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# Nitrate accumulation in plants, factors affecting the process, and human health implications. A review

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**Abstract** – Leafy vegetables occupy a very important place in the human diet, but unfortunately constitute a group of foods which contributes maximally to nitrate consumption by living beings. Under excessive application of nitrogen fertilizer, these vegetables can accumulate high levels of nitrate and, upon being consumed by living beings, pose serious health hazards. Therefore, efforts are warranted to minimize the accumulation of nitrate in leafy vegetables and its ingestion by human beings. This review focuses on (i) the contribution of vegetables towards dietary nitrate intake by humans, (ii) the nutritional, environmental and physiological factors affecting nitrate accumulation in plants, (iii) the harmful and beneficial effects of nitrate on human health, and (iv) the strategies that may be followed to minimize the nitrate content in plants and its subsequent consumption by human beings. The risk to human health due to nitrate consumption may be minimized by harvesting vegetables at noon, removal of organs rich in nitrate content and cooking of vegetables in water with a low nitrate content. The European Commission Regulation No. 1822/2005 needs to be followed in order to ensure safe levels of nitrate in plants for human consumption.

**nitrate toxicity / nitrate reductase / spinach / human health / leafy vegetables**

## 1. INTRODUCTION

The anthropogenic activities aimed at enhancing food production may facilitate accumulation of undesirable substances in plants and affect the quality of soil and water resources adversely. Excessive amounts of nitrogenous fertilizers are applied to crops, considering that it is a reasonable insurance against yield losses and their economic consequences. However, when input of nitrogen exceeds the demand, plants are no longer able to absorb it, and nitrogen then builds up in the soil, mostly as nitrates (Nosengo, 2003). This causes imbalance of nutrients in the soil and increases the nitrate level in ground-water supplies (NAAS, 2005) which influences the nitrate content of plants (Dapoigny et al., 2000; Vieira et al., 1998), especially the leafy vegetables. Nitrate has long been one of the highly emotive anions, always being talked about, whether with pride or with horror (Hill, 1999). There is conflicting evidence regarding the potential long-term health risks associated with nitrate levels encountered in the human diet. That reduction in dietary nitrate is a desirable preventive measure (Santamaria, 2006) stands undisputed.

Vegetables are the major source of the daily intake of nitrate by human beings, supplying about 72 to 94% of the total intake (Dich et al., 1996). Therefore, the European Union prescribed, almost a decade ago, the maximum limits for nitrate in lettuce and spinach, which became the foundation stone for the subsequent European Commission Regulation (No. 1822/2005). Investigations have indicated that a high nitrate accumulation

in plants results in nitrite production, which is converted into nitric oxide (NO) which, together with  $O_2^-$ , could be rapidly catalyzed by nitrate reductase into peroxynitrite (ONOO<sup>-</sup>) which is highly toxic to plants (Durner and Klessig, 1999; Lamattina et al., 2003). Therefore, high nitrate accumulation in plants is harmful to human health (Ikemoto et al., 2002; Ishiwata et al., 2002) as well as to plant growth (Reddy and Menary, 1990).

The factors responsible for nitrate accumulation in plants are mainly nutritional, environmental and physiological. Nitrogen fertilization and light intensity have been identified as the major factors that influence the nitrate content in vegetables (Cantliffe, 1973). Diurnal changes in light intensity lead to a diurnal pattern of nitrate accumulation in plants. Many nutrients, such as chloride, calcium, potassium, sulfate and phosphorus, are involved in the nitrate accumulation process in plants. The nitrate content varies in various parts of a plant (Anjana et al., 2006; Santamaria et al., 1999) and with the physiological age of the plant (Anjana et al., 2006; Maynard et al., 1976). A reduction in nitrate content can add value to vegetable products already very popular for their nutritional and therapeutic properties (Santamaria, 2006). Therefore, it is important to adopt appropriate strategies and determine the role of individual physiological factors in the process in order to limit accumulation of nitrate in vegetables, optimize the use of fertilizer nitrogen and reduce the potential degradation of soil and water resources.

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**Table I.** Summary of the maximum levels in European Commission (EC) Regulation No. 1822/2005.

Product	Harvest Period	Maximum level (mg NO <sub>3</sub> /kg)
Fresh spinach <sup>1</sup> ( <i>Spinacia oleracea</i> )	1 October to 31 March	3000
	1 April to 31 September	2500
Preserved, deep-frozen or frozen spinach		2000
Fresh lettuce ( <i>Lactuca sativa</i> L.) (protected and open-grown lettuce) excluding 'iceberg'-type lettuce	1 October to 31 March	
	– grown under cover	4500
	– grown in open air	4000
	1 April to 30 September	
	– grown under cover	3500
	– grown in open air	2500
'Iceberg'-type lettuces	Lettuce grown under cover	2500
	Lettuce grown in the open air	2000
Baby foods and processed cereal-based foods for infants and young children <sup>2</sup>		200

<sup>1</sup> The maximum levels for fresh spinach do not apply to fresh spinach to be subjected to processing and which is directly transported in bulk from the field to the processing plant.

<sup>2</sup> The maximum levels apply to the products as proposed ready for consumption or as reconstituted according to the instructions of the manufacturers.

**Table II.** Signs and symptoms of methaemoglobinemia.

Methaemoglobin Concentration (%)	Clinical Findings
10–20	Central cyanosis of limbs/trunk; usually asymptomatic
20–45	Central nervous system depression (headache, dizziness, fatigue, lethargy, syncope), dyspnea
45–55	Coma, arrhythmias, shock, convulsions
>70	High risk of mortality

Source: Dabney et al. (1990).

This review focuses on the contribution of vegetables towards the dietary nitrate intake, factors responsible for nitrate accumulation in plants, and the effects of nitrate on human health. It also suggests ways of minimizing the nitrate content in plants and its subsequent consumption by human beings.

## 2. VEGETABLES AS A SOURCE OF NITRATE

Vegetables, an important component of the human diet and a major source of nitrate, constitute nearly 72 to 94% of the average daily human dietary intake (Dich et al., 1996). Unfortunately, leafy vegetables grown under different agro-ecological conditions accumulate nitrate to potentially harmful concentrations. Generally, nitrate-accumulating vegetables belong to the families Brassicaceae (rocket, radish and mustard), Chenopodiaceae (beetroot, Swiss chard and spinach), Amaranthaceae (*Amaranthus*), Asteraceae (lettuce) and Apiaceae (celery and parsley) (Santamaria, 2006).

