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REVIEW

Leaf senescence in response to elevated atmospheric CO_2 concentration and low nitrogen supply

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Abstract

This review reports the physiological and metabolic changes in plants during development under elevated atmospheric carbon dioxide concentration and/or limited-nitrogen supply in order to establish their effects on leaf senescence induction. Elevated CO_2 concentration and nitrogen supply modify gene expression, protein content and composition, various aspects of photosynthesis, sugar metabolism, nitrogen metabolism, and redox state in plants. Elevated CO_2 usually causes sugar accumulation and decreased nitrogen content in plant leaves, leading to imbalanced C/N ratio in mature leaves, which is one of the main factors behind premature senescence in leaves. Elevated CO_2 and low nitrogen decrease activities of some antioxidant enzymes and thus increase H_2O_2 production. These changes lead to oxidative stress that results in the degradation of photosynthetic pigments and eventually induce senescence. However, this accelerated leaf senescence under conditions of elevated CO_2 and limited nitrogen can mobilize nutrients to growing organs and thus ensure their functionality.

Additional key words: antioxidants, C/N ratio, gene expression, oxidative stress, photosynthesis, sugars.

Introduction

Senescence is the last stage of leaf development, after a period of active photosynthesis and biomass production. Although it is widely assumed as the final stage in the plant's life cycle, senescence is not the result of a degenerative process but of a programme aimed at the orderly degradation and recycling of certain structures and molecules that, at a given moment, cease to be of use to the plant (Lim *et al.* 2007). Leaf senescence involves not only a redistribution process through which nitrogen and other nutrients are transported to growing organs, but also cell death (Wiedemuth *et al.* 2005). Senescence processes occur under a strict genetic control. During senescence, mesophyll cells are dismantled in a programmed manner and undergo changes in structure, metabolism, and gene expression. The sequential degeneration of cell structures during senescence takes place in an orderly manner, starting with chloroplast dismantling (Lee and Chen 2002). As chloroplasts contain most protein in leaves, they provide a major source of nitrogen; thus, chloroplast degradation contributes more than 80 % of nitrogen received by grains in case of oilseed rape (Girondé *et al.* 2015). Contrasting with rapid chloroplast degradation, mitochondria and cell nucleus remain intact until the final stages of senescence since catabolic processes taking place in senescent tissue require the *de novo* synthesis of hydrolytic enzymes (proteases, nucleases, lipases, and chlorophyll degradation enzymes) (Lee and Chen 2002).

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Abbreviations: APX - ascorbate peroxidase; Asn - asparagine; Asp - aspartic acid; GDH - glutamate dehydrogenase; Glu - glutamic acid; Gln - glutamine; GS1 - cytololic glutamine synthetase; GS2 - chloroplastic glutamine synthetase; IPCC - intergovernmental panel on climate change; LHCP - light-harvesting chlorophyll-binding proteins; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase; ROS - reactive oxygen species; SAG - senescence associated gene; SLM - specific leaf mass; SOD - superoxide dismutase.

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Senescence is associated with a slower rate of photosynthesis, a decrease in content of photosynthetic pigment and proteins (Ougham *et al.* 2008). A loss of photosynthetic pigments was observed, and chlorophylls were more susceptible to degradation than carotenoids. Also, chlorophyll *a* is more affected than chlorophyll *b,* probably because the catabolic pathway is specific to chlorophyll *a* whereas chlorophyll *b* must be converted into chlorophyll *a* before it can be catabolized (Cabello *et al.* 2006). One biomarker widely used to determine the onset of senescence is the rapid loss of chlorophyll associated with the degeneration of chloroplast ultrastructure (Lim *et al.* 2007). Although chlorophyll degradation is one of the first symptoms of senescence, leaf yellowing is not a suitable marker of early senescence, since it is observed when the process has reached an advanced stage (Diaz *et al.* 2005).

