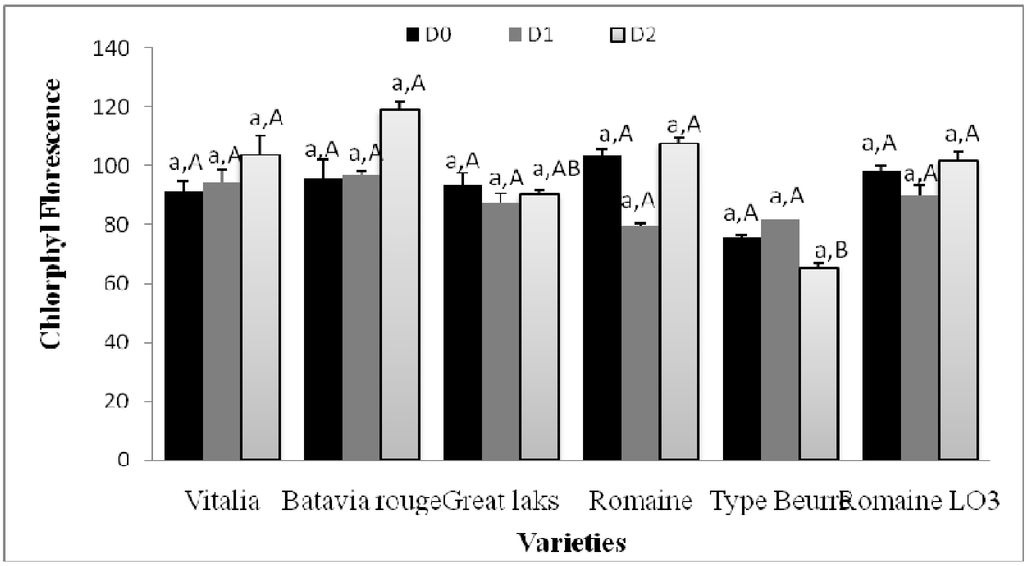

Fig .8. Effect of nitrogen level on Chlorophyll fluorescence. Means followed by the same letters are not significantly different (P<0.05) according to Duncan test. The first letter indicates the effects of nitrogen level within the same variety and the second varietal differences within the same dose.

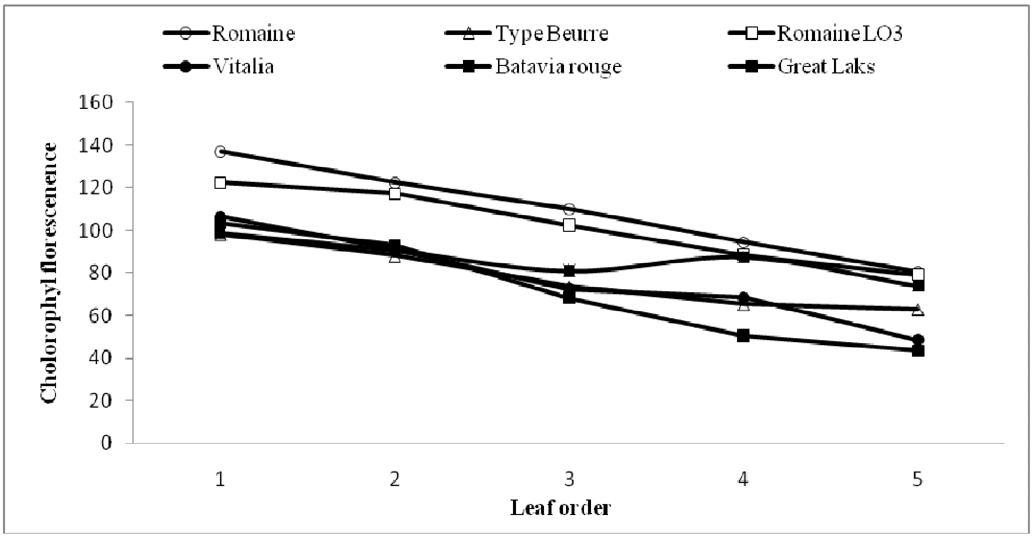

Fig.9. Chlorophyll fluorescence distribution in the first five leaves of six lettuce varieties

Table 1. Statistical analysis of the interaction between variety and nitrogen level in lettuce variety for agronomic parameters

	Fresh weight	Dry weight	Shoot fresh weight	Diameter
Var	S	S	S	S
N	S	NS	NS	NS
Var * N	S	NS	S	S

Var: lettuce variety; N: nitrogen fertilization level; S: difference statistically significant (P < 0.05); NS: difference statistically no significant (P<0.05)

Table 2. Statistical analysis of single effect and interaction between varieties, nitrogen level and leaf order in lettuce variety for physiological parameters

