



**HAL**  
open science

## Legume nodule senescence: a coordinated death mechanism between bacteria and plant cells

Théophile Kazmierczak, Li Yang, Eric Boncompagni, Eliane Meilhoc, Florian Frugier, Pierre Frenedo, Claude Bruand, Véronique Gruber, Renaud Brouquisse

► **To cite this version:**

Théophile Kazmierczak, Li Yang, Eric Boncompagni, Eliane Meilhoc, Florian Frugier, et al.. Legume nodule senescence: a coordinated death mechanism between bacteria and plant cells. Regulation of Nitrogen-Fixing Symbioses in Legumes, 94, Academic press, Elsevier, 2020, Advances in Botanical Research, 978-0-08-102798-1. 10.1016/bs.abr.2019.09.013 . hal-02493529

**HAL Id: hal-02493529**

**<https://hal.science/hal-02493529>**

Submitted on 17 Dec 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Legume nodule senescence: a coordinated death mechanism between**  
2 **bacteria and plant cells**

3

4 Authors: Kazmierczak Théophile<sup>1,4</sup>, Yang Li<sup>2</sup>, Boncompagni Eric<sup>2</sup>, Meilhoc Eliane<sup>3</sup>, Frugier  
5 Florian<sup>1</sup>, Frendo Pierre<sup>2</sup>, Bruand Claude<sup>3</sup>, Gruber Véronique<sup>1</sup>, Brouquisse Renaud<sup>2</sup>

6

7 <sup>1</sup> *Institute of Plant Sciences Paris Saclay (IPS2), CNRS, INRA, Univ Paris Diderot, Univ*  
8 *Paris-Sud, Univ d'Evry, Univ Paris-Saclay, Bâtiment 630, 91190 Gif-sur-Yvette, France*

9 <sup>2</sup> *INRA, CNRS, Université Côte d'Azur, ISA, France*

10 <sup>3</sup> *Laboratoire des Interactions Plantes-Microorganismes (LIPM), Université de Toulouse,*  
11 *INRA, CNRS, INSA, Castanet-Tolosan, France*

12 <sup>4</sup> *BIOtransfer, 41 rue Emile Zola, 93100 Montreuil*

13

14 Corresponding author: Renaud Brouquisse  
15 [renaud.brouquisse@inra.fr](mailto:renaud.brouquisse@inra.fr)  
16 +33 (0) 492 38 66 38  
17 ISA, 400 route des Chappes, BP 167, 06 903 Sophia-Antipolis,  
18 France

19

20 **Contents**

21 1. Introduction

22 2. The regulation of nodule senescence

23 2.1 Physiological and structural modifications

24 2.2 Redox state, ROS and RNS

25 2.3 Hormones

26 2.4 Proteases associated with bacteroid and plant cell degradation

27 3. Transcriptomic characterization of nodule senescence

28 4. Concluding remarks and future prospects

29 Acknowledgements

30 References

31

32 Keywords: hormones, legumes, proteases, Rhizobium, reactive oxygen species, reactive  
33 nitrogen species, senescence.

34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55

**Abstract**

In the legume-rhizobia symbiosis, atmospheric dinitrogen (N<sub>2</sub>) fixation by rhizobia takes place in a specific root organ, called the nodule. During biological N<sub>2</sub> fixation, the plant provides photosynthetic carbohydrates to bacteria and, in exchange, bacteria feed the plant with reduced nitrogen (N) under ammonia (NH<sub>3</sub>) form. Like most other organs, the root nodule has a limited lifespan, and eventually enters a senescence process characterized by a decline of N<sub>2</sub> fixation and the coordinated death of both bacteria and plant cells. This senescence process characterized by a metabolic switch from a carbon sink to a nutrient source either results from nodule aging (developmental nodule senescence) or can be triggered prematurely by adverse environmental conditions. Accumulating evidence suggests that redox and hormone signaling contribute to the triggering of senescence. Indeed, nodule senescence is a developmentally programmed process in which antioxidants, reactive oxygen and N species (ROS/RNS), hormones and proteinases play key roles. This chapter reviews recent knowledge on nodule senescence and proposes models by which ROS, RNS, and likely hormones intervene in the orchestration of this process.

## 56 1. Introduction

57

58 Plant-microbe interactions represent a major aspect of the formation and of the  
59 development of ecosystems. Plants often establish symbiotic relationships with specific fungi  
60 and bacteria to acquire the nutrients they need to support growth and development (Wang et  
61 al., 2017; Smith and Smith, 2011; van der Heijden et al., 2016; Venkateshwaran et al., 2013).  
62 Amongst the different nutrients, nitrogen (N) has a major importance as it represents a crucial  
63 constituent of proteins, nucleic acids and chlorophyll and is often in insufficient amounts in  
64 the soil under nitrate and/or ammonia forms. As a result, plants belonging to the legume  
65 family have developed a symbiotic association with soil bacteria collectively called rhizobia  
66 to increase their N nutrition via the biological fixation of atmospheric dinitrogen (N<sub>2</sub>) (BNF).  
67 In BNF, the plant provides photosynthetic carbohydrates to the bacteria and, in exchange,  
68 bacteria feed the plant with reduced N under ammonia (NH<sub>3</sub>) form. Legumes are a family of  
69 dicotyledonous plants of the *Fabales* order. It is the third most important family of flowering  
70 plants after the *Orchidaceae* and *Asteraceae* with more than 19,500 species (Christenhunz &  
71 Byng, 2016). Economically, *Fabaceae* is the second largest family after *Poaceae* and is a  
72 valuable source of plant proteins for human food and animal feeding.

73 The success of the symbiotic interaction relies on the molecular dialog between the  
74 plant and the bacteria (Oldroyd, 2013). The plant root exudates which contain flavonoids and  
75 isoflavonoids induce the production by rhizobia of lipochitooligosaccharides, called  
76 Nodulation Factors (NFs; Fergusson et al., 2010). NFs are usually essential for the bacterial  
77 infection process and the organogenesis of the root nodule, the organ which hosts the N<sub>2</sub>-  
78 fixing bacteria. During the infection process, rhizobia attach to root hairs in the susceptible  
79 zone of the host roots. The recognition of the NFs by the plant induces the root hair curling  
80 and the formation of an infection pocket (Fig. 1). Then, bacteria enter into the root hairs  
81 through a membrane invagination and the formation of tubular structures, the infection  
82 threads (Fig. 1), which allow the transit of the bacteria from the root surface to the cells of the  
83 neo-formed nodule (Timmers et al., 1999). Later, the bacteria are endocytosed into the plant  
84 cell in a new organelle-like structure, called symbiosome. Bacteria inside the symbiosome,  
85 called bacteroids, become able to reduce N<sub>2</sub> to NH<sub>3</sub> thanks to the activity of a specific  
86 enzymatic complex, the nitrogenase (Peters et al., 1995).

87 Two predominant nodule types exist in legumes (Fig. 1). The nodule type depends on  
88 the host plant and is determined by the nature of the nodule meristem which could be either  
89 transient or persistent (Oldroyd, 2013). Determinate nodules, with a transient meristem, are

90 spherical since cell divisions occur only during a short period of time. The final growth of the  
91 nodule is achieved by cell enlargement. In contrast, indeterminate nodules, with a persistent  
92 meristem, are cylindrical as they grow during the whole nodule life by increasing the number  
93 and the size of cells. Determinate nodules are generally formed in tropical legumes such as  
94 *Phaseolus*, *Vigna* and *Glycine*, whereas indeterminate nodules are found in temperate legumes  
95 such as *Medicago*, *Trifolium* or *Pisum*.

96 Beside the nodule types, the bacteroids possess also three different morphotypes  
97 named U, S and E (Kondorosi et al., 2013; Oono et al., 2010). The U-morphotype (for  
98 Undifferentiated) corresponds to bacteroids with minor morphological modifications  
99 compared to the free-living bacteria and the ability to reverse from the symbiotic lifestyle to  
100 the free-living state. The S- and E- morphotypes correspond to large Spherical and large  
101 Elongated bacteroids, respectively. These bacteroids cannot return to free-living life and are  
102 termed “terminally differentiated”. This terminal differentiation is associated with the increase  
103 of permeability of the bacteroid membrane and of DNA content compared to free-living  
104 bacteria (Mergaert et al., 2006). The bacteroid differentiation is driven by the plant host via  
105 the production of a class of peptides called nodule-specific cysteine-rich peptides (NCRs; Van  
106 de Velde et al., 2010). To date, NCR peptides have been found to control the terminal  
107 differentiation of endosymbionts in inverted repeat-lacking clade (IRLC) legumes and in  
108 *Aeschynomene* spp. (Mergaert et al., 2003; Alunni et al., 2007; Czernic et al., 2015). At the  
109 functional level, NCRs induce membrane permeability (Lamouche et al., 2019) and have  
110 intracellular bacteroid targets such as the cell division protein FtsZ and the chaperone protein  
111 GroEL, suggesting that NCRs interact with the bacterial metabolism to induce the terminal  
112 differentiation (Farkas et al., 2014).