Plastid proteins are mostly degraded during senescence (Hendry *et al.* 1987), thus confirming that a primary function of leaf senescence is to recycle nutrients, especially nitrogen remobilization (Himelblau and Amasino 2001). In several plant species, over-expression of NAC (No apical meristem - Arabidopsis transcription activation factor - Cup shaped cotyledon) transcription factor gene (*e.g.*, *HvNAC005)*, is involved in stress responses, senescence, and nutrient remobilization (Christiansen *et al.* 2016). Some proteins are degraded entirely in the chloroplast (*e.g.*, those of light harvesting chlorophyll-binding proteins, LHCP) whereas ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and other chloroplastic proteins may be broken down *via* a hybrid pathway involving both chloroplasts and extraplastidic compartments such as the central vacuole and small senescence-associated vacuoles (Martínez *et al.* 2008).

During senescence, carbon metabolism is replaced by catabolism of cell organelles and macromolecules. In plants containing low content of sugars, proteins are used as alternative substrates for respiration. In this way, available free amino acids allow senescence leaves to ensure maintenance of the growing organs; also, amino acids can be carried to sink organs, such as grains, in order to fuel protein synthesis and facilitate nitrogen storage in tissues (Aranjuelo *et al*. 2011).

In *Arabidopsis*, leaves also exhibit substantial lipid recycling and loose at least 80 % of total fatty acids during senescence (Yang and Ohlrogge 2009). Because the phosphorus content of soil is usually deficient, senescent plants develop effective mechanisms to mobilize phosphates stored in their tissues (Himmelblau and Amasino 2001), mainly through degradation of DNA and RNA in cell organs. A lower mobility of phosphate in leaves decreases the total phosphate content in seeds and so their ability to germinate (Robinson *et al.* 2012).

An oxidative stress is generated during senescence, as it is revealed by an increased cell-membrane lipid peroxidation (Srivalli and Khanna-Chopra 2004, Agüera *et al*. 2010) and accumulation of reactive oxygen species (ROS). ROS are continuously produced in different cell compartments during plant metabolism and can damage and even kill cells (Apel and Hirt 2004). They are produced by the metabolic activity in chloroplasts and/or peroxisomes (Rosenwasser *et al.* 2011). ROS production is elevated when plants are subjected to different stresses (Zulfugarov *et al.* 2011). Although ROS are removed by antioxidant defence systems under stable physiological conditions (Sharma *et al.* 2012), stressful environmental factors may disturb the equilibrium between production and scavenging of ROS (Vanacker *et al.* 2006). ROS can be removed *via* enzymatic and non-enzymatic mechanisms. Major ROS-scavenging enzymes included superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) (Mittler 2002). These enzymes play a major role in regulating free-radical content during leaf senescence (Procházková and Wilhelmová 2007, Liu *et al*. 2016). When defence systems fail to detoxify ROS, biological processes and cell structures are impaired (Sharma *et al.* 2012, Liu *et al.* 2016).

Nitrogen and carbon metabolism plays a fundamental role in the senescence process, and both external (CO_2 concentration, nitrogen availability, irradiance) and internal (regulating metabolites, C/N ratio) factors are involved in leaf senescence regulation (Wingler *et al*. 2006, Wingler and Roitsch 2008). The characterization of leaf senescence can be of major agronomic and economic importance, since acceleration of this process shortens plant life and diminishes crop yield (Cantamutto and Poverene 2007). Senescence develops differently among plant species (Cabello *et al*. 2006). In this paper, we briefly review physiological and metabolic changes in the leaf during development under conditions of elevated CO_2 and low nitrogen in order to identify their effects on leaf senescence.

Factors that determine the leaf senescence

When plants grow in a medium with adequate nutrition and they are free from biotic and abiotic stresses, leaf senescence starts and progresses in an age-dependent manner (Quirino *et al.* 2000). However, the senescence process can be prematurely induced when a plant is exposed to some nutritional imbalances (especially nitrogen deficiency) and environmental stresses. Elevated CO_2 and low N have been found to alter the expression of proteins and also to influence various aspects of photosynthesis, saccharide metabolism, nitrogen metabolism, and redox state in plants (Yousuf *et al.* 2016). In *Solanum lycopersicum*, Albacete *et al*. (2014)

observed that impaired growth in response to stress, results from assimilate accumulation in source leaves due to reduced sink activity, thereby triggering premature senescence.

Elevated ambient CO_2 concentration as a cause of early leaf senescence: Carbon dioxide is a natural, essential component of the earth's atmosphere. However, human activities and burning fossil fuels considerably raised atmospheric CO_2 concentration and this trend will continue in future years. Sustained emissions of CO_2 are in fact one source of climate change because this gas has the ability to absorb infrared radiation and raise temperature as a result (Taylor and MacCracken 1990). According to Gruissem *et al.* (2012), it is advisable to identify plants flexible enough to adapt to the changes derived from climate change.