	Nitrate leaves	Nitrate roots	Fluorescence	Sugar content
Var	S	S	S	NS
N	S	S	NS	NS
Var *N	S	NS	S	
Leaf order	S		S	NS
Var* N* Leaf order	S		NS	NS

Var: lettuce variety; N: nitrogen fertilizer level; S: difference statistically significant (P<0.05); NS: difference statistically no significant (P<0.05)

Table 3. Total sugars content (mg / g FW) in 6 lettuce varieties

Varieties	Sugar content
Vitalia	957.67 a* ± 0,073
Batavia rouge	819.68 b ± 0,009
Great Laks	825.22 b ± 0,013
Romaine	832.09 b ± 0,023
Type Beurre	830.75 b ± 1,014
Romaine LO3	828.10 b ± 0,009

* Means followed by the same letters did not differ significantly (P<0.05) according to Duncan test.

Root weight

Root weight of 'Batavia rouge' was the lowest in N0 and N2 levels, contrary to 'type beurre' and 'Romaine' which showed the greatest root weight in N0 and N2 levels, respectively. The root weight, in N0 level, was higher for all cultivars (Figure 3). Statistical analyses showed significant effect of the interaction cultivar * nitrogen level (Table 1).

Dry matter

Nitrogen level doesn't affect significantly the dry matter. 'Great Laks' showed the driest matter content contrary for 'Type beurre' which had the lowest value (Figure 4).

Physiological parameters

Nitrate concentration of leaves

The increase in nitrogen level from 0 to 240 Kg ha^{-1} resulted in a significant increase in the nitrate concentration for all the cultivars except 'Romaine LO3'. However, statistical analyses didn't show any significant difference between cultivars with the different nitrogen levels. The lowest (1421.77 mg Kg^{-1}) and greatest (2944.44 mg Kg^{-1}) leaf nitrates concentration were obtained with 'Vitalia' respectively with N0 and N2 levels. With N0 and N1 treatments, nitrate concentration of all cultivars was lower than the maximum acceptable level for human consumption. In contrast, with N2 level the legislative levels were exceeded by all cultivars (Figure 5).

Nitrates distribution on lettuce leaves

Statistical analysis showed significant difference in the distribution of nitrate throughout the leaves. The highest nitrate concentration was recorded in external leaves whereas the lowest in the central leaves for all the cultivars and with the three nitrogen treatments (Figure 6).

Nitrate concentration of roots

Our results showed significant differences between all cultivars and not nitrogen treatment. The greatest and lowest nitrate shoot concentration was obtained with 'Vitalia' and 'Type beurre' with N2 and N0 treatment, respectively (Figure 7).

Chlorophyll fluorescence

The effect of cultivars and the interaction between cultivar and nitrogen level on the chlorophyll fluorescence was significant. The different nitrogen levels didn't affect significantly this parameter. Regardless the fertilizer rate, 'Batavia rouge' showed the highest chlorophyll fluorescence whereas 'type beurre' showed the lowest chlorophyll fluorescence with N0 and N2 (Figure 8). Also, the variation of chlorophyll fluorescence between the leaves showed that regardless the nitrogen treatment and cultivar, the greatest value is obtained with the first leaf and thereafter decrease in the other inner leaves (Figure 9).

Table 4. Pearson Correlation between nitrate concentration of leaves and roots, agronomic and physiological parameters of 6 lettuce varieties.