113 The cellular differentiation of both symbiotic partners is required to allow the  
114 symbiotic N<sub>2</sub> fixation. The production of the nitrogenase enzymatic complex in the bacteroid  
115 is a prerequisite for the reduction of N<sub>2</sub> to NH<sub>3</sub>. This complex needs high amounts of energy,  
116 using sixteen ATP for each N<sub>2</sub> reduced to NH<sub>3</sub>, and as a result demands a high respiration rate  
117 in the nodule. However, the nitrogenase activity is extremely sensitive to dioxygen (O<sub>2</sub>)  
118 implying that free O<sub>2</sub> should be present at a very low level in bacteroids (Peters et al., 1995).  
119 Multiple strategies allow to deal with this paradox. To decrease the O<sub>2</sub> level in the infected  
120 cells, the nodule inner cortex cell layer acts as an oxygen barrier (Tjepkema and Yocum,  
121 1974). In parallel, leghemoglobin (Lb), an efficient O<sub>2</sub>-binding protein, present at millimolar  
122 concentration in the cytoplasm of infected cells (Appleby, 1992), delivers O<sub>2</sub> to the  
123 mitochondria and the bacteroids for respiration and decreases free O<sub>2</sub> to the nanomolar range

124 in the N<sub>2</sub>-fixing zone (Soupène et al., 1995; Ott et al., 2005). Finally, the bacteroid is able to  
125 function at a very low oxygen concentration thanks to a specific high affinity *cbb3*  
126 cytochrome oxidase (Preisig et al., 1996). The reduction of N<sub>2</sub> to NH<sub>3</sub> is accompanied by the  
127 interruption of NH<sub>3</sub> assimilation into bacteroid amino acids. NH<sub>3</sub> is secreted from the bacteroid  
128 into the plant cytosol, for assimilation into the amides glutamine and asparagine, or into  
129 ureides (Day et al., 2001). In return, the plant provides carbon and energy sources to  
130 bacteroids in the form of dicarboxylic acids, particularly malate and succinate, which are  
131 produced from sucrose via sucrose synthase and glycolytic enzymes. Thus, the nodule  
132 presents multiple peculiarities, involving a plant-microbe crosstalk associated with a  
133 metabolism switch which needs a high energy level under microoxic conditions. These  
134 working conditions involve a specific cellular redox control in both symbiotic partners. The  
135 contribution of reactive oxygen and N species (ROS and RNS) and the importance of the  
136 antioxidant defence in the formation and the functioning of root nodules have been largely  
137 demonstrated (Alloing et al., 2018; Damiani et al., 2016; Frendo et al., 2013; Puppo et al.,  
138 2013).

139 Whereas formation and functioning of the root nodule has been extensively analysed  
140 the rupture of the interaction leading to the nodule senescence has not been much  
141 characterized and only few reviews have been dedicated to nodule senescence (Puppo et al.,  
142 2005; Dupont et al., 2012; Serova and Tsyganov, 2014; Bruand and Meilhoc, 2019). Some  
143 investigators have used the initial decline in nitrogenase activity and BNF efficiency as an  
144 indication that nodule senescence has begun (Klucas, 1974). Others authors have defined  
145 nodule senescence as the period of rapid nodule decay which occurs at a plant physiological  
146 maturity stage, i.e. at leaf yellowing (Streeter, 1981), after flowering has finished (Serova and  
147 Tsyganov, 2014), or at pod filling (Lawn and Brun, 1974; Bethlenfalvay and Phillips, 1977).  
148 The nodule senescence represents a strong agronomical interest through the possible  
149 improvement of N<sub>2</sub> fixation efficiency under adverse environmental conditions to maintain or  
150 even increase the yield of legume crops. In this review, we summarize the different  
151 modifications of nodule functioning observed during the set up and the progress of nodule  
152 senescence, and we focus on the role of antioxidants, ROS/RNS, hormones and proteases in  
153 regulating this process.

154

## 155 **2. The regulation of nodule senescence**

156

### 157 ***2.1 Physiological and structural modifications***

158

159           Although the lifespan of nodules of most legumes is 10-12 weeks, their ability to fix N  
160 starts to decline 3-5 weeks post-inoculation (Puppo et al., 2005). Nodule senescence is a  
161 highly organized and genetically controlled process that begins with metabolic changes. As  
162 nodules and leaves are tightly connected in a reciprocal sink-to-source relationship for carbon  
163 and N, any defect in photosynthetic activity will indeed impair the metabolic functions of the  
164 nodule associated with N assimilation and consequently provoke nodule senescence (Fig. 2).  
165 Developmental senescence (also known as natural senescence) occurs because of organ aging,  
166 but exposure to various environmental changes also induces nodule senescence (Fig. 2), e.g.  
167 when plants are submitted to drought (Gogorcena et al., 1997; Aranjuelo et al., 2013; Gil-  
168 Quintana et al., 2015), cadmium stress (Balestrasse et al., 2004; Redondo et al., 2009),  
169 photosynthesis inhibitors (Müller et al., 2001), prolonged darkness (Matamoros et al., 1999;  
170 Hernandez-Jimenez et al., 2002; Perez-Guerra et al., 2010; Pierre et al., 2014), defoliation  
171 (Vance et al. al., 1979), or treatments with argon (Curioni et al., 1999), nitrate (Matamoros et  
172 al., 1999; Pierre et al., 2014) or abscisic acid (ABA; Gonzalez et al., 2001). Alternately,  
173 senescence can result from a deficient recognition between the two partners (Banba et al.,  
174 2001). Depending on the type of triggering factor, the senescence process is more or less rapid  
175 and implement variable responses (Van de Velde et al., 2006), but a number of common  
176 typical features are found in most nodule senescence processes.

177           During developmental senescence, the first sign of nodule senescence is the drop in  
178 measurable N<sub>2</sub> fixation activity that occurs in parallel with a decrease in Lb content (Dalton et  
179 al., 1986; Lahiri et al., 1993). However, during induced senescence, such as after dark  
180 treatment, drought stress, or ABA treatment, the rapid decline in N<sub>2</sub> fixation occurs well  
181 before a decrease in Lb transcripts or amounts is observed (Escuredo et al., 1996; Gogorcena  
182 et al., 1997; Matamoros et al., 1999; Gonzalez et al., 2001). The most visible sign of  
183 senescence is a change of the nodule color, from pink to green, caused by bilirubin and  
184 biliverdin accumulation after disruption of the Lb structure (Lehtovaara and Pettilä, 1978;  
185 Swaraj and Bishnoi, 1996). In senescent soybean nodules, the green color comes partially  
186 from Lb nitration, which highlights the critical role of ROS and RNS in the senescence  
187 process (Meakin et al., 2007; Navascués et al, 2012; Sainz et al., 2015).

188           In nodules of the determinate type, senescence develops radially (Fig. 1), starting from  
189 the center of the nodule and then progressively extending to the peripheral tissues in a few  
190 weeks (Puppo et al., 2005). In nodules of indeterminate type, the N<sub>2</sub> fixation zone is  
191 constantly renewed and the senescence zone (zone IV) grows as the nodule grows (Fig. 1); it

192 increases from the root proximal zone towards the apical zone of the nodule (Puppo et al.,  
193 2005; Perez-Guerra et al., 2010; Montiel et al., 2016). Because this zonation is not strictly  
194 defined, a senescence inter-zone is sometimes described between zone III and zone IV.  
195 Histologically, early nodule senescent cells exhibit a less dense cytoplasm containing  
196 numerous vacuoles, altered peroxisomes, mitochondria, starch granules, and late senescent  
197 cells ultimately accumulate damaged “ghost” membranes and cell walls (Puppo *et al.*, 2005;  
198 Pérez-Guerra *et al.*, 2010). In common bean nodule developmental senescence, mitochondria  
199 were found to be early targets of oxidative modifications and a potential source of ROS/RNS  
200 (Matamoros et al., 2013). A dynamic evolution of both plant and bacteroid cell structures is  
201 observed: bacteroids are first degraded whereas plant cells remain alive during the early  
202 senescence stage, allowing nutrient remobilization, even though at later stages plant cells are  
203 also degraded (Vasse et al., 1990; Van de Velde *et al.*, 2006). In senescent cells, the  
204 degradation of symbiosomes is correlated with the loss of the acidity of the peribacteroid  
205 space (Pierre *et al.*, 2013), this acidification being an indicator of the bacteroid functioning in  
206 the nodule N fixing zone (Pierre *et al.*, 2013).

207 The senescence process was analyzed at gene expression level in both leaves and  
208 nodules, particularly in *Medicago truncatula* (Van de Velde et al., 2006, De Michele et al.,  
209 2009). It appeared that nodule senescence has a significant degree of overlap with leaf  
210 senescence, arguing for the recruitment of similar pathways. A strong redox regulation,  
211 through antioxidants decrease and ROS/RNS accumulation, as well as hormonal regulations  
212 such as abscisic acid, ethylene or jasmonic acid, was evidenced to correlate with catabolic and  
213 remobilization processes during nodule senescence (Puppo et al., 2005; Van de Velde et al.,  
214 2006).