Some studies using wheat and sunflower have shown that plants growing in elevated CO_2 exhibit increased photosynthetic rate, biomass and crop yield (Carlisle *et al.* 2012, De la Mata *et al*. 2012). Therefore, elevated CO_2 concentration might have a fertilizer effect, especially under favourable water and nutrient conditions. De la Mata *et al.* (2013) has found that sunflower plants grown under elevated CO_2 conditions display greater growth, evident in increased specific leaf mass (SLM) in young leaves. Leaf growth is controlled by cell division and expansion, which are controlled in a coordinated manner by some endogenous factors (including plant hormones), in response to environmental conditions (Riikonen *et al.* 2010). Increased cell expansion is due to increased activity of the enzyme xyloglucan endotrans-glycosydase, which leads to enhanced cell-wall extensibility (Ferris *et al.* 2001). Research in soybean and *Betula papyrifera* leaves has shown that elevated CO_2 increases the expression of genes involved in cell expansion and cell division (Kontunen-Soppela *et al.* 2010). However, prolonged exposure to elevated CO_2 reduces photosynthetic ability in some plant species (*e.g*., spring wheat). It also causes stomatal closure, reduces transpiration rate and Rubisco activity, increases leaf sugar content, and decreases content of proteins and nitrogen (Gutierrez *et al.* 2013).

Amounts of photosynthetic pigments decline during development of sunflower primary leaves; the fact that the decrease is more marked under elevated CO_2 suggests that it accelerates chlorophyll degradation and possibly also leaf senescence (De la Mata *et al.* 2012, Lotfiomran *et al.* 2016). Reduced activities of some antioxidant enzymes are reported during leaf development under elevated CO_2, while H_2O_2 content increases (Gillespie *et al.* 2011). These changes lead to oxidative stress, which prompts the degradation of photosynthetic pigments (Geissler *et al.* 2009). Srivalli and Khanna-Chopra (2009) showed that inappropriate production of oxidants and carbonyl groups is related to plant age and also to decreased mitochondrial APX and SOD activities, which may additionally enhance carbonylation in wheat leaf. In *Arabidopsis* and soybean, elevated CO_2 leads to an increase in carbonyl groups and a loss of leaf chlorophyll (Qiu *et al.* 2008). Protein carbonylation increases concomitantly with a stimulation of endoproteolytic activity and a decrease in protein content, suggesting a possible link between protein oxidation and proteolysis during natural leaf senescence (Havé *et al.* 2015).

Elevated CO_2 concentrations increases photosynthetic CO_2 fixation during development of sunflower plants. Stomatal conductance and transpiration rate in sunflower leaves decrease during leaf ontogeny (De la Mata *et al.* 2012). The stomatal response to elevated CO_2 varies considerably among species (Easlon *et al.* 2015), and in some species (*e.g*., *Helianthus annuus*), it appears to be unaffected. The lack of stomatal response to elevated CO_2 may be genetically determined, or may reflect adaptation to an atmosphere with high relative humidity (Haworth *et al.* 2016). Elevated CO_2 increases photosynthetic CO_2 fixation by increasing the Rubisco activity and reducing the photorespiration (Bloom 2015, Noguchi *et al.* 2015).

In sunflower, De la Mata *et al.* (2012) found that the hexose/sucrose ratio increases at the start of senescence, especially under elevated CO_2, while the starch content decreases. Increased expression of sucrose synthase and starch degradation may be associated with greater availability of saccharides under elevated CO_2 (Buchner *et al.* 2015). The increase in soluble sugars might also be a result of senescence, boosting membrane lipid metabolism, and increasing production of sugars by gluconeogenesis (Lim *et al.* 2007). Accumulation of soluble sugars at the onset of senescence has been observed in several plant species, although changes in sucrose content associated with leaf development remain unclear, and may vary among plant species (Diaz *et al.* 2005, Wingler *et al.* 2006). There is evidence that the sugar signalling pathway plays a major role in the regulation of senescence, although other pathways, such as those resulting from biotic and abiotic stresses, may also be involved (Wingler and Roitsch 2008, Schippers *et al.* 2015). Elevated CO_2 concentration usually causes accumulation of sugars and decrease in nitrogen content, thereby leading to an imbalanced C/N ratio in mature leaves (Pérez *et al.* 2005, Vicente *et al.* 2016).