			NF	PF	PS	F	S	PFR	NR
Vitalia	N0	NF	1	-0,129	-0,332	0,409	0,337	0,093	0,163
		NR	0,163	-0,120	0,059	-0,130	-0,148	0,396	1
	N1	NF	1	-0,116	-0,224	-0,116	-,0361	-0,585*	-0,235
		NR	0,235	0,338	0,124	0,338	0,171	-0,360	1
	N2	NF	1	0,253	0,364	-0,899**	0,811**	0,364	-0,282
		NR	-0,282	-0,115	0,063	0,286	-0,185	0,063	1
Batavia rouge	N0	NF	1	-0,262	-0,319	0,955**	0,198	0,154	0,051
		NR	0,051	-0,075	0,061	-0,055	0,355	-0,289	1
	N1	NF	1	-0,012	0,236	0,622**	0,465	-0,084	0,134
		NR	0,134	-0,438	-0,036	-0,078	0,244	-0,198	1
	N2	NF	1	-0,290	-0,198	-0,900**	-0,418	0,362	-0,161
		NR	-0,161	-0,035	-0,346	0,353	-0,365	-0,270	1
Great Laks	N0	NF	1	-0,330	-0,336	0,862**	-0,622**	-0,388	-0,054
		NR	-0,054	0,205	0,044	-0,161	0,211	0,038	1
	N1	NF	1	-0,094	-0,085	-0,316	-0,994**	0,307	0,286
		NR	0,286	0,235	-0,100	-0,074	-0,287	0,156	1
	N2	NF	1	-0,117	-0,103	0,873**	0,646**	-0,200	-0,095
		NR	-0,095	-0,490*	-0,248	0,022	0,103	-0,235	1
Romaine	N0	NF	1	0,052	0,328	0,710**	-0,988**	0,425	0,187
		NR	0,187	0,370	0,035	0,038	-0,164	-0,208	1
	N1	NF	1	-0,216	-0,202	0,990**	-0,627**	-0,180	-0,080
		NR	-0,080	0,160	-0,243	-0,095	-0,034	-0,439	1
	N2	NF	1	0,212	-0,128	-0,987**	0,080	0,162	0,021
		NR	0,021	-0,532*	-0,135	0,017	0,234	-0,134	1
Type Beurre	N0	NF	1	-0,319	0,296	0,546*	0,222	-0,141	-0,177
		NR	-0,177	-0,179	0,134	0,215	-0,016	0,270	1
	N1	NF	1	-0,229	0,185	-0,376	-0,406	0,113	-0,191
		NR	-0,191	0,398	0,025	0,249	-0,165	0,294	1
	N2	NF	1	-0,396	-0,348	0,742**	-0,679**	-0,330	-0,384
		NR	-0,384	0,419	0,642**	-0,495*	0,491*	0,299	1
Romaine LO3	N0	NF	1	0,223	-0,087	0 ,2	0,975**	0,072	0,249
		NR	,249	,147	,213	-,661**	,076	-0,068	1
	N1	NF	1	-0,403	-0,138	-0,440	-0,665**	-0,137	-0,053
		NR	-,053	-,308	-,264	,025	,037	-0,056	1
	N2	NF	1	0,109	-0,124	0,980**	0,949**	0,279	0,109
		NR	0,109	-0,280	-0,609**	0,132	0,144	0,328	1

NO: 0 Kg ha $^{-1}$, N1: 120 Kg ha $^{-1}$, N2: 240 Kg ha $^{-1}$, NF: Nitrate concentration at leaves, NR: Nitrate concentration of shoot, PF: fresh weight, PS: dry weight, F: Chlorophyll fluorescence, S: sugar content, PFR: fresh weight of shoots. * Statistically significant at p= 0.01, ** Statistically significant at p= 0001.

Sugar content

Different levels of nitrogen didn't affect significantly the sugar content of all cultivars (Table 2). Independently of nitrogen level, 'Vitalia' cultivar showed the most sugar concentration (Table 3).

Correlations between nitrate concentration and agronomic and physiologic parameters

We found a significant correlation between nitrate concentration of leaves and chlorophyll fluorescence and sugar content (Table 4). These correlations varied positively or negatively and depend on nitrogen level and the cultivar used. Also, we observed a negative correlation between nitrate in leaves and shoot weight for 'Vitalia' at N1 treatment. Correlation between root nitrate concentrations and the other variables measured didn't show significant correlation except for head fresh weight with 'Great laks' and 'Romaine' cultivars (at N2 level), chlorophyll florescence and sugar content with 'Type beurre' (at N2 treatment) and dry weight with 'Romaine LO3' (at N2 level).

Discussion

Increasing nitrogen fertilization does not always increase lettuce fresh weight. In our study, N1 treatment (120 Kg ha^{-1}) gives the greatest fresh weight for all cultivars (except Vitalia). Fresh weight of all cultivar except 'Vitalia' decreased with N2 nitrogen treatment (240 Kg ha^{-1}). Same results were also reported by Sadeghi (2009); Bozkurt et al. (2009). Boroujerdnia and Ansari (2007) showed that increasing nitrogen, increases fresh weight. Indeed, testing 5 levels of nitrogen, the fresh weight increase to reach the maximum at 200 Kg ha^{-1}. Beyond this level, fresh weight decrease, this might be due to plant toxicity or no attraction of nitrogen by plant which resulted from consumption of excess nitrogen fertilizer (Tabatabie and Malakotie, 1997). Also, high levels of nitrogen fertilization affect head diameters. With N2 treatment, all cultivar's diameter decreased. Acar (2008) showed that increasing nitrogen level, the equatorial diameter was larger. However, increasing the dose to 300 Kg ha^{-1} decreased the diameter from 31.70 cm to 30.87 cm. Same results were obtained by Lastre et al. (2009) who reported a decrease in the equatorial diameter in 'Brisa' cultivar associated with an increase in nitrogen dose. Moreover, we observed that root weight was higher without nitrogen fertilizer for all cultivar tested. This can be explained by the adaptation of roots to search nutritive elements for plants in the case of its absence in soil.