215

## 216 **2.2 Redox state, ROS and RNS**

217

218 Changes in the redox balance, which modify the antioxidant defence and the level of  
219 ROS and RNS, is directly associated with the transition between N<sub>2</sub> fixation and senescence  
220 in nodules (Fig. 2; Puppo et al., 2005).

221 Ascorbate (ASC) and glutathione (GSH) are the main reported soluble antioxidants  
222 within nodules and their regulation was supposed to control the organ lifespan (Loscos *et al.*,  
223 2008; Becana *et al.*, 2010). In soybean plants, drought-induced nodule senescence is  
224 characterized by a decrease in ASC reduction state and an increase in GSH content (Marquez-  
225 Garcia *et al.*, 2015). In bean nodules, the progression of developmental senescence correlates



226 with a decrease in both ASC and GSH concentrations and reduction states (Loscos *et al.*,  
227 2008). Moreover, the decrease in ASC and GSH concentrations in mitochondria correlates to  
228 an increase of oxidative modifications of lipids and proteins, supporting the hypothesis that  
229 the mitochondria is an early target of senescence in nodules (Matamoros *et al.*, 2013). In  
230 contrast, an increased level of GSH in the N<sub>2</sub>-fixing zone is correlated with a higher N<sub>2</sub>  
231 fixation efficiency (El Msehli *et al.*, 2011). GSH content is notably involved in the control of  
232 glutathione *S*-transferases (GSTs) activities, which are enzymes involved in the cell protection  
233 from an oxidative damage, by catalyzing the conjugation of GSH with oxidative degradation  
234 products. The level of the GST9 protein, the most prevalent glutathione-*S*-transferase in  
235 soybean nodules, is accordingly increased during senescence (Dalton *et al.*, 2009). A decrease  
236 in N<sub>2</sub> fixation associated with an increase of the oxidatively damaged protein content is  
237 observed in GST9-RNAi silenced soybean nodules, illustrating the role of GSTs in nodule  
238 senescence through the regulation of antioxidant defences (Dalton *et al.*, 2009). In parallel,  
239 the importance of GSH during symbiosis of *Sinorhizobium meliloti* and *Rhizobium tropici* has  
240 been also demonstrated (Harrison *et al.*, 2005; Muglia *et al.*, 2008). The *S. meliloti* and *R.*  
241 *tropici* strains deficient in *gshB*, the gene encoding the enzyme catalysing the second step of  
242 GSH synthesis, have a reduced N<sub>2</sub> fixation capacity associated with early nodule senescence.  
243 Similarly, mutation in the *S. meliloti* glutathione reductase gene, *gor*, impaired N<sub>2</sub> fixation  
244 efficiency (Tang *et al.*, 2018).

245 During the legume-rhizobium symbiosis, both ROS and RNS, and especially nitric  
246 oxide (NO), appear to play crucial roles in the establishment and functioning of the interaction  
247 (Puppo *et al.*, 2013; Hichri *et al.*, 2015). ROS including superoxide radicals (O<sub>2</sub><sup>•-</sup>) and  
248 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) are produced in high amounts in nodules due to elevated rates of  
249 respiration in bacteroids and plant mitochondria (Matamoros *et al.*, 2013). Plant NADPH  
250 oxidases, known as Respiratory Burst Oxidase Homologues (RBOHs), are plasma membrane  
251 enzymes catalyzing the reduction of O<sub>2</sub> to generate O<sub>2</sub><sup>•-</sup> using the reducing power of NADPH.  
252 Seven *Rboh* genes have been identified in legume genomes (Marino *et al.*, 2011) and these  
253 oxidases are involved in legume-rhizobium symbioses (Arthikala *et al.*, 2014; Marino *et al.*,  
254 2011; Montiel *et al.*, 2016). Other possible sources for H<sub>2</sub>O<sub>2</sub> are cell wall peroxidases,  
255 germin-like oxalate oxidases, and diamine oxidases (Smirnoff and Arnaud, 2019). On the  
256 other hand, NO is a reactive diffusible gas known to have important functions as inter- or  
257 intra-cellular signaling molecules in plant growth, development and defence against abiotic or  
258 biotic stresses (Brouquisse, 2019, and references therein). For a decade, NO has been also

259 identified at all stages of legume-rhizobium symbiotic interactions (Hichri et al., 2015, 2016;  
260 Berger et al., 2019). The way NO is produced in legumes is not completely clear. The best  
261 characterized sources of NO are the nitrate reductase (NR) and the mitochondrial transfer  
262 chain (Horchani et al., 2011). Interestingly, it has been shown in soybean and *M. truncatula*  
263 nodules that the micro-symbiont accounts for 30% up to 90% of NO production thanks to a  
264 nitrite reductase (Nir) belonging to the denitrification pathway (Calvo-Begueria et al., 2018;  
265 Horchani et al., 2011; Sánchez et al., 2010). Even though ROS and NO are different types of  
266 molecules issuing from distinct synthesis pathways, some parallels can be established when  
267 looking at their occurrence during symbiosis. Strikingly, NO and ROS have comparable  
268 production patterns during symbiosis such as an early production burst in the root in response  
269 to rhizobia inoculation, later in the infection thread, then during nodule organogenesis and  
270 finally in mature nodules (Hichri et al., 2016; Montiel et al., 2016).

271 Interestingly, NO and ROS play a role in nodule senescence. At a late stage of the  
272 nodulation process (e.g. 6 weeks post-inoculation), H<sub>2</sub>O<sub>2</sub> is detected in ultrathin sections of  
273 nodules (Puppo et al., 2013). At the ultrastructural level, the senescent nodules of *Glycine*  
274 *max* and *Pisum sativum* plants display alterations in the symbiosome structure accompanied  
275 by the presence of H<sub>2</sub>O<sub>2</sub>, supporting the idea that ROS have roles also during senescence  
276 (Montiel et al., 2016). In addition, it has been suggested that NO is present in higher amounts  
277 during the late symbiotic stages than at earlier stages (Bruand and Meilhoc, 2019). Indeed,  
278 NO detected by means of a bacterial biosensor is more abundant in the root-proximal part of  
279 *M. truncatula* nodules, where senescence is known to initiate (Cam et al., 2012). Similarly,  
280 NO detected by means of a fluorescent probe accumulates in old *Lotus japonicus* nodules and  
281 Lbs with nitrated heme groups are found in old soybean nodules, indicating the presence of  
282 NO in ageing nodules (Chungopast et al., 2017; Navascués et al., 2012; Sainz et al., 2015).  
283 Remarkably, exogenous addition of a NO donor on mature nodules of *M. truncatula* lead to  
284 an accelerated senescence of the nodules (Cam et al., 2012). Also, several *S. meliloti* mutant  
285 strains, altered on *hmp*, *nnrS1*, *nnrS2* and *norB* genes (Table 1) and showing either increased  
286 or decreased NO degradation capacity compared to the wild-type, were used to modulate NO  
287 levels within nodules and, strikingly, strains deficient in NO degradation induced an early  
288 nodule senescence, whereas strains with higher degradation activity led to a delayed nodule  
289 senescence (Blanquet et al., 2015; Cam et al., 2012; Meilhoc et al., 2013). NO is also  
290 involved in stress-induced *M. truncatula* nodule senescence (Cam et al., 2012). Comparable  
291 results were recently obtained in determinate *L. japonicus* nodules (Fukudome et al., 2019).

292 Indeed, overexpression of a phytolegumin gene *LjGlb1-1* (Table 1) decreased NO levels in  
293 nodules and delayed nodule senescence.

294 By analogy, Redondo et al. (2009, 2012) reported that overexpression in *S. meliloti* of  
295 a cyanobacterial flavodoxin involved in the response to oxidative stress in bacteria and  
296 cyanobacteria improves the oxidative balance, delays developmental nodule senescence in  
297 *Medicago sativa*, and enhances tolerance to cadmium. Overexpression of this flavodoxin also  
298 permitted to avoid the decline in nitrogenase activity associated with a salinity stress whether  
299 the flavodoxin was expressed in *S. meliloti* or in *M. truncatula* (Coba de la Peña et al., 2010).  
300 In *S. meliloti*, various ROS scavenging enzymes (katA and katC catalases particularly) are  
301 expressed in the N<sub>2</sub>-fixing zone (Roux et al., 2014). The *S. meliloti katA katC* double mutant  
302 displays a reduced N<sub>2</sub> fixation activity and bacteroids underwent faster senescence (Puppo et  
303 al., 2013). *R. etli* and *B. japonicum* possess only one catalase gene and corresponding mutants  
304 were not affected in nodule functioning, but ROS scavenging enzymes other than catalases  
305 might reduce H<sub>2</sub>O<sub>2</sub> (Puppo et al., 2013). This might also suggest a different role for ROS and  
306 antioxidant defence components in the senescence of determinate and indeterminate nodules.