Stitt and Krapp (1999) reported that various plant species grown under elevated CO_2 exhibit greater growth and increased nitrogen assimilation rate. However, studies in wheat, *Arabidopsis*, and sunflower have revealed a decrease in nitrogen assimilation under elevated CO_2 (Bloom *et al.* 2010, De la Mata *et al.* 2013, Bloom 2015, Buchner *et al*. 2015). Growth at elevated CO_2 and sufficient nitrogen supply results in increased plant biomass and sugar and starch content in sunflower plants (Canales *et al*. 2016). However, under elevated CO_2 and limited nitrogen, chlorosis and anthocyanin accumulation are observed, together with increased expression of senescence associated genes (SAGs).

Moreover, similar results are reported for plants grown in a high-sugar and limited-nitrogen medium, suggesting that an altered C/N ratio affects the senescence (Lotfiomra *et al.* 2016) since under these conditions, plants are nitrogen- rather than carbon-limited (Aoyama *et al.* 2014, Chen *et al.* 2015).

Elevated CO_2 concentrations reduce photorespiration rates and cytosolic NADH availability (Igamberdiev *et al.* 2001) thus impairing nitrate reduction (De la Mata *et al.* 2013, Bloom 2015, Vicente *et al.* 2016). Low cytosolic NADH availability diminishes the transport of malate from chloroplast to cytosol *via* the dicarboxylic acid translocator (Backhausen *et al.* 1998), and decreases nitrate assimilation (Quesada *et al.* 2000). Mariscal *et al.* (2006) observed that transporters of the nitrate assimilation related component 1 (Nar1) family are involved in transporting nitrite from the cytosol to the chloroplast in *Chlamydomonas*, and some of them transport both nitrite and HCO_3^-. An analogous system has been postulated in higher plants (Bloom *et al.* 2010), in which HCO_3^- inhibits nitrite influx into isolated wheat and pea chloroplasts. Decreased nitrite influx into the chloroplast may be the cause of the reduced glutamine synthetase (GS) activity observed in wheat and sunflower plants under elevated CO_2 (De la Mata *et al.* 2013, Buchner *et al.* 2015). Elevated CO_2 enhances a number of processes leading to the acceleration of sunflower leaf senescence, including the degradation of chloroplasts containing GS2 (McNally and Hirel 1983), which would account for the low amounts of GS2 transcripts under these conditions and for an increase in the relative expression of the GS1 isoform (De la Mata *et al.* 2013). SAGs induced during senescence include those encoding hydrolytic enzymes such as proteases, ribonucleases, and lipases (Buchanan-Wollaston *et al.* 2005). Other kinds of SAGs encode proteins involved in mobilizing degradation products, such as the GS1 isoform, which is responsible for recycling nitrogen from senescent tissue (Guo *et al.* 2004). In sunflower and tobacco, the activity and expression of genes encoding glutamate dehydrogenase (GDH) and GS1 are induced during senescence, while GS2 activity and expression show a continuous decrease with leaf ageing (De la Mata *et al.* 2013, Uzelac *et al.* 2016). In C3 plants, most of the ammonium assimilated under ambient CO_2 conditions arises from photorespiration, and it is assimilated by isoform GS2 (Stitt and Krapp 1999). Thus, elevated CO_2 alter nitrogen and carbon metabolism during leaf development and so raise the C/N ratio, which is a factor leading to premature leaf senescence (De la Mata *et al.* 2013, Buchner *et al.* 2015, Lotfiomaran *et al.* 2016).