Nitrogenous fertilizers, mainly of the nitrate variety, are used widely in vegetable agriculture, resulting in accumulation of nitrate in plants, if the rate of its uptake exceeds the rate of its reduction to ammonium (Luo et al., 1993). As suggested by McCall and Willumsen (1998), high rates of nitrate application increase the plant nitrate content without increasing the yield. Therefore, growers who apply excessive fertilizers to ensure that nitrogen is not limiting for plant growth are unlikely to achieve any gain in terms of yield, but increase the nitrate

content of crops to the levels potentially toxic to humans. Ysart et al. (1999) estimated for the adult human population a total nitrate intake of 93 mg/day, normally through potatoes (33%), green vegetables (21%), other vegetables (15%), beverages (8.5%), meat products (4.2%), fresh fruit (3.5%), dairy (3.1%), milk (2.9%), miscellaneous cereals (2.1%), bread (1.6%) and others (5.1%).

The European Commission (EC)'s Scientific Committee for Food (SCF) established, in 1995, the Acceptable Daily Intake (ADI) of nitrate ion as 3.65 mg kg<sup>-1</sup> body weight (equivalent to 219 mg/day for a person weighing 60 kg) (SCF, 1995), whereas the Joint Expert Committee of the Food and Agriculture (JECFA) Organization of the United Nations/World Health Organization (WHO) established the Acceptable Daily Intake of nitrate as 0–3.7 mg kg<sup>-1</sup> body weight (Speijers, 1996). Therefore, assuming a 60-kg body weight, ingestion of only 100 g of fresh vegetables with a nitrate concentration of 2500 mg kg<sup>-1</sup> fresh weight exceeds the Acceptable Daily Intake for nitrate by approximately 13%. For a real assessment, however, nitrate content in all other sources as well as their average daily consumption amount must be taken into account. On the other hand, the USA's Environmental Protection Agency (EPA)'s Reference Dose (RfD) for nitrate is 1.6 mg nitrate-N kg<sup>-1</sup> body weight per day (equivalent to about 7.0 mg NO<sub>3</sub> kg<sup>-1</sup> body weight per day) (Mensinga et al., 2003). On November 8, 2005, the European Commission adopted EC Regulation No. 1822/2005 (Tab. I) and set the harmonized maximum levels for

nitrate in lettuce, spinach, baby foods and processed cereal-based foods. The limits vary depending on the season, with higher nitrate levels permitted in winter-grown vegetables.

### 3. FACTORS RESPONSIBLE FOR NITRATE ACCUMULATION

Plant nitrate content is commonly viewed as an outcome of imbalance between its net absorption and assimilation rates (Cardenas-Navarro et al., 1999). The effect of endogenous and exogenous factors seems to be straightforward: on one hand, both uptake and assimilation systems are genetically determined (Ferrario-Mery et al., 1997; Ourry et al., 1997), explaining the variability in plant nitrate content among species and cultivars, whereas on the other hand, nitrate absorption depends on availability of nutrition, and nitrate assimilation depends independently on climate, as it is partly a photosynthetic process in many plants (Ferrario-Mery et al., 1997). Alternatively, plant nitrate content might be either fixed through osmotic potential regulation (McIntyre, 1997) or regulated *per se* through negative feedback on its transport systems (Cardenas-Navarro et al., 1999).

#### 3.1. Nutritional factors

Nazaryuk et al. (2002) have studied the role of agricultural chemicals in regulating the nitrate accumulation in plants and shown that the process of nitrate accumulation depends on three major groups of factor: application of mineral fertilizers, treatment with physiologically active substances and sorbents, and the natural and anthropogenic changes in the soil environment. With respect to their impact on nitrate accumulation, these factors may be arranged in the following descending order: fertilizers > physiologically active substances > soil. To explore the possibility of controlling the nitrate accumulation in plant tissues, it is important to estimate the effect of an exogenous nitrogen supply on the degree of utilization of nitrogen from soil as well as fertilizers (Nazaryuk et al., 2002).

Nitrogen fertilization facilitates accumulation of nitrate in plant tissues as a result of an excess of nitrogen uptake over its reduction. When taken up in excess of immediate requirement, it is stored as free nitrate in the vacuole and can be remobilized subsequently when nitrogen supply is insufficient to meet the demand (van der Leij et al., 1998). Nitrate accumulation in vegetables often depends on the amount and kind of nutrients present in the soil and is closely related to the time of application, and the amount and composition of the fertilizers applied (Zhou et al., 2000). An adequate fertilization program may ensure sufficient plant growth without any risk of plant nitrate levels going too high (Vieira et al., 1998). Plants accumulate more nitrate as the nitrogen fertilization level increases (Chen et al., 2004; Nazaryuk et al., 2002; Santamaria et al., 1998a, b), whereas limiting the nitrogen availability reduces nitrate content significantly (McCall and Willumsen, 1999). Applying nitrogen once at the beginning of the cropping cycle is effective at controlling nitrate accumulation, since the plant and soil nitrate concentrations decrease as plants reach a marketable size (Vieira et al., 1998). The use of fertilizers based on ammonia or a mixture of nitrate and ammonium can reduce the nitrate

content in plants (Inal and Tarakcioglu, 2001; Santamaria et al., 2001); it is worthy of further consideration in soilless growth systems because ammonium reduces the need for adding acids to the nutrient solution to lower its pH (Santamaria and Elia, 1997). Depending on which plant part is to be consumed, appropriate fertilizer can be selected (Zhou et al., 2000), e.g. the order of nitrate accumulation in the cole leaves, upon application of different nitrogenous fertilizers, is: urea > ammonium carbonate > ammonium nitrate > ammonium sulfate; for nitrate accumulation in petioles, it is: urea > ammonium nitrate > ammonium sulfate > ammonium carbonate.