307 A drastic reduction in N<sub>2</sub> fixation was observed in *Phaseolus vulgaris* nodules silenced  
308 in NADPH oxidase (*Rboh*) genes probably linked to an early senescence phenotype  
309 (Arthikala et al., 2014). By contrast, overexpression of *RbohB* delays senescence of the  
310 nodule. Hence, these elements suggest that ROS produced by RBOHs may have either a  
311 promoting, or an inhibiting role during nodule senescence in *P. vulgaris*. Two *Rboh* genes  
312 (*MtRbohA* and *MtRbohB*) were found specifically expressed in the N<sub>2</sub>-fixing zone of *M.*  
313 *truncatula* nodules (Marino et al., 2011; Montiel et al., 2016; Roux et al., 2014). A decrease in  
314 *MtRbohA* expression via a RNA interference (RNAi) approach led to a reduction in the N<sub>2</sub>  
315 fixation capacity, showing again the link between MtRBOHA and nodule function (Marino et  
316 al., 2011). However, none of these genes displays any detectable promoter activity in the  
317 senescence zone of indeterminate nodules, suggesting that they do not directly act in nodule  
318 senescence. In addition, RBOHs might have a more prominent role in determinate nodules  
319 than in indeterminate nodules, even though the possibility that they participate in nodule  
320 senescence should not be eliminated before each gene has been studied in detail in different  
321 plants.

322 Hypotheses on mechanisms by which NO and ROS could trigger nodule senescence  
323 are multiple. These molecules can both act at the transcriptional and post-translational levels.  
324 Indeed, NO is known to activate the expression of bacterial as well as plant genes (Boscari et  
325 al., 2013a; Ferrarini et al., 2006; Meilhoc et al., 2010). However, it is unknown yet which

326 genes or transcription factors (TFs) could be involved at the onset of nodule senescence. One  
327 of the main downstream effects of NO is the post-translational modification of proteins such  
328 as S-nitrosation of cysteine residues or nitration of tyrosine residues, which might cause  
329 conformational changes in the protein with concomitant changes in their activities, turnover or  
330 localization. About 80 proteins were found to be S-nitrosated in *M. truncatula* nodules;  
331 among them 20 and 8 plant and bacterial proteins, respectively, were also found to be  
332 sulfenylated by ROS (Puppo et al., 2013). These protein targets remain to be characterized to  
333 establish a potential link with the nodule senescence. However, the activity of many of them,  
334 involved either in the glycolysis, the tricarboxylic acid cycle, the energy regeneration or the  
335 N<sub>2</sub> assimilation, were found to be inhibited by NO, suggesting that an increased NO  
336 production could directly down-regulate N, carbon and energy metabolism in senescent  
337 nodules (Boscari et al., 2013b). Interestingly, the enzymatic activity of the plant glutamine  
338 synthetases (GS) involved in N assimilation decreases when GS is subjected to NO-mediated  
339 tyrosine nitration (Melo et al., 2011). Nevertheless, methionine sulfoxidation of GS is also  
340 important for its regulation in response to ROS, showing again that both NO and ROS can be  
341 involved in the regulation of protein activity (Matamoros et al., 2013). It has also been shown  
342 that the nitrogenase activity is inhibited by NO (Trinchant and Rigaud, 1982).

343 NO and ROS are generated in cells at the same time and in response to the same cues,  
344 so that it is important to consider their possible interaction and associated consequences. One  
345 of the best known interaction between NO and ROS is the production of peroxynitrite  
346 (ONOO<sup>-</sup>) from NO and superoxide anion (O<sub>2</sub><sup>-</sup>). This has two potentially important outcomes:  
347 first, the reaction removes both O<sub>2</sub><sup>-</sup> and NO from the cell environment, thereby reducing their  
348 bioavailability; and second, there is a new compound produced (ONOO<sup>-</sup>) which itself can act  
349 as a signaling/regulatory molecule, potentially giving different responses than that resulting  
350 from ROS or NO signaling alone. As an example, peroxynitrite is more potent than NO alone  
351 to trigger tyrosine nitration (Astier and Lindermayr, 2012).

352 NO/ONOO<sup>-</sup>-dependent modifications may also alter signaling pathways mediated by  
353 other reactive signals. Indeed, Tyr nitration of superoxide dismutase can inhibit its activity  
354 and hence alter ROS signaling (Holzmeister et al., 2015). NO has also been proposed as a  
355 negative regulator of RBOH activity in nodulation (Damiani et al., 2016). Indeed, it has been  
356 reported that the AtRBOHD enzyme is S-nitrosated which thus reduces its ROS generating  
357 activity (Yun et al., 2011). Therefore, NO has a role in controlling ROS levels and hence their  
358 associated downstream signaling. In addition, the *B. japonicum* key regulator of the N<sub>2</sub>-fixing

359 enzymes FixK<sub>2</sub> is reversibly susceptible to oxidation, which might indirectly have an  
360 influence on the expression of bacterial genes involved in the NO metabolism regulated by  
361 FixK<sub>2</sub> (Mesa et al., 2009). Similarly, LsrB, a TF involved in the nodulation and redox  
362 regulation, is also regulated by its redox state and might be involved in the senescence process  
363 (Tang et al., 2017).

364

### 365 **2.3 Hormones**

366

367 Plant hormones are other obvious candidate regulatory molecules to control the  
368 transition between N<sub>2</sub> fixation and nodule senescence, some of these signals being previously  
369 associated with leaf senescence, such as cytokinins that delay leaf senescence (Zwack &  
370 Rashotte, 2013). In white lupin, expression of the cytokinin receptor *LaHK1*, closely related  
371 to *AHK4/CRE1* in *Arabidopsis thaliana*, is increased during nodule senescence (Coba de la  
372 Peña et al., 2008). In *L. japonicus*, expression of the *LjIPT3* (*IsoPentenyl-adenine Transferase*  
373 *3*, Table 1) gene encoding a rate-limiting cytokinin biosynthetic enzyme, increases during  
374 nodule aging and is associated with vascular bundles of mature nodules. *LjIPT3* down-  
375 regulation by an RNAi approach triggers a decrease in N<sub>2</sub> fixation, consequently associated  
376 with premature nodule senescence (Chen et al., 2014). In addition, a maintained N<sub>2</sub> fixation  
377 associated with delayed nodule senescence was observed in drought-stressed nodules of  
378 alfalfa inoculated with an *S. meliloti* strain engineered to overexpress an *IPT* cytokinin  
379 biosynthesis gene and therefore producing increased levels of cytokinins (Xu et al., 2012).  
380 Cytokinin may therefore act as a positive regulator of N<sub>2</sub> fixation (Boivin et al., 2016; Reid et  
381 al., 2016), and possibly as a negative regulator of root nodule senescence as reported during  
382 leaf senescence (Zwack et al., 2013).

383 Besides cytokinins, several gene expression analyses revealed putative functions for  
384 other plant hormones (Van de Velde et al., 2006; De Michele et al., 2009). Among those,  
385 genes encoding enzymes of the ethylene biosynthesis pathway, such as an 1-  
386 aminocyclopropane-1-carboxylate (ACC) oxidase, are induced in senescent nodules (Van de  
387 Velde et al., 2006). Interestingly, the lack of an ACC deaminase activity in the *Sinorhizobium*  
388 strain sp. BL3 accelerates nodule senescence in mung bean, suggesting a positive role for  
389 ethylene in nodule senescence similarly to what is observed during leaf senescence (Tittabutr  
390 et al., 2015). Lipoxygenase genes associated with Jasmonic Acid (JA) biosynthesis and GA2-  
391 oxidase genes associated with Gibberellic Acid (GA) degradation are also up-regulated during  
392 nodule senescence, suggesting that JA may positively and GA negatively regulate nodule

393 senescence (Van de Velde *et al.*, 2006; Benedito *et al.*, 2008). Abscisic acid (ABA) could be  
394 also involved in nodule senescence since some ABA responsive genes such as *ABR17* (*ABA*  
395 *Responsive 17*) are induced during nitrate-induced senescence (Liese *et al.*, 2017). In addition,  
396 ABA, which is associated with various environmental stress responses, improves nodule N<sub>2</sub>  
397 fixation capacity in alfalfa salt-stressed nodules where antioxidant defenses mediated by the  
398 polyamine spermidine and spermine are induced (Palma *et al.*, 2014), and might therefore  
399 regulate senescence progression. Such putative negative effect of ABA on nodule senescence  
400 is however not observed in leaves where ABA is a positive regulator of senescence (Lee *et*  
401 *al.*, 2011).

402         Considered together, if each of these studies suggests that hormones can intervene in  
403 the regulation of senescence, in most cases, functional analyses are lacking to clearly identify  
404 how these hormones regulate nodule senescence and more specifically how they may interact  
405 with ROS and RNS signaling pathways that are the most extensively linked to nodule  
406 senescence. These limited data may nevertheless suggest that common hormonal regulations  
407 between leaf and nodule senescence may exist, which could be surprising considering their  
408 differences in carbon *versus* N metabolic status.