Low nitrogen supply accelerates leaf senescence: Nitrogen is an essential element for plants and only C, H, and O occur in greater amounts in plant tissues. For this reason, nitrogen is considered to be the main factor governing plant growth and also crop yield (Marschner 2012). Thus, limited nitrogen supply affects both cell metabolism and plant growth by decreasing protein and chlorophyll content, and altering nitrogen and carbon metabolism (Agüera *et al.* 2010, Zhao *et al*. 2015). Plants usually acquire nitrogen from the soil in the form of nitrate or ammonium, but especially nitrate is the prevalent form in warm and well-aerated fields (Carlisle 2012). Under limited nitrogen supply, plants trigger senescence in order to mobilize N through degradation of proteins (Masclaux *et al.* 2000). Protein degradation in leaves is effected by proteases through the proteasome-ubiquitin system (Kurepa and Smalle 2008). Some proteases present in senescence-related vacuoles are used to degrade chloroplast-derived proteins (Carrion *et al*. 2013). The drop in chlorophyll content starts earlier under limited nitrogen indicating that low N content accelerates leaf senescence (Agüera *et al*. 2010). Generally, in plants grown under limited N supply, photosynthetic activity decreases during leaf development faster than chlorophyll content (Aranjuelo *et al.* 2013). Wingler *et al.* (2004) reported that low photosynthetic activity in conjunction with high chlorophyll content due to the imbalance between energy capture and dispersion, could induce oxidative stress by promoting O_2 photoreduction to superoxide anion radical.

In some plant species, low nitrogen prompts change that induce and accelerate leaf senescence (Wei *et al.* 2016) by activating SAG transcription (Buchanan Wollaston *et al.* 2005). It also causes sugar accumulation in *Arabidopsis* plants (Wingler *et al.* 2006, Wingler *et al.* 2009, Noguchi *et al.* 2015). In sunflower, Agüera *et al.* (2010) reported that senescence regulation by sugars is dependent on nitrogen supply, also supporting the hypothesis that leaf senescence is regulated by carbon and nitrogen signals. A high sugar to nitrogen ratio could act as a signal to promote the degradation of photosynthetic proteins in the old leaves to release nitrogen that would become available for the growth of young leaves (Wingler *et al.* 2004, Havé *et al.* 2017).

Agüera *et al.* 2010 found in sunflower plants grown under both low and enriched nitrogen supply, that the content of ammonium and free amino acids is high in young leaves, and decreases progressively in senescent leaves. In both treatments, content of asparagine (Asn) and in a lower degree content of glutamine (Gln) increase during the senescence. The drop in the ratio (Glu+aspartic acid)/(Gln+Asn) observed during leaf development indicates greater nitrogen mobilization. Moreover, the decline occurs earlier and more rapidly in nitrogen-deficient plants, suggesting that the N-mobilization rate correlates with leaf senescence.

Low nitrogen supply leads to decreased production of organic acids and most amino acids in *Arabidopsis* plants (Noguchi *et al.* 2015). *Arabidopsi*s growth at a variable C/N ratio revealed that this ratio plays a central role in the regulation of seedling growth, the mobilization of stored lipids, and the expression of photosynthetic genes

(Palenchar *et al*. 2004). In addition, leaf senescence can be triggered by a high availability of C and a low availability of N causing an imbalanced C/N ratio (Wingler *et al.* 2006, Aoyama *et al.* 2014).

Considerable oxidative stress is generated during sunflower primary leaf senescence in nitrogen-deficient plants, as revealed by the formation of ROS (particularly hydrogen peroxide) and by lipid peroxidation (Agüera *et al.* 2010). ROS are known to play a key role in regulating the physiological response to biotic and environmental stresses (Del Rio *et al.* 2015). In senescent leaves, the increase in hydrogen peroxide content occurs in tandem with a decline in antioxidant enzyme activities, which decreases earlier in nitrogen-deficient plants (Srivalli and Khanna-Chopra 2004, Agüera *et al.* 2010). Thus, accelerated senescence in nitrogen-deficient plants may be due to oxidative damage caused by the decline in antioxidant enzyme activities. Accelerated senescence of the sunflower primary leaves probably takes place in order to maintain the functionality of young leaves.

All this suggests that a set of signals including an increase in content of soluble sugars, C/N ratio, and oxidative stress may interact to accelerate leaf senescence under elevated CO_2 concentrations and low nitrogen supply, in order to mobilize nutrients to growing organs and preserve their functionality (Fig. 1).