Vegetables supplied with organic fertilizers have a low nitrate content, compared with minerally fertilized (Raupp, 1996) or conventionally grown vegetables, and this effect is independent of site conditions. Nitrate accumulation in products is a function of increasing supply of nitrate by fertilization and mineralization of soil organic matter on the one hand, and the reduced availability of assimilates on the other hand. Therefore, the higher the nitrogen availability (mineral fertilizer > liquid manure = slurry > manure > compost) and the lower the assimilation intensity (e.g. by site conditions and season effects), the greater would be the nitrate accumulation. Moreover, the poorly controlled flux of soil nitrogen resulting from active mineralization of organic matter may lead to excessive accumulation of nitrate in plants (Nazaryuk et al., 2002).

To have a quality yield with low nitrate content is possible by manipulating the nitrogen nutrition of plants (Izmailov, 2004). Apart from nitrogen fertilization, a balanced nutrition of plants is particularly important in intensive agricultural production where high fertilizer applications are aimed at improving the yield and quality. Nutrient balance may be a critical factor affecting the nutrient status of plants (Ahmed et al., 2000). Proper application of nitrogenous, phosphate, potassium fertilizers, as well as the green and farmyard manures, could materially reduce the nitrate accumulation in vegetables (Zhou et al., 2000).

Seginer (2003) developed a dynamic lettuce model, NICOLET, for predicting plant growth and nitrate content. This was valid for the initial stage of plant growth, where composition of plants with respect to reduced nitrogen and water contents is time-invariant. The model was later modified (Seginer et al., 2004) to extend its validity to a late stage of vegetative growth to accommodate the ontogenetic changes in the reduced nitrogen and water contents. According to the modified model, prediction of nitrogen uptake for the substantial nitrate pool of lettuce depends on the water content. These models have the potential to improve the estimates of nitrogen uptake, thus leading to a more accurate calculation of fertilizer needs.

Nitrate concentration in plants can also be manipulated by stopping the nitrogen supply for some days before crop harvesting (Santamaria et al., 2001). In this way, nitrate will be removed from vacuoles and plants will guard the organic vacuoles needed to make up for the decreased osmotic value. Nitrate concentration can also be reduced by replacing nitrate-N with chloride, sulfate, ammonium or amino acids a few days prior to crop harvesting (Inal and Tarakcioglu, 2001; Santamaria et al., 1998b).

Chloride ( $\text{Cl}^-$ ) and nitrate ( $\text{NO}_3^-$ ) ions play an interchangeable role in osmoregulation; the former are able to prevent

excessive nitrate concentration by replacing the latter and may have a positive effect on nitrogen content in plant organs (Dorais et al., 2001). Interaction of chloride uptake with the uptake of nitrate and other nutrients has been reviewed by Xu et al. (2000). Chapagain et al. (2003) have shown that fruit nitrate decreases and chloride increases upon increasing the chloride concentration in the nutrient solution; this agrees with the earlier findings on antagonism between the chloride and nitrate uptake in plants, especially in the foliar tissues. As the total nitrogen content of plants did not decrease in response to chloride treatment, Liu and Shelp (1996) believed that chloride absorption did not compete directly with nitrate absorption. Addition of moderate amounts of chloride to the growing medium of broccoli plants decreased their nitrate content by increasing accelerating nitrate reduction. The authors suggested that chloride application could be used as a strategy to decrease nitrate content of vegetables, particularly of those such as spinach, lettuce and cabbage which are classified as nitrate accumulators (Maynard et al., 1976). However, inhibition of nitrate uptake by chloride depends on the plant species and the concentrations of both nitrate and chloride in the medium (Cerezo et al., 1997). In root cells, the high-affinity, saturable system for nitrate uptake that operates at small nitrate concentrations (Siddiqi et al., 1990) is inhibited by high external chloride, whereas the low-affinity, linear system that operates at high nitrate concentrations seems to be inhibited by internal chloride (Cerezo et al., 1997). Urrestarazu et al. (1998) have suggested that replacing nitrate with chloride in the nutrient solution during the last week before the crop harvest decreases nitrate content in leafy vegetables.

Increase in the rate of potassium application facilitates uptake and transport of nitrate towards the aerial parts of the plant, promotes the metabolism and utilization of nitrate and, ultimately, reduces nitrate accumulation in some vegetable crops (Ahmed et al., 2000; Ruiz and Romero, 2002). However, some studies have indicated that the soil potassium resources have no effect on nitrate accumulation (Drlik and Rogl, 1992).

Ahmed et al. (2000) have shown a reduction in nitrate content due to increasing phosphorus fertilization. Inorganic phosphorus within the plant is necessary for the metabolism and storage of nitrate, but high concentrations inhibit enzyme reactions, create abnormal pressure in the cell, and accelerate senescence, and the advancing plant age is accompanied by a reduced nitrate uptake and accumulation (Ahmed et al., 2000). Growth reduction associated with limiting phosphate results in increases in the root-shoot ratio, dry matter content, concentrations of sugars and organic acids, and reduction in the concentration of nitrate in the shoots (Buwalda and Warmenhoven, 1999).

Nitrate accumulation has also been studied in relation to some other chemicals. Foliar application of salicylic acid (Ahmed et al., 2000), molybdenum fertilizers (Zhou et al., 2000) and nitrification inhibitors (Xu et al., 2005; Zhou et al., 2000) and calcium (Tzung et al., 1995) can materially reduce the nitrate content of plants. In lettuce leaves sugar levels and free amino acid concentrations increase under high supply of calcium and replace nitrate in the vacuole of the lettuce cell (Ahmed, 1996). There exists a negative correlation between nitrate content and sulfate content in the lettuce plant (Blom-Zandstra and Lampe, 1983) and, therefore, sulfur deficiency

might lead to an increase in nitrate content (Maynard et al., 1976). The contents of nitrate in most crops (cabbage, carrot, beet, tomato, onion and potato) decreased significantly under the influence of humic acid as well as zeolite (Nazaryuk et al., 2002).

A supply of boron in concentrations that were either deficient or just sufficient for the plant's normal metabolism did not affect the nitrate content, but the toxic boron rate significantly increased the nitrate content of plants (Inal and Tarakcioglu, 2001). Cultivation of vegetables on soil contaminated with sewage sludge may also lead to accumulation of nitrate in edible plant parts due to very high levels of soil nitrogen caused by the sludge (Nazaryuk et al., 2002).

Accumulation of salt in the soils can alleviate nitrate accumulation in vegetable crops (Chung et al., 2005). Due to high fertilization and poor water supply, the water potential of the soil may become so negative that salination takes place and plants take up and accumulate nitrate as an osmoticum to adapt to water conditions. In such a situation, potassium compensates for the increased nitrate in maintaining the electroneutrality of the plant.