409

#### 410 ***2.4 Proteases associated with bacteroid and plant cell degradation***

411

412         A hallmark of cell aging is the progressive decline in proteostasis and the  
413 accumulation of misfolded, oxidized and damaged proteins (López-Otín *et al.*, 2013).  
414 Therefore, proteolytic activities are under strict regulation to ensure protease activation and  
415 degradation at appropriate time and space. In plants, proteases have been associated with  
416 global protein turnover, protein processing, degradation of regulatory proteins, cell growth  
417 and differentiation, stress response and developmental senescence, but to date functional  
418 evidence of their mode of action remains still limited (Buono *et al.*, 2019). There are hundreds  
419 of protease-encoding genes; for example, more than 800 have been identified in Arabidopsis  
420 (van der Hoorn, 2008). Such a large number of proteases can be grouped into families  
421 according to their catalytic residues, aspartic-, cysteine-, serine-, threonine-, glutamic-,  
422 asparagine-, or metalloproteases (Oda, 2012). A description of the protease classification can  
423 be retrieved in the MEROPS database (<https://www.ebi.ac.uk/merops/>; Rawlings *et al.*, 2010).

424         To date, many reports have elucidated the roles of cysteine proteases (CPs) in nodule  
425 senescence and in response to detrimental environments (Cilliers *et al.* 2018; Perez-Guerra *et*  
426 *al.* 2010; Pierre *et al.* 2014; Pladys *et al.*, 1991; Pladys and Vance, 1993; Van de Velde *et al.*

427 2006). The synthesis of CP precursors is orchestrated by a complex transcriptional regulation  
428 network. The post-translational activation of many CPs relies on either self-processing or on  
429 the interaction with other enzymes. Their activity is also modulated by inhibitors and co-  
430 factors. CPs encoding genes are among the most strongly and specifically induced nodule  
431 senescence genes, either developmentally or environmentally triggered (Van de Velde *et al.*,  
432 2006; Seabra *et al.*, 2012; Chungopast *et al.*, 2014; Cabeza *et al.*, 2014). Their activity is  
433 directly correlated to the protein degradation occurring during senescence, and was  
434 documented in various legume plants including soybean (Alesandrini *et al.*, 2003), *Astragalus*  
435 *sinicus* (Naito *et al.*, 2000), pea (Kardailsky & Brewin, 1996), and *M. truncatula* (Pierre *et al.*,  
436 2014).

437 In soybean, 18 cysteine proteases were identified as actively transcribed in nodules  
438 during the developmental senescence (van Wyk *et al.*, 2014). This expression was increased  
439 at specific time points of the senescence process with a majority of CP genes highly expressed  
440 in 14 week-old nodules. In *Trifolium repens*, the identified CP encoding genes were  
441 specifically expressed in the senescent zone of the nodule as revealed by a spatio-temporal  
442 analysis (Asp *et al.*, 2004). In *A. sinicus* indeterminate nodules, the papain cysteine protease  
443 *AsNodF32* expressed in senescent tissues of the nodule was identified from a differential  
444 screening of a nodule cDNA library (Naito *et al.*, 2000). An RNAi silencing of *AsNodF32*  
445 extended the period of the active N<sub>2</sub>-fixation, delaying nodule senescence (Li *et al.*, 2008). In  
446 *M. truncatula* nodules, the Cysteine Protease 6 (*MtCP6*), (MEROPS family C1A;  
447 <https://www.ebi.ac.uk/merops/>), and a Vacuolar Processing Enzyme (*MtVPE*; MEROPS  
448 family C13), belonging respectively to papain and legumain families, were strongly up-  
449 regulated in response to both developmental and environmentally-induced nodule senescence  
450 (Van de Velde *et al.*, 2006; Pierre *et al.*, 2014; Table 1). A detailed spatio-temporal analysis  
451 of the *MtCP6* and *MtVPE* expression patterns during nodule developmental senescence  
452 revealed an expression associated with the transition region between the N<sub>2</sub>-fixing and  
453 senescence zones (Fig. 3 A and B, Pierre *et al.*, 2014). In nitrate-induced senescent nodules,  
454 *MtCP6* and *MtVPE* expression was detected in the whole nodule except in the basal  
455 senescence zone (Fig. 3 C and D). Subcellular localization of both *MtCP6* and *MtVPE* in  
456 senescent nodules was associated with vacuoles of cells non-infected by rhizobia and to  
457 symbiosomes of infected cells, suggesting a role of these CPs in symbiosomes and plant cell  
458 degradation (Pierre *et al.*, 2014) Silencing of *MtCP6* or *MtVPE* genes delayed nodule  
459 senescence and increased N<sub>2</sub> fixation, while their ectopic expression in the N<sub>2</sub>-fixing zone  
460 promoted nodule senescence and hindered N<sub>2</sub> fixation (Pierre *et al.*, 2014). In pea, both

461 *PsCyp1* and *PsCyp15a* (CP) genes transcript levels are increased during nodule aging.  
462 However, laser capture microdissection of pea nodule zones III and IV revealed a different  
463 pattern of expression: the *PsCyp1* gene is activated at an earlier stage of nodule senescence as  
464 transcripts are found exclusively in zone III, whereas the *PsCyp15a* gene is expressed in both  
465 zone III and senescent zone IV of pea nodule (Serova et al., 2017).

466 CPs were reported to interact with CP inhibitors called cystatins, which act as  
467 proteolysis regulators (Benchabane *et al.*, 2010; Martínez *et al.*, 2012; van Wyk *et al.*, 2014;  
468 Kunert *et al.*, 2015; Quain *et al.*, 2015). In soybean developmental senescent nodules, three  
469 cystatin genes are co-expressed with CPs (van Wyk *et al.*, 2014). Remarkably, despite no  
470 direct evidence for a role of cystatins during nodule senescence was yet reported, an ectopic  
471 overexpression of a rice oryzacystatin in soybean leads to a strong decrease in cathepsin L-  
472 like CP activities that are linked to nodule senescence (Quain et al., 2015). The coordinated  
473 expression of transcripts encoding cystatins and CPs interacting partners suggests their  
474 involvement as a fine-tuning control of proteolysis events associated with nodule senescence.  
475 In *M. truncatula* nodules, serpins (serine protease inhibitors) have also been found to be  
476 activated in drought-induced senescence and might play a role as modulators of papain-like  
477 CPs (Dhanushkodi *et al.*, 2018).

478 Increasing data show that the symbiotic establishment relies on the suppression of  
479 plant immunity (Liang et al. 2013, Yu et al. 2018). Transcriptomic studies have revealed  
480 transient elicitation of legume plant defence related genes upon *rhizobium* inoculation (Lohar  
481 et al. 2006; Libault et al. 2010; Puciariello et al., 2009), followed by their suppressions in the  
482 subsequent symbiotic stages (Aslam et al. 2008). Besides being known as an ending stage of  
483 organ development, nodule senescence is also proposed to involve a reversion of the plant  
484 innate immunity (Puppo et al. 2005). CPs have also been shown to be involved in salicylic  
485 acid induced plant defence to cope with microbial pathogens (Shindo et al. 2012; van der  
486 Linde et al. 2012; Müller et al. 2013). Thus, we could hypothesize that activation of CPs  
487 during nodule senescence might be linked to increasing plant defence responses linked to the  
488 declining nodule N<sub>2</sub> fixation capacity.

489 Genes encoding components of the SCF (skp1-cullin-F-Box protein) complex, also  
490 known as the E3 ubiquitin ligase complex, and genes encoding 26S proteasome AAA-ATPase  
491 subunits and the ubiquitin carrier protein 4 are up-regulated during *M. truncatula*  
492 developmental nodule senescence (De Michele et al., 2009; Perez-Guerra et al., 2010; Van de  
493 Velde et al., 2006). This suggests that the 26S proteasome pathway may be acting during  
494 nodule senescence.



495

### 496 **3. Transcriptomic characterization of nodule senescence**

497

498 To get insights into transcriptional regulations associated with symbiotic nodule  
499 senescence, several transcriptomic approaches were performed initially on *M. truncatula*  
500 nodules. First, a cDNA-AFLP (Amplified Fragment Length Polymorphism) technique was  
501 applied on developmental senescent nodules (Van de Velde *et al.*, 2006). A second approach  
502 used microarrays on nodules treated for 8h with phosphinotricin, an inhibitor of GS activity  
503 and therefore affecting N<sub>2</sub> fixation (Seabra *et al.*, 2012). More recently, an RNAseq was  
504 performed on nodules exposed to nitrate for 4h or 28h (Cabeza *et al.*, 2014). Similar studies  
505 have been also performed in other legumes species than *M. truncatula*, such as in *L. japonicus*  
506 using microarrays and in soybean using RNAseq, at different time-points during nodule aging  
507 (Chungopast *et al.*, 2014; Yuan *et al.*, 2017).

508 Among the differentially expressed genes identified, the downregulation of specific N<sub>2</sub>  
509 fixation associated genes, such as Lb, late nodulins and NCR peptide encoding genes attests  
510 the inhibition of the N<sub>2</sub> fixation at the molecular level (Van de Velde *et al.*, 2006; Seabra *et*  
511 *al.*, 2012; Cabeza *et al.*, 2014). Genes related to energy production, such as mitochondrial  
512 ATP synthase subunit encoding genes, or amino-acid biogenesis encoding genes, were  
513 accordingly repressed (Seabra *et al.*, 2012; Cabeza *et al.*, 2014). An upregulation of genes  
514 encoding proteins involved in macromolecule degradation, such as the CPs previously  
515 mentioned, and nutrient remobilization (phosphate, amino-acid, ion transporters and ABC  
516 [ATP-Binding Cassette] transporters) was observed, whereas ammonium, potassium and most  
517 of the sulfate transporters were down-regulated (Van de Velde *et al.*, 2006; Cabeza *et al.*,  
518 2014). Genes associated with redox stress responses such as catalases, oxidoreductases,  
519 hydrolases, peroxidases, esterases, glutathione transferases were also upregulated, showing  
520 the relevance of redox regulations during nodule senescence (Van de Velde *et al.*, 2006;  
521 Seabra *et al.*, 2012), as previously described.