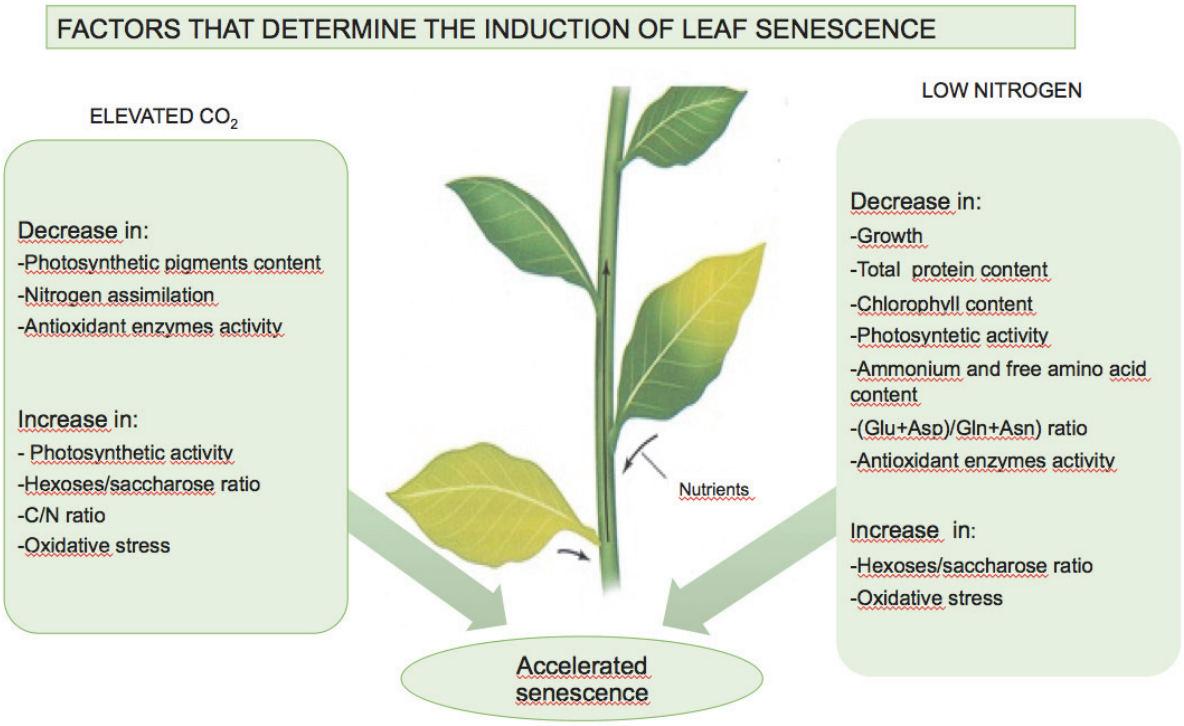

Fig. 1. Factors determining the induction of leaf senescence.

Future perspectives

The unceasing population growth and ongoing climate change have raised the crucial challenge of finding more productive crops for this century. Crop yields depend largely on the amount of assimilates that were formed and stored during the vegetative growth stage, as well as the onset of the senescence processes. An expanding body of studies on the effects of senescence on crop yield and quality is being aimed at identifying species capable of adapting more efficiently to an altered environment (Thomas and Ougham 2014). Some plants can adjust their life cycle to adverse environmental conditions even before their integrity and viability are compromised by degenerative processes related to senescence (Thomas 2013). Plants acquire the ability to senesce during leaf development, and the internal and external factors are integrated with age to determine the beginning of senescence. There is the widespread belief that delayed senescence facilitates prolonged formation of assimilates and improves crop productivity as a result. Thus, Liang *et al.* (2014) found that reduced *OsNAP* (*Oryza sativa* - NAC like - activated - apetala3/pistillata) expression in *Oryza sativa* delays leaf senescence and extends the grain-filling period, ultimately resulting in a 6 - 10 % increase in grain yield. In wheat, overexpression of *NAC* transcription factor, which delays leaf senescence, may increase not only grain yield, but also grain protein

content. On the other hand, early senescence may make plants more suitable for agricultural purposes under adverse conditions. Thus, early senescence in wheat has been associated to an increase in the content of nutrients, such as iron and zinc in grains, and hence to an improved nutritional value (Uauy *et al.* 2006). As a rule, appropriate mobilization of nutrients increases the efficiency in their use and can, to some extent, reduce the need for crop fertilization.

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