### 3.2. Environmental factors

Nitrate accumulation in plants is affected greatly by environmental factors. Santamaria et al. (2001) observed an interaction between light intensity, nitrogen availability and temperature on nitrate accumulation in rocket. Under conditions of low light availability, an increase in temperature increases the nitrate accumulation. Under high light intensity, an increase in temperature increases the nitrate content mainly when the nitrogen supply is high. Chadjaa et al. (2001) have studied the effect of artificial lighting in greenhouses on nitrate accumulation in lettuce. High-pressure sodium vapor lamps were more effective than metal halide lamps at increasing the nitrate reductase (NR) activity and reducing the nitrate accumulation. The effect of climate on nitrate accumulation was studied by Grzebelus and Baranski (2001), who found that nitrate content was less in the year that had a high rainfall. In warm and wet years, increased accumulation of nitrate is possible, regardless of whether the nitrogen originates from organic or mineral sources (Custic et al., 2003). Plant nitrate levels were influenced by weather conditions more significantly than by the form and application rates of fertilizers (Custic et al., 2003). Nitrate accumulation varies with the season (Vieira et al., 1998), being higher in autumn-winter than in spring (Santamaria et al., 1999). It is accepted that plants in winter are not able to use all the nitrogen available in the soil due to less favorable light and temperature conditions. Gruda (2005) has extensively reviewed the effect of different environmental factors on the nitrate content of greenhouse vegetables.

### 3.3. Physiological factors

#### 3.3.1. Genotypic variability

The nitrate content varies markedly with plant species, cultivars of the same species, and even genotypes with different ploidy (Anjana et al., 2006; Grzebelus and Baranski, 2001; Harada et al., 2003). The shoot nitrate content is genetically

determined and likely to be controlled by several genes (QTLs) (Harrison et al., 2004). The causal factors might include genetic differences among genotypes in enzymes of the nitrogen metabolic pathway (nitrate reductase/nitrite reductase), the rate of nitrate uptake, the rate of uptake of other elements needed for enzyme activity, or differences in generation of electron donors needed in the assimilative pathway that might lead to the observed variation in nitrate accumulation. In contrast, Blom-Zandstra and Eenink (1986) found no evidence to conclude that nitrate accumulation was caused by a low assimilation rate of nitrate in lettuce genotypes which differed significantly in nitrate content. The differing capacities of nitrate accumulation can also be correlated with differing locations of nitrate reductase activity (Andrews, 1986), and with differences in photosynthetic capacity (Behr and Wiebe, 1992), ability to generate and translocate respiratory substrate and reducing equivalents, or differences in capacity to translocate the absorbed nitrate to reduction sites. Nitrate accumulation decreases with increasing carbohydrate concentration in the vacuoles. Nitrate accumulation is negatively correlated to sugar concentrations (Blom-Zandstra and Lampe, 1983) and dry matter content (Reinink et al., 1987), while the latter two parameters are positively correlated to each other in different genotypes. Thus, genotypes with high dry matter content could have a high carbohydrate content in their vacuoles and thus need little nitrate to maintain their osmotic value (Reinink et al., 1987). All these factors need to be studied intensively to determine the causal factors for differences in nitrate accumulation between genotypes.

As discussed by Harada et al. (2004), studies using mutants and transgenic plants have revealed a number of genes that can affect the concentration of nitrate in a plant, e.g., genes encoding nitrate reductase (Scheible et al., 1997a), a putative anion channel, At CLC-a (Geelen et al., 2000), glutamine synthetase, and ferredoxin-dependent glutamate synthase (Hausler et al., 1994). However, other genes must also be involved. Loudet et al. (2003), using *Arabidopsis* Bay-O and Shahdara recombinant inbred lines (RILs), identified eight quantitative trait loci (QTLs) for nitrate content on a dry matter basis. In maize, Hirel et al. (2001) identified five quantitative trait loci for nitrate content in dry matter, one of which included a gene encoding glutamine synthetase. Nitrate storage in vacuoles is affected by multiple processes including the relative rates of nitrate uptake, nitrate reduction and assimilation, nitrate transfer to the vacuole and its export from there. Therefore, numerous gene products can potentially influence the naturally occurring variation in free nitrate levels at the whole tissue level (Harada et al., 2004).

Harrison et al. (2004) have studied genotypic variability in shoot nitrate content in *Lotus japonicus* and found that it was mainly due to an increase in the ion uptake regardless of biomass production. The positive correlation between the shoot nitrate content and the steady state level of mRNA encoding high-affinity nitrate transporters suggests that the higher nitrate flux is due to enhanced expression of transporters. In contrast, neither the level of nitrate reductase mRNA, nor the potential enzyme activity *in vivo* in the different lines was correlated with shoot nitrate content. This indicates that nitrate transport is one of the main checkpoints controlling shoot nitrate accumulation and that it is possible to lower the nitrate content through breeding strategies without affecting the biomass pro-

duction. According to Harrison et al. (2004), the concept that nitrogen accumulation in crops under suboptimal nitrogen feeding conditions is highly related to crop growth rate and biomass accumulation via internal plant regulation (Gastal and Lemaire, 2002) needs to be reconsidered. It is necessary to take into account that the control of nitrate uptake and its accumulation in the plant may be subjected to genetic variability regardless of the plant's demand. This variability allows for an adaptive regulatory control mechanism depending on soil nitrate availability.

Thus, selection of genotypes that accumulate less nitrate may contribute significantly to reduction in the nitrate consumption by humans through vegetables and the subsequent risk of nitrate poisoning. Another method of circumventing the problems associated with nitrate accumulation may be to breed cultivars that do not accumulate nitrate even under heavy nitrogen fertilization.