522 In order to distinguish at the molecular level environmentally induced-senescence  
523 from developmental senescence, selected genes up-regulated during a *M. truncatula*  
524 developmental nodule senescence were tested by qRT-PCR in nodules exposed to darkness  
525 for 3 to 5 days (Van de Velde *et al.*, 2006; Pérez-Guerra *et al.*, 2010). Only 50% of the genes  
526 selected as up-regulated during developmental senescence were up-regulated during a dark-  
527 induced nodule senescence. Genes not regulated in the dark-induced nodule senescence were  
528 notably related to the 26S proteasome-mediated protein degradation (Pérez-Guerra *et al.*,

529 2010), suggesting either a less organized, or more probably an accelerated cellular  
530 degradation in dark-induced conditions. To refine the spatio-temporal expression profiles  
531 during nodule senescence, *M. truncatula* nodules were sectioned into apical *versus* basal  
532 zones and pea nodules were microdissected (Van de Velde et al., 2006; Serova et al., 2017).  
533 In this way, the *M. truncatula* or pea *ATB2* genes, encoding a bZIP (basic leucine ZIPper)  
534 transcription factor (TF), were identified as reaching a peak in fully senescent nodules (Van  
535 de Velde *et al.*, 2006; D'haeseleer *et al.*, 2010; Serova *et al.*, 2017).

536 To evaluate if genes were specifically regulated during the senescence of nodule  
537 organs, a transcriptomic analysis was performed using a cDNA-AFLP approach in *M.*  
538 *truncatula* senescent leaves (de Michele et al., 2009). The overlap between nodule and leaf  
539 senescence associated genes was relatively low (23%). When excluding N-metabolism related  
540 genes, a higher degree of overlap was nevertheless observed in the early phase, including  
541 specific MAP kinases, ubiquitin cycle components, phosphatases, glutathione transferases,  
542 and transcription factors; as well as in the late phase, i.e. several CPs and components of the  
543 26S proteasome (Van de Velde *et al.*, 2006; de Michele *et al.*, 2009).

544 Based on transcriptomic analyses previously described, transcription factors (TF)  
545 encoding genes differentially regulated during nodule senescence were identified, mainly  
546 from MYB (MYeloBlastosis), bHLH (basic Helix-Loop-Helix), and NAC (NAM ATAF  
547 CUC) families (Van de Velde *et al.*, 2006; Seabra *et al.*, 2012; Cabeza *et al.*, 2014;  
548 Chungopast *et al.*; 2014; Yuan *et al.*, 2017). Among those, only few TFs were selected for  
549 further functional analyses, with the aim of identifying regulatory pathways controlling the  
550 transition between N<sub>2</sub> fixation and nodule senescence, either during aging or in response to  
551 environmental stress conditions. The NAC TF family was previously largely associated with  
552 leaf senescence based on transcriptome and genetic studies performed in *Arabidopsis* (Breeze  
553 *et al.*, 2011), and is also enriched in nodule senescence transcriptomes. Based on RNAseq or  
554 microarray experiments, approximately one third of *NAC* genes expressed in nodules are  
555 induced by nitrate (16 / 59 in Cabeza *et al.* 2014; and 21 / 57 in Benedito *et al.*, 2008) whereas  
556 over 30 out of 90 *NAC* genes are upregulated during *Arabidopsis* leaf senescence (Breeze *et*  
557 *al.*, 2011). Expression of the *M. truncatula* TF *MtNAC969*, closely related to the well-known  
558 *Arabidopsis* leaf senescence associated gene *AtNAP* (*NAC-LIKE ACTIVATED BY AP3/PI*), is  
559 induced by nitrate and associated with the central region of symbiotic nodules including the  
560 N<sub>2</sub>-fixation zone (de Zélicourt et al., 2012). In addition, both *AtNAP* and *MtNAC969* genes are  
561 induced during *Arabidopsis* and *M. truncatula* leaf senescence, respectively (de Michele et  
562 al., 2009; Balazadeh *et al.*, 2010). An RNAi silencing of the *MtNAC969* TF promotes nodule

563 senescence (Table 1), based on the increased expression of *CP* genes used as senescence  
564 markers, concomitantly to an accumulation of amyloplasts suggestive of an altered nodule  
565 metabolism (de Zélicourt *et al.*, 2012). Expression of the *M. truncatula MtATB2* bZIP TF is  
566 increased during nodule aging senescence, as well as by sucrose but not by a nitrate treatment  
567 (Van de Velde *et al.*, 2006; D'haeseleer *et al.*, 2010). Despite this transcriptional regulation  
568 pattern, ectopic expression of *MtATB2* reduced the number of nodules formed but no  
569 senescence phenotype could be observed (D'haeseleer *et al.*, 2010). This highlights that an  
570 increased expression in senescent nodules is not a sufficient criteria to identify genes  
571 functionally linked to nodule senescence. As *MtATB2* is expressed in the peripheral region of  
572 nodules containing the vascular bundles and not in the N<sub>2</sub> fixation or senescence central  
573 zones, and because of its regulation by sucrose and not by nitrate, this suggests that this TF  
574 might sense carbon sources availability and regulate remobilization processes linked to  
575 senescence (D'haeseleer *et al.*, 2010). Finally, no TF was yet directly linked to the  
576 upregulation of genes encoding CPs or other cell degradation related enzymes, or to the  
577 downregulation of Lb or N-assimilation metabolic enzymes encoding genes, or to components  
578 of redox pathways. Similarly, a symbiotic bacterial contribution to the regulation of nodule  
579 senescence associated genes has not been explored even if some toxin/antitoxin bacterial  
580 systems, such as the vapBC-5 (Table 1), have been showed to alter nodule senescence  
581 progression (Oláh *et al.*, 2001; Lipuma *et al.*, 2014).

582

#### 583 **4. Concluding remarks - future prospects**

584

585 Senescence affecting the legume-rhizobia symbiotic nodule organ during aging or in  
586 response to environmental conditions remains poorly documented. The best functionally  
587 characterized senescence mechanisms are related to the redox dependent inhibition of the N<sub>2</sub>  
588 fixation and assimilation. Physiological and molecular approaches show that nodule  
589 senescence is followed by a decrease in the level and/or reduction state of the main cellular  
590 antioxidants ASC and GSH, and by an increase in the production of ROS and RNS, such as  
591 H<sub>2</sub>O<sub>2</sub> and NO. This results in an increase of oxidized/denatured proteins and subsequently in  
592 an inhibition of N, carbon and energy metabolisms. In addition from being harmful to cellular  
593 processes, both ROS and NO production are also important components of the signaling  
594 network used by legumes and their symbionts to communicate at the various stages of the  
595 symbiotic interaction. The evolution of efficient ROS/NO degrading systems has most likely  
596 enabled plant cells and bacteroids inside nodules to overcome ROS and NO toxicity. Diverse

597 bacterial and plant systems contribute to the control of NO and ROS levels in nodules. Only  
598 when these systems are overcome by NO and ROS, these molecules can act as signals to  
599 trigger senescence (Puppo et al., 2013). Alternatively, senescence could be a consequence of  
600 NO-induced modifications of proteins essential for N<sub>2</sub> fixation. Further studies are needed to  
601 link the modifications of these proteins by NO or ROS to nodule senescence.

602 An increased proteolysis and the induction of plant vacuolar proteases such as CPs and  
603 VPEs during senescence are also well documented, and used as a hallmark to follow the  
604 senescence progression. Identifying how these nodule specific proteases are regulated and  
605 what are their targets remains a challenge to understand better nodule senescence. Both 26S  
606 and 20S proteasome proteolytic pathways are additionally induced during nodule senescence,  
607 and their contribution should be established. Finally, the nature of bacterial proteases as well  
608 as their contribution to the degradation of bacteroid proteins remains to be better documented.