In our experiments, nitrogen fertilizer didn't affect dry matter cultivars. Several studies showed that increasing nitrogen level results in an increase of dry matter content in many plants especially lettuce (Marsic and Osvald, 2002). This increase in dry matter was explained by Takebe et al. (1995) by the combination of nitrogen with the products of photosynthesis such as glucose and ascorbic acid. Also, Tei et al. (2000) explained this increase by the fact that nitrogen stimulates plant growth and more specifically increases the leaf surface, resulting in higher photosynthetic capacity and consequently in better dry matter content.

Our results showed the importance of nitrogen level as a determinant factor of nitrate accumulation in lettuce leaf as reported previously (Chen et al., 2004; Kostantopoulou et al., 2004; Mantovani et al., 2005). We showed, that increasing nitrogen level, nitrate concentration in leaves increased consequently. The highest lettuce fresh weight for all varieties except 'vitalia' was obtained with N1 treatment. Increasing this level resulted in higher nitrate concentration that exceeds the limit (2500 mg Kg^{-1}) with no positive effect on the fresh weight and yield. Exceeding this limit is harmful for consumer health and the environment.

Nitrate seems to be accumulated more in the outer leaves, and its concentration decreased in the other leaves. In previous studies, it has been asserted that nitrates is accumulated more in the older, outer leaves of lettuce than in the inner leaves (Abu-Rayyan et al., 2004; Boroujerdnia et al., 2007). Kostantopoulou et al. (2004) showed that applying high nitrogen levels, nitrate accumulation was higher in the outer leaves. However, at the lowest level (20 mg NL^{-1}), nitrate concentration was higher in the inner younger leaves.

Also, increasing nitrogen level results in nitrate accumulation in roots since lettuce plants accumulates the higher nitrate concentration in roots with N2 treatment for all cultivars. This may explain that the absorption and assimilation of nitrate is under leaves control and depends on climatic conditions. Indeed, when these are ideal for plant growth, it increased demand and occurs an assimilation of nitrates. Otherwise, the nitrates absorbed in the roots will not be transported to the leaves and remain stored in the roots (Buwalda, 1999). Therefore, it is not necessary to apply more fertilizer for plants, because their assimilation is dependent on leaf growth which is dependent on other factors. It seems that nitrate was accumulated in the more photosynthetically active leaves. The same distribution is obtained with chlorophyll fluorescence which reduces progressively as the order of the leaves increases. The chlorophyll florescence indirectly reflects the photosynthetic activity. Behr and Wiebe (1992) showed a negative correlation between nitrate concentration and photosynthetic ability of cultivars. In our study, correlation between nitrate accumulation and chlorophyll fluorescence is dependent on nitrogen level. It seems to be negative with all cultivars except for 'Great Laks' at N2 level, but it is positive for NO and N1 treatments.

The sugar content was similar for most cultivars. These products have as source photosynthesis. They are used for the breath, or to be stored inside vacuoles and increase the dry matter content (Blom-Zandstra, 1989). The dry matter consists of all proteins, sugars and organic acids accumulated in vacuoles. Therefore, varieties that accumulate fewer nitrates have a better use of these molecules in the transforming protein. Unlike the varieties accumulating higher nitrate, which has poor efficiency and didn't invest nitrates absorbed to protein. Also, correlation between nitrate accumulation and sugar content of cultivars is dependent on nitrogen level and cultivars. Indeed, with N2 treatment this correlation is positive in 'Vitalia' cultivar and negative with 'Batavia rouge'. Previous studies showed that nitrate concentration is inversely proportional to sugar content. This was explained by the fact that sugars such as glucose, fructose accumulates in vacuoles and therefore the cells do not need nitrates to ensure turgor (Behr and Wieb, 1992).

Comparing nitrate accumulation in leaves and roots, there was no significant correlation between them. But we observed that 'Great Laks' which accumulated the most nitrates in N2 treatment had the less nitrate concentration in roots. Reinink and Eeninik (1988) showed that lettuce roots accumulate nitrate and that root nitrate content is closely correlated with shoot nitrate content. No correlations were found between fresh weight and nitrate content contrary to studies of Reinink et al., (1987) who showed a negative correlation between nitrate and dry weight and explained that plants which had high dry matter content, had a large amount of organic solutes in the vacuoles. Therefore, these plants do not need large amounts of nitrates to ensure cell turgor.

Nitrogen fertilization is an important factor determining nitrate accumulation in lettuce leaves. So, it is important to choose the best nitrogen fertilization level so that nitrate concentration in leaves doesn't exceed the international norms and to ensure the quality of lettuce. More studies are required for the comprehension of the mechanisms of nitrate accumulation in leafy vegetables especially for the potential role of roots and its relationship with shoots.

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