### 3.3.2. Nitrate distribution within the plant

In general, vegetables that are consumed with their roots, stems and leaves have a high nitrate accumulation, whereas those with only fruits and melons as consumable parts have a low nitrate accumulation (Zhou et al., 2000). Nitrate content of various parts of a plant differs (Santamaria et al., 1999). Indeed, the vegetable organs can be listed by decreasing nitrate content as follows: petiole > leaf > stem > root > inflorescence > tuber > bulb > fruit > seed (Santamaria et al., 1999). In lettuce and 'head chicory', inner leaves accumulate less nitrate than outer leaves and in parsley and spinach, leaf blades accumulate less nitrate than petioles (Santamaria et al., 1999, 2001). Nitrate concentration in the petiole was more than double that in the lamina of rocket leaf (Elia et al., 2000); the difference was as high as 6.6 times greater in spinach (Anjana et al., 2006). Similarly, nitrate concentration in the petiole stems was higher than in the leaves, with the lowest in the roots in leafy vegetables (Chen et al., 2004). Therefore, increasing the blade/petiole ratio of spinach plants at harvesting (Santamaria et al., 1999) and separation of those parts of a vegetable that accumulate high nitrate concentrations, prior to processing or preparation of vegetable food, may appreciably reduce the extent of nitrate consumption by humans. Chen et al. (2004) have also shown that nitrate supply has a significant effect on nitrate distribution, both in the metabolic pool and the storage pool of leaf blades. The comparison of the nitrate storage pool with the nitrate concentration in the whole leaf (nitrate metabolic pool + nitrate storage pool) suggests that about 90% of the nitrate accumulates in the storage pool. Functional diversity of nitrate compartmentation in the cells of various species has been discussed by Izmailov (2004).

The concentration of nitrate in various plant parts could be a measure to evaluate the balance between adequate and excessive available nitrogen for optimum growth of plants during a growth season. Typically, however, this balance assumes two markedly different modes depending on the vegetable crop under consideration. Plants that develop fruit or storage organs, such as potato and tomato, usually have low nitrate-N concentrations in petioles as the crop approaches the harvest. The decline in the petiolar nitrate concentration depends on the translocation of soluble nitrogen to the developing storage

organ, and on the gradual decrease in the available soil nitrogen. Vegetables that do not develop storage organs as the foodstuff have a different pattern of nitrate accumulation wherein nitrate often continues accumulating with the growing plant's age (Maynard et al., 1976). The findings of Anjana et al. (2006) were similar, showing that nitrate concentration in spinach was higher at later stages of plant growth. Therefore, the optimum physiological age for harvesting needs to be standardized for different leafy vegetable crops. Since nitrate continues to accumulate in spinach plants until maturity, the extent of nitrate intake by humans through spinach leaves may be reduced by harvesting the crop during the vegetative stage.

### 3.3.3. Diurnal effects

Nitrate accumulation is a complex process involving many physiological steps. Being involved in photosynthesis as well as in uptake, translocation and reduction of nitrate, the intensity of light has a crucial role in the regulation of nitrate accumulation (Merlo et al., 1994). Light intensity is inversely correlated to the nitrate content of plants; therefore, diurnal changes in light intensity might cause a diurnal nitrate accumulation pattern. The diurnal variation in nitrate content can be kept low by selecting a proper harvest time. That a high light intensity reduces nitrate accumulation has been shown in several studies (Blom-Zandstra et al., 1988; van der Boon et al., 1990). Marked diurnal patterns showing a decrease in nitrate content during the day followed by an increase during the night have also been demonstrated (Cardenas-Navarro et al., 1998; Delhon et al., 1995a, b). Various explanations, depending on the uptake and assimilation of nitrate, are put forth for the diurnal changes in the nitrate content of plants.

**Nitrate uptake and accumulation:** Light intensity can have a profound effect on nitrate acquisition by roots and restrict the rate of nitrate uptake in darkness (Le Bot and Kirkby, 1992). The differences in nitrate content of butterhead lettuce cultivars are due to differences in nitrate uptake (Behr and Wiebe, 1992). On the contrary, several studies have indicated that nitrate uptake does not change under dark conditions (Macduff and Wild, 1988). These discrepancies demonstrate that characterization of the effect of light intensity on nitrate uptake is still incomplete. In particular, it is not yet clear whether inhibition of net nitrate uptake in darkness results from a decreased influx (Macduff and Jackson, 1992) and/or an increased efflux (Pearson et al., 1981) across the plasmalemma of the root cells. Beevers et al. (1965) described the role of light in nitrate accumulation to be indirect in that light enhances nitrate uptake as a result of increased permeability of the tissue and this, in turn, stimulates enzymatic activity and assimilation of nitrate. There exists a strong correlation between the net uptake of nitrate by the roots and the vacuolar nitrate concentration in the leaf blades (Steingrover et al., 1986a). The increase in nitrate concentration of leaf blades during the night results from increased nitrate uptake by roots, and from reduction of nitrate to organic solutes that are metabolized in the cytoplasm. During the daytime, nitrate leaves the vacuoles partly to be replaced by soluble carbohydrates and organic acids synthesized in the light. When light is limiting, synthesis of organic acids and soluble carbohydrates declines and their role as an osmoticum is taken up readily by nitrate.

Scaife (1989) and Scaife and Schloemer (1994) have developed a model, assuming a concentration-dependent nitrate efflux in which net nitrate uptake (influx minus leakage) and nitrogen assimilation (considered as a constant fraction of the photosynthetic rate) can be calculated. This could provide precise details of the diurnal variation in nitrate uptake dependent on radiation. Another similar model assumes that nitrate influx is adjusted to reduction flux by means of a negative feedback control, which is proportional to the internal nitrate concentration (Cardenas-Navarro et al., 1998). In both these models, the internal nitrate concentration itself is involved directly in the regulation mechanism as the regulatory signal. By this hypothesis, low nitrate concentrations in plants result from an increased nitrate demand, associated with higher growth rates at high daily integrals, and correspond with a low level of feedback control over nitrate influx in the Cardenas-Navarro et al. model (1998), with a low rate of concentration-driven efflux in the Scaife model (1989). Thus, these models suggest that nitrate concentration would result from a simple proportional regulation mechanism. This hypothesis is supported by experiments in which nitrogen nutrition or assimilation was altered (King et al., 1993), and the negative correlation obtained between uptake rate and endogenous nitrate (Laine et al., 1995; Cardenas-Navarro et al., 1998, 1999). Furthermore, a direct measurement of cytosolic nitrate concentration shows that it is regulated in root cortical cells (Miller and Smith, 1996) and there is no such evidence of homeostasis of nitrate in other compartments/organs, or at the whole-plant level. The recent simulation models based on this hypothesis seem to be able to predict the whole-plant nitrate content adequately (Cardenas-Navarro et al., 1998).