609 Large-scale transcriptomic analyses from the last decade allowed identifying new  
610 candidate genes that may act in the senescence processes. These approaches were however  
611 focused on the plant transcriptome, and it would be interesting to analyze in parallel the  
612 transcriptional response of symbiotic bacteria to determine if they could contribute to the  
613 regulation of nodule senescence progression. In the model legume *M. truncatula*, partially  
614 overlapping plant regulatory pathways were identified between environmental, developmental  
615 and leaf senescence. This suggests that a core of common genes is conserved between the  
616 senescence of different organs, but also that a subset of genes may specifically regulate  
617 senescence in nodules, which may be notably related to the symbiotic and metabolic features  
618 of this organ hosting numerous N<sub>2</sub> fixing bacteria. Such putative plant-microbe gene network  
619 interactions remain however to be investigated. Only few regulatory genes directly regulating  
620 nodule senescence have been yet functionally characterized, such as TFs and hormone-related  
621 genes. A future prospect would be to integrate how the diversity of bacteria hosted inside the  
622 nodules, beyond rhizobia, could control senescence progression. Indeed, promising recent  
623 data (Rodrigues *et al.*, 2013) reported that other soil bacteria than rhizobia might indeed  
624 modify the nodule lifespan, as cowpea nodules co-inoculated with *Bradyrhizobium* sp. and  
625 *Paenibacillus graminis* or *Paenibacillus durus* plant growth-promoting bacteria present an  
626 increased N availability and delayed nodule senescence. Finally, an applied perspective of  
627 studying nodule senescence would be, as achieved with “staygreen” leaves, to engineer  
628 “staypink” nodules that would have maintained, and even improved, capacities to fix N<sub>2</sub> under  
629 environmental stress conditions.

630

631 **Acknowledgments**

632 The three laboratories involved (IPS2, ISA and LIPM) are funded by a common “Agence  
633 Nationale de la Recherche” (ANR) project (STAYPINK ANR-15-CE20-0005). The IPS2  
634 institute benefits from the support of the LabEx “Saclay Plant Sciences” (SPS) and of the  
635 Lidex “Plant Phenotyping Pipeline” (3P). T.K. was supported by Université Paris-Saclay and  
636 ANR fellowships. The ISA was supported by the Institut National de la Recherche  
637 Agronomique, the Centre National de la Recherche Scientifique, the University of Nice–  
638 Sophia-Antipolis, and the French Government (National Research Agency, ANR) through the  
639 LABEX SIGNALIFE program (ANR-11-LABX-0028-01). L.Y. was supported by a doctoral  
640 fellowship from China Scholarship Council (CSC). The LIPM was supported by the French  
641 Laboratory of Excellence project TULIP (ANR-10-LABX-41). E.M. was supported by the  
642 National Institute for Applied Science (INSA-Toulouse).

643

644 **References**

645

- 646 Alesandrini, F., Mathis, R., Van de Sype, G., Hérouart, D., & Puppo, A. (2003). Possible roles  
647 for a cysteine protease and hydrogen peroxide in soybean nodule development and  
648 senescence. *New Phytologist*, 158, 131-138.
- 649 Alloing, G., Mandon, K., Boncompagni, E., Montrichard, F., & Frendo, P. (2018).  
650 Involvement of Glutaredoxin and Thioredoxin Systems in the Nitrogen-Fixing  
651 Symbiosis between Legumes and Rhizobia. *Antioxidants (Basel)*, 7, 182.
- 652 Alunni B., Kevei Z., Redondo-Nieto M., Kondorosi A., Mergaert P. & Kondorosi E. (2007).  
653 Genomic organization and evolutionary insights on GRP and NCR genes, two large  
654 nodule-specific gene families in *Medicago truncatula*. *Molecular Plant-Microbe*  
655 *Interactions*, 20, 1138–1148.
- 656 Appleby, C. A. (1992). The origin and functions of haemoglobin in plants. *Science Progress*  
657 76, 365-398.
- 658 Aranjuelo, I., Tcherkez, G., Molero, G., Gilard, F., Avice, J. C., & Nogués, S. (2013).  
659 Concerted changes in N and C primary metabolism in alfalfa (*Medicago sativa*) under  
660 water restriction. *Journal of Experimental Botany*, 64, 885-897.
- 661 Arthikala, M. K., Sánchez-Lopez, R., Nava, N., Santana, O., Cardenas, L., & Quinto, C.  
662 (2014). RbohB, a *Phaseolus vulgaris* NADPH oxidase gene, enhances symbiosome  
663 number, bacteroid size, and nitrogen fixation in nodules and impairs mycorrhizal  
664 colonization. *New Phytologist*, 202, 886-900.
- 665 Aslam, S. N., Newman, M.-A., Erbs, G., Morrissey, K. L., Chinchilla, D., Boller, T., et al.  
666 (2008). Bacterial polysaccharides suppress induced innate immunity by calcium  
667 chelation. *Current Biology*, 18, 1078-1083.
- 668 Asp, T., Bowra, S., Borg, S., & Holm, P. B. (2004). Cloning and characterisation of three  
669 groups of cysteine protease genes expressed in the senescing zone of white clover  
670 (*Trifolium repens*) nodules. *Plant science*, 167, 825-837.
- 671 Astier J., & Lindermayr, C. (2012). Nitric oxide-dependent posttranslational modification in  
672 plants: an update. *International Journal of Molecular Sciences*, 13, 15193-15208.

- 673 Balazadeh, S., Siddiqui, H., Allu, A. D., Matallana-Ramirez, L. P., Caldana, C., Mehrnia, M.,  
674 et al. (2010). A gene regulatory network controlled by the NAC transcription factor  
675 ANAC092/AtNAC2/ORE1 during salt-promoted senescence. *The Plant Journal*, 62,  
676 250-264.
- 677 Balestrasse, K., Gallego, S., & Tomaro, M. (2004). Cadmium-induced senescence in nodules  
678 of soybean (*Glycine max* L.) plants. *Plant and Soil*, 262, 373-381.
- 679 Banba, M., Siddique, A. B., Kouchi, H., Izui, K., & Hata, S. (2001). *Lotus japonicus* forms  
680 early senescent root nodules with *Rhizobium etli*. *Molecular Plant-Microbe*  
681 *Interactions*, 14, 173-180.
- 682 Baudouin, E., Pieuchot, L., Engler, G., Pauly, N., & Puppo, A. (2006). Nitric oxide is formed  
683 in *Medicago truncatula*-*Sinorhizobium meliloti* functional nodules. *Molecular Plant-*  
684 *Microbe Interactions* 19, 970-975.
- 685 Becana, M., Matamoros, M. A., Udvardi, M., & Dalton, D. A. (2010). Recent insights into  
686 antioxidant defences of legume root nodules. *New Phytologist*, 188, 960-976.
- 687 Benchabane, M., Schluter, U., Vorster, J., Goulet, M. C., & Michaud, D. (2010). Plant  
688 cystatins. *Biochimie*, 92, 1657-1666.
- 689 Benedito, V. A., Torres-Jerez, I., Murray, J. D., Andriankaja, A., Allen, S., Kakar, K., et al.  
690 (2008). A gene expression atlas of the model legume *Medicago truncatula*. *The Plant*  
691 *Journal*, 55, 504-513.
- 692 Berger, A., Boscari, A., Frendo, P., & Brouquisse, R. (2019). Nitric oxide signaling,  
693 metabolism and toxicity in nitrogen-fixing symbiosis. *Journal Experimental Botany*,  
694 70, in Press. DOI: 10.1093/jxb/erz159.
- 695 Bethlenfalvay, G. J., & Phillips, D. A. (1977). Ontogenetic Interactions between  
696 Photosynthesis and Symbiotic Nitrogen Fixation in Legumes. *Plant Physiology*, 60,  
697 419-421.
- 698 Blanquet, P., Silva, L., Catrice, O., Bruand, C., Carvalho, H., & Meilhoc, E. (2015).  
699 *Sinorhizobium meliloti* Controls Nitric Oxide-Mediated Post-Translational  
700 Modification of a *Medicago truncatula* Nodule Protein. *Molecular Plant-Microbe*  
701 *Interactions*, 28, 1353-1363.
- 702 Boivin, S., Kazmierczak, T., Brault, M., Wen, J., Gamas, P., et al. (2016). Different cytokinin  
703 histidine kinase receptors regulate nodule initiation as well as later nodule  
704 developmental stages in *Medicago truncatula*. *Plant, Cell & Environment*, 39, 2198-  
705 2209.
- 706 Boscari A, Del Giudice J, Ferrarini A, Venturini L, Zaffini A-L, Delledonne M, & Puppo A.  
707 (2013a). Expression dynamics of the *Medicago truncatula* transcriptome during the  
708 symbiotic interaction with *Sinorhizobium meliloti*: which role for nitric oxide? *Plant*  
709 *physiology*, 161, 425-39.
- 710 Boscari A, Meilhoc E, Castella C, Bruand C, Puppo A, & Brouquisse R. (2013b). Which role  
711 for nitric oxide in symbiotic N<sub>2</sub>-fixing nodules: toxic by-product or useful  
712 signaling/metabolic intermediate? *Frontiers in Plant Science*, 4, 384.
- 713 Breeze, E., Harrison, E., McHattie, S., Hughes, L., Hickman, R., Hill, C., et al. (2011). High-  
714 resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals  
715 a distinct chronology of processes and regulation. *The Plant Cell*, 23, 873-894.
- 716 Brouquisse, R. (2019). Multifaceted roles of nitric oxide in plants. *Journal of Experimental*  
717 *Botany*, 70, in press. DOI: 10.1093/jxb/erz352.
- 718 Bruand, C., & Meilhoc, E. (2019). Nitric oxide in plants: pro- or anti-senescence. *Journal of*  
719 *Experimental Botany*, 70, in press. DOI: 10.1093/jxb/erz117.
- 720 Buono, R. A., Hudecek, R., & Nowack, M. K. (2019). Plant proteases during developmental  
721 programmed cell death. *Journal of Experimental Botany*, 70, 2097-2112.