Macduff and Bakken (2003) have provided a framework for considering diurnal co-regulation of nitrate uptake by the amino acid and nitrate levels in species assimilating nitrate predominantly in the shoots. According to this model, diurnal regulation of nitrate influx is affected by nitrate levels throughout the dark period (downregulation) and the first half of the light period (upregulation), but by amino acid levels during the second half of the light period (downregulation). The sudden light/dark transitions affect the transpiration rate and hence xylem nitrogen flux, which in turn affects the concentration of nitrate in the cytoplasmic compartment of the roots, the rate of nitrate assimilation in the shoot and the phloem amino acid flux, once the shoot demand for amino acids associated with protein synthesis and vacuolar storage has been met.

On the other hand, according to the turgor maintenance concept, nitrate level is negatively correlated with the level of soluble, non-structural carbon compounds (SNC; mainly sugars and organic acids) as observed in lettuce and some other vegetables (Blom-Zandstra et al., 1988; Steingrover et al., 1986a, b). Veen and Kleinendorst (1985) demonstrated that nitrate and soluble carbohydrates play complementary roles in the maintenance of cell turgor. This concept was generalized by Seginer et al. (1998), who assumed that the pool size of soluble, non-structural carbon compounds is determined by the balance between source activity (supply by photosynthesis) and sink activity (demand by growth and maintenance). They also hypothesized that a regulation mechanism adjusts the nitrate concentration to the soluble, non-structural carbon compound level according to the plant's requirements for osmotically

active solutes to maintain turgor. This means the low summer nitrate level in lettuce is caused by the increased rate of photosynthesis at high daily integrals, which leads to accumulation of soluble, non-structural carbon compounds, thereby diminishing the need for nitrate as a cellular osmoticum. In this concept, maintaining a constant level of turgor by adjusting the nitrate concentration to fluctuating levels of soluble, non-structural carbon compounds would require a rather complex regulation. Therefore, the carbon balance, rather than nitrate homeostasis, is the primary factor that determines nitrate accumulation in lettuce (Buwalda and Warmenhoven, 1999).

Light intensity determines the production of carbohydrates and affects nitrate assimilation (by supply of NADH and induction of nitrate reductase). This may also affect the leakage of nitrate from the vacuoles (Aslam et al., 1976). Organic acids and sugars play an important role in the osmotic adjustment. There is an inverse relationship between nitrate compounds and organic compounds, suggesting that nitrate also has a role as an osmoticum, which may explain its relative unavailability for reduction in the metabolic pool (Blom-Zandstra, 1989). Concentrations of nitrate, organic acids and sugars change significantly with varying light intensity. Variation in nitrate concentration is fully compensated for by a change in the concentration of organic acids and sugars to maintain electroneutrality and osmotic pressure. At low light intensities, production of organic acids is low due to the low rate of photosynthesis (Blom-Zandstra and Lampe, 1985) and less organic compounds are thus available for storage in the vacuoles and, therefore, nitrate can serve as an alternative. This will require low energy costs, because nitrate uptake, transfer through the xylem, and storage in the vacuoles do not require as much energy, derived from ATP, as the production and accumulation of organic compounds (Lambers and Steingrover, 1978). The plant seems to shift the balance from carbohydrates to nitrate so as to maintain turgor for cell expansion during growth, and the availability of organic compounds for storage affects the need for nitrate as an osmoticum, which in turn affects nitrate uptake. Thus, the ratio between accumulation of organic compounds and accumulation of nitrate is light-dependent.

Under light-limiting conditions, assimilate production increases with increasing light intensity, which is invested in structural growth rather than in osmoregulation if nitrate is available at a higher level than is required to meet the plant's demand for reduced nitrogen. It, therefore, appears likely that increasing light intensity will result in a substantial replacement of nitrate with organic osmotica only where growth is not limited by light intensity or where nitrate availability becomes growth-limiting (McCall and Willumsen, 1999). Moreover, a negative correlation exists between nitrate accumulation and dry matter content, as reported for different genotypes (Reinink et al., 1987). It implies that when a plant accumulates nitrate as an osmoticum, it can utilize more carbohydrates for growth since the osmotic requirement is fulfilled by nitrate.

Nitrate content can be reduced significantly both by limiting the nitrogen availability and by increasing the light intensity (McCall and Willumsen, 1999). Reducing the nitrogen availability significantly reduces the contribution of nitrate to the osmotic potential and increases that of chloride, glucose and sucrose. Furthermore, contribution of nitrate to the osmotic potential becomes significantly low and that of sucrose signif-

icantly high at a high light intensity. As the reduction in nitrate content with supplementary light becomes relatively slight, growth is greatly enhanced. The reduced nitrate content under high light intensity is accompanied by an increased content of sucrose, suggesting an increased rate of photosynthesis under high light intensities (McCall and Willumsen, 1999).

**Nitrate assimilation and accumulation:** As discussed in detail by Stitt et al. (2002), nitrogen metabolism undergoes drastic diurnal changes, which are driven by a transient imbalance between the rate of nitrogen assimilation and the rate of nitrate uptake and ammonium assimilation in the first part of the light period. It has been shown in tobacco plants growing in a high nitrate and favorable light regime that their leaves contain high levels of the *NIA* transcript at the end of the night. Illumination stimulates translation of the transcript and inhibits degradation of *NIA* protein (Kaiser et al., 1999), leading to an approximately threefold increase of *NIA* protein during the first hours of the light period (Scheible et al., 1997b). A rapid post-translational activation of *NIA* and a high rate of nitrate assimilation are achieved during the first part of the light period. These exceed the rate of nitrate uptake by a factor of 2 (Matt et al., 2001), leading to a rapid depletion of the leaf nitrate pool. During the second part of the light period, nitrate assimilation is progressively inhibited by mechanisms that act at several levels to decrease *NIA* activity. These include a dramatic decrease in the *NIA* transcript level, which commences soon after illumination (Matt et al., 2001; Scheible et al., 1997b) and results in a decline in *NIA* protein and activity as well as post-translational activation of *NIA* after darkening (Scheible et al., 1997b). In consequence, the rate of nitrate reduction falls about twofold in the second part of the light period, and is negligible during the night (Matt et al., 2001). By contrast, nitrate uptake in the roots and movement of nitrate to the shoot remain high during the entire light period and fall by only 30% during the night. The nitrate that is taken up during the night is used almost exclusively to replenish the leaf nitrate pool (Matt et al., 2001).

**Nitrate reductase activity and nitrate accumulation:** It is noteworthy that during a diurnal cycle, nitrate reductase activity in leaves often decreases during the late light phase, although the sugar level in the leaves is still increasing, or at least is not decreasing (Kaiser et al., 2002). This "afternoon depression" of nitrate reductase may reflect its degradation (Man et al., 1999) and/or a block in its synthesis, and is usually paralleled by decreasing nitrate concentrations in the leaves. This effect of light is reflected in fluctuations in the carbohydrate level and in the corresponding supply of reducing equivalents (ferredoxin and NADPH). In addition, however, leaves of plants grown under low light intensity usually have low levels of nitrate reductase; the enzyme activity increases when the plants have been transferred to conditions of high light intensity. Light intensity also affects the stability of the enzyme. The rate of nitrate reduction in leaves is thus affected by light in a variety of ways.

Anjana et al. (2006) have reported that nitrate concentration was lowest at noon on a sunny day in spinach leaves. However, the time at which plants contain the lowest nitrate concentration may vary with the environmental conditions in different geographical regions of the world. Therefore, harvesting schedules need to be standardized for different geographical regions and

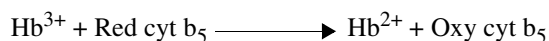


the farmers advised to harvest vegetables at the recommended time of day and supply them to the market as soon as possible so that deterioration in their quality due to nitrite formation does not take place during the post-harvesting period. Moreover, by cooking vegetables in water (with low nitrate concentration), at least 50% of accumulated nitrate can be removed (Meah et al., 1994).

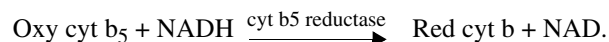
#### 4. EFFECT OF NITRATE INGESTION ON HUMAN HEALTH

##### 4.1. Adverse effects

Several human health hazards due to nitrate toxicity have been identified. The toxicity of nitrate is thought to be due to its reduction to nitrite and conversion to nitrosamines and nitrosamides through reaction with amines and amides, whose carcinogenic action is well known (Walker, 1990). The principal mechanism of nitrite toxicity is the oxidation of the ferrous iron ( $\text{Fe}^{2+}$ ) in haemoglobin to the ferric ( $\text{Fe}^{3+}$ ) valence state, producing methaemoglobin. As a consequence of methaemoglobin formation, oxygen delivery to human tissues is impaired (Knobeloch et al., 2000; Mensinga et al., 2003). The methaemoglobin that is formed is reduced by the following reaction:



The reduced cytochrome  $\text{b}_5$  (Red cyt b) is regenerated by the enzyme cytochrome  $\text{b}_5$  reductase:



The enzyme cytochrome  $\text{b}_5$  reductase plays a vital role in counteracting the effects of nitrate ingestion.

The percentage of total methaemoglobin in oxidized form determines the clinical picture of oxygen deprivation with cyanosis, cardiac dysrhythmias and circulatory failure, and progressive central nervous system (CNS) effects. The CNS effects can range from mild dizziness and lethargy to coma and convulsions (Agency for Toxic Substances and Disease Registry, 2001). Clinical findings vary with methaemoglobin concentrations (Tab. II). Methaemoglobinemia, earlier believed to occur in infants only, has been reported by Gupta et al. (2000a) in people of different ages with a high nitrate ingestion, the infant and above 45 age groups being most susceptible to nitrate toxicity.

Of the total nitrate absorbed from the diet or produced endogenously, 25% is taken up by the salivary glands and secreted into the mouth (McColl, 2005). Bacteria on the dorsum of the tongue convert 10 to 90% of this nitrate in saliva into nitrite. Upon being swallowed, when the saliva meets acidic gastric juice, it is converted into nitrosating species (that is,  $\text{N}_2\text{O}_3$  and  $\text{NOSCN}$ ) and by further reacting with ascorbic acid in the gastric juice into nitric oxide (NO) (McColl, 2005), which is potentially mutagenic and carcinogenic (Iijima et al., 2003; Moriya et al., 2002). Excessive local production of nitric oxide leads to functional abnormalities associated with gastroesophageal reflux diseases (McColl, 2005).

In the early weeks of life, before the establishment of the gastric acid barrier, the infant's gut is colonized by bacteria throughout its length and, therefore, the nitrate in the feed is readily reduced to nitrite in the stomach and small intestine by the action of bacterial nitrate reductase (E.C. 1.6.6.1). This is then absorbed and reacts with haemoglobin to form methaemoglobin; the latter greatly reduces the oxygen-binding capacity of blood and consequently, the infant's tissues are starved of oxygen and ultimately cyanosis results (Hill, 1999). In pregnant women, the level of methaemoglobin increases from the normal (0.5 to 2.5% of total haemoglobin) to a maximum of 10.5% at the 30th week of gestation and subsequently declines to normal after delivery. Thus, pregnant women might be more sensitive to the induction of clinical methaemoglobinemia by nitrites or nitrates in or around the 30th week of gestation (Agency for Toxic Substances and Disease Registry, 2001).

Another concern pertains to human cancer as a result of the bacterial production of N-nitroso compounds in the colonized hypochlorhydric stomach. N-nitroso compounds have been shown to be carcinogenic in the more than 40 animal species tested, including mammals, birds, reptiles and fish (Hill, 1999) and in humans (Michaud et al., 2004). Endogenously-formed nitrogen and oxygen free radicals are believed to be involved in human cancer etiology (Szaleczky et al., 2000). Elevated risks for cancer of the urinary bladder (Michaud et al., 2004), esophagus, nasopharynx and prostate have been reported due to high nitrate ingestion (Eicholzer and Gutzwiller, 1990). Other health problems associated with nitrate toxicity include oral cancer (Badawi et al., 1998), cancer of the colon, rectum or other gastrointestinal regions (Knekt et al., 1999; Turkdogan et al., 2003), Alzheimer's disease, vascular dementia of Biswanger type or multiple small infarct type (Tohgi et al., 1998), multiple sclerosis (Giovannoni et al., 1997), spontaneous abortion or congenital defects (Fewtrell, 2004), anencephaly (Croen et al., 2001), non-Hodgkin's lymphoma (Michal, 1998) and cardiovascular disorders (Morton, 1971).