- 722 Cabeza, R., Koester, B., Liese, R., Lingner, A., Baumgarten, V., Dirks, J., et al. (2014). An  
723 RNA sequencing transcriptome analysis reveals novel insights into molecular aspects  
724 of the nitrate impact on the nodule activity of *Medicago truncatula*. *Plant Physiology*,  
725 164, 400-411.
- 726 Calvo-Begueria, L., Rubio, M. C., Martinez, J. I., Perez-Rontome, C., Delgado, M. J.,  
727 Bedmar, E. J., & Becana, M. (2018). Redefining nitric oxide production in legume  
728 nodules through complementary insights from electron paramagnetic resonance  
729 spectroscopy and specific fluorescent probes. *Journal of Experimental Botany*, 69,  
730 3703-3714.
- 731 Cam, Y., Pierre, O., Boncompagni, E., Herouart, D., Meilhoc, E., & Bruand, C. (2012). Nitric  
732 oxide (NO): a key player in the senescence of *Medicago truncatula* root nodules. *New*  
733 *Phytologist*, 196, 548-560.
- 734 Chen, Y., Chen, W., Li, X., Jiang, H., Wu, P., Xia, K., Yang, Y., & Wu, G. (2014).  
735 Knockdown of LjIPT3 influences nodule development in *Lotus japonicus*. *Plant &*  
736 *Cell Physiology*, 55, 183-193.
- 737 Christenhunz, M. J., & Byng, J. W. (2016). The number of known plants species in the world  
738 and its annual increase. *Phytotaxa*, 261, 201-217.
- 739 Chungopast, S., Duangkhet, M., Tajima, S., Ma, J. F., & Nomura, M. (2017). Iron-induced  
740 nitric oxide leads to an increase in the expression of ferritin during the senescence of  
741 *Lotus japonicus* nodules. *J Plant Physiology*, 208, 40-46.
- 742 Chungopast, S., Hirakawa, H., Sato, S., Handa, Y., Saito, K., Kawaguchi, M., Tajima, S., &  
743 Nomura, M. (2014). Transcriptomic profiles of nodule senescence in *Lotus japonicus*  
744 and *Mesorhizobium loti* symbiosis. *Plant Biotechnology*, 31, 345-349.
- 745 Cilliers, M., van Wyk, S. G., van Heerden, P. D. R., Kunert, K. J., & Vorster, B. J. (2018).  
746 Identification and changes of the drought-induced cysteine protease transcriptome in  
747 soybean (*Glycine max*) root nodules. *Environmental and Experimental Botany*, 148,  
748 59-69.
- 749 Coba de la Peña, T., Carcamo, C. B., Almonacid, L., Zaballos, A., Lucas, M. M., Balomenos,  
750 D., & Pueyo, J. J. (2008). A salt stress-responsive cytokinin receptor homologue  
751 isolated from *Medicago sativa* nodules. *Planta*, 227, 769-779.
- 752 Coba de la Peña, T., Redondo, F. J., Manrique, E., Lucas, M. M., & Pueyo, J. J. (2010).  
753 Nitrogen fixation persists under conditions of salt stress in transgenic *Medicago*  
754 *truncatula* plants expressing a cyanobacterial flavodoxin. *Plant Biotechnol Journal*, 8,  
755 954-965.
- 756 Curioni, P. M., Hartwig, U. A., Nosberger, J., & Schuller, K. A. (1999). Glycolytic flux is  
757 adjusted to nitrogenase activity in nodules of detopped and argon-treated alfalfa  
758 plants. *Plant Physiology*, 119, 445-454.
- 759 Czernic, P., Gully D., Cartieaux F., Moulin L., Guefrachi I., Patrel D. et al. (2015).  
760 Convergent evolution of endosymbiont differentiation in Dalbergioid and IRLC  
761 legumes mediated by nodule-specific cysteine-rich peptides. *Plant Physiology*,  
762 169,1254-1265.
- 763 D'haeseleer, K., De Keyser, A., Goormachtig, S., & Holsters, M. (2010). Transcription factor  
764 MtATB2: about nodulation, sucrose and senescence. *Plant & Cell Physiology*, 51,  
765 1416-1424.
- 766 Dalton, D. A., Boniface, C., Turner, Z., Lindahl, A., Kim, H. J., Jelinek, L., et al. (2009).  
767 Physiological roles of glutathione s-transferases in soybean root nodules. *Plant*  
768 *Physiology*, 150, 521-530.
- 769 Dalton, D. A., Russell, S. A., Hanus, F. J., Pascoe, G. A., & Evans, H. J. (1986). Enzymatic  
770 reactions of ascorbate & glutathione that prevent peroxide damage in soybean root  
771 nodules. *Proceedings of the National Academy of Sciences*, 83, 3811-3815.

772 Damiani, I., Pauly, N., Puppo, A., Brouquisse, R., & Boscari, A. (2016). Reactive Oxygen  
773 Species and Nitric Oxide Control Early Steps of the Legume - Rhizobium Symbiotic  
774 Interaction. *Frontiers in Plant Science*, 7, 454.

775 Day DA, Poole PS, Tyerman SD, Rosendahl L (2001). Ammonia and amino acid transport  
776 across symbiotic membranes in nitrogen-fixing legume nodules. *Cellular and*  
777 *Molecular Life Sciences : CMLS*, 58,61-71.

778 De Michele, R., Formentin, E., Todesco, M., Toppo, S., Carimi, F., Zottini, M., et al. (2009).  
779 Transcriptome analysis of *Medicago truncatula* leaf senescence: similarities and  
780 differences in metabolic and transcriptional regulations as compared with Arabidopsis,  
781 nodule senescence and nitric oxide signalling. *New Phytologist*, 181, 563-75.

782 de Zelicourt, A., Diet, A., Marion, J., Laffont, C., Ariel, F., Moison, M., et al. (2012). Dual  
783 involvement of a *Medicago truncatula* NAC transcription factor in root abiotic stress  
784 response and symbiotic nodule senescence. *The Plant Journal*, 70, 220-230.

785 del Giudice, J., Cam, Y., Damiani, I., Fung-Chat, F., Meilhoc, E., Bruand, C., et al. (2011).  
786 Nitric oxide is required for an optimal establishment of the *Medicago truncatula*-  
787 *Sinorhizobium meliloti* symbiosis. *New Phytologist*, 191, 405-417.

788 Dhanushkodi, R., Matthew, C., McManus, M. T., & Dijkwel, P. P. (2018). Drought-induced  
789 senescence of *Medicago truncatula* nodules involves serpin and ferritin to control  
790 proteolytic activity and iron levels. *New Phytologist*, 220, 196-208.

791 Dror, I., Golan, T., Levy, C., Rohs, R., & Mandel-Gutfreund, Y. (2015). A widespread role of  
792 the motif environment in transcription factor binding across diverse protein families.  
793 *Genome Research*, 25, 1268-1280.

794 Dupont L, Alloing G, Pierre O, El Msehli S, Hopkins J, Hérouart D, et al. (2012). The legume  
795 root nodule: from symbiotic nitrogen fixation to senescence. In: Nagata T, ed.  
796 *Senescence*, 8, Shanghai, China: InTech, 137-168.

797 El Msehli S, Lambert A, Baldacci-Cresp F, Hopkins J, Boncompagni E, Smiti SA, et al.  
798 (2011). Crucial role of (homo)glutathione in nitrogen fixation in *Medicago truncatula*  
799 nodules. *New Phytologist*, 192, 496-506.

800 Escuredo, P. R., Minchin, F. R., Gogorcena, Y., Iturbe-Ormaetxe, I., Klucas, R. V., & Becana,  
801 M. (1996). Involvement of Activated Oxygen in Nitrate-Induced Senescence of Pea  
802 Root Nodules. *Plant Physiology*, 110, 1187-1195.

803 Farkas, A., Maroti, G., Durgo, H., Gyorgypal, Z., Lima, R. M., Medzihradzsky, K. F., et al.  
804 (2014). *Medicago truncatula* symbiotic peptide NCR247 contributes to bacteroid  
805 differentiation through multiple mechanisms. *Proceedings of the National Academy of*  
806 *Sciences*, 111, 5183-5188.

807 Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M.-H., Lin, Y.-H., Reid, D. E., &  
808 Gresshoff, P. M. (2010). Molecular analysis of legume nodule development and  
809 autoregulation. *Journal of Integrative Plant Biology*, 52, 61-76.

810 Ferrarini, A., de Stefano, M., Baudouin, E., Pucciariello, C., Polverari, A., Puppo, A., &  
811 Delledonne, M. (2008). Expression of *Medicago truncatula* genes responsive to nitric  
812 oxide in pathogenic and symbiotic conditions. *Molecular Plant-Microbe Interactions*,  
813 21, 781-790.

814 Frendo, P., Matamoros, M. A., Alloing, G., & Becana, M. (2013