A high percentage of acute respiratory tract infection with a history of recurrence has been reported in children consuming high nitrate content (Gupta et al., 2000b). Recurrent diarrhoea in children up to 8 years of age (Gupta et al., 2001) and recurrent stomatitis (Gupta et al., 1999) are also associated with high nitrate ingestion. It also affects the human immune system (Ustyugova et al., 2002). Some other reported effects are infant mortality, early onset of hypertension, hypothyroidism, diabetes and an adverse effect on cardiac muscles, alveoli of the lungs and adrenal glands (Gupta, 2006).

In animals, nitrate toxicity varies according to species. In general, ruminant animals develop methaemoglobinemia while monogastric animals exhibit severe gastritis (Bruning-Fann and Kaneene, 1993).

##### 4.2. Beneficial effects

Various studies suggest that nitrate is harmless and rather beneficial. It has been postulated as a useful nutrient (Dykhuisen et al., 1996). Bjorne et al. (2004) have suggested that dietary nitrate may serve important gastroprotective functions. Dietary nitrate is converted into nitrite by a symbiotic relationship involving nitrate-reducing bacteria on the tongue's surface. This relationship is designed to provide a host defense against

microbial pathogens in the mouth and lower gut (Duncan et al., 1995). The host provides nitrate, which is an important nutrient for many aerobic bacteria. In return, bacteria help the host by generating the substrate (nitrite) necessary for generation of nitric oxide in the stomach (Bjorne et al., 2004). A high concentration of nitrite present in saliva may, upon acidification, generate nitrogen oxides in the stomach (Duncan et al., 1995). Nitrite-derived nitric oxide and related compounds play an important role in gastric host defense by enhancing the acid-dependent killing of swallowed pathogens (Benjamin et al., 1994; Duncan et al., 1995; Dykhuizen et al., 1996; Xu et al., 2001). The high plasma nitrate levels in patients suffering from infective gastroenteritis may protect against the faecal-oral route of reinfection via increased generation of salivary nitrite (Dykhuizen et al., 1996). According to McKnight et al. (1999), nitrate in the diet is an effective host defense against gastrointestinal pathogens. It acts as a modulator of platelet activity, gastrointestinal motility and microcirculation.

It has also been suggested that the reactive nitrosating chemistry that occurs when saliva meets acidic gastric juice may also have biological benefit (McCull, 2005). Since this chemistry possesses antimicrobial activity (Duncan et al., 1997), it appears to be primarily designed to kill pathogenic microbes entering the body via the upper gastrointestinal tract (McCull, 2005). The beneficial effects of nitrate also include reduction of hypertension and cardiovascular diseases (McKnight et al., 1999). The various effects of nitrate on human health have been covered extensively by L'hirondel and L'hirondel (2002) in their book "Nitrate and man: Toxic, harmless or beneficial?".

Thus, evidence regarding the effect of nitrate on human health is conflicting. However, in view of the large number of its harmful effects on human health, it seems reasonable to take preventive measures so as to reduce accumulation of nitrate in plants and its subsequent consumption by human beings.

## 5. CONCLUSION

Vegetables are the major source of dietary nitrate intake of humans and nitrate has many detrimental and some beneficial effects on human health. Our understanding of the specific role of nitrate and its derivatives with respect to health is incomplete, for which further studies need to be carried out. The various approaches that may be adopted to reduce nitrate levels in vegetables are summarized below:

- A balanced fertilization program for vegetable crops should be chalked out to ensure an adequate, but not excessive supply of nutrients for optimum yield and quality, and avoid or minimize nitrogen losses to the environment. The supply and release of nutrients must be in synchrony with the need of the plant. Therefore, finding out an exact adjustment of a balanced fertilizer application to nitrate uptake and reduction capability of plants should be the priority for research by agronomists. For this purpose, strategies can be evolved with simulation models. Moreover, agronomic indicators need to be implemented at farm level to provide yardsticks for nutrient management by the farmers.
- Nitrate concentration in plants may be reduced by partial replacement of nitrate in the nutrient solution with ammo-

nium, urea, mixed amino acids, chloride or sulfate a few days prior to crop harvesting. Rational application of organic manure instead of inorganic nutrients, use of physiologically active substances, proper spray of nitrification inhibitors and molybdenum fertilizers, and growing plants under controlled environmental conditions may materially reduce nitrate accumulation in plants. Effects of other micronutrients and cultural conditions also need to be investigated. Selection among the available genotypes/cultivars and breeding of new cultivars that do not accumulate nitrate even under heavy fertilization may also limit human consumption of nitrate through vegetables.

- While there exists a large body of published research on strategies for reducing nitrate accumulation in plants, it remains to be translated into practice due to lack of information for farmers. Therefore, there is a need to bridge the gap between research laboratories and farmers' fields. Decision-makers must formulate relevant agricultural policies encompassing education and training of farmers to make them understand the effects of nitrate on human health and the importance of nutrient management and other strategies for minimizing the nitrate content in plant tissues. Consumers also need to be educated regarding the nitrate content in vegetables and its health implications. They must be motivated to adopt practices that help in minimizing nitrate consumption, e.g., harvesting of leafy vegetables at noon, removal of the plant organ rich in nitrate content, cooking vegetables in water containing less nitrate content, and use of fresh vegetables, etc.

- It is important to determine the exact physiological mechanism responsible for nitrate accumulation in plants. Moreover, the effect of fertilization and other cultivation practices on the physiological parameters, and their significance for enhancing crop production and quality need to be investigated. Molecular tools may be applied to reduce nitrate concentration in plants. Even though the literature suggests that genetic manipulation of activities of nitrogen assimilation enzymes may not increase yield and/or nitrogen-use efficiency of plants, we understand that overexpression of nitrate reductase genes may prove helpful in reducing the nitrate content of plants and, in consequence, improve the quality of plants for human consumption.

Therefore, an integrated collaboration among agronomists, physiologists, molecular biologists, farmers, consumers and policy-makers is the need of the hour and may yield satisfactory results towards minimizing accumulation of nitrate in plants and its subsequent consumption by human beings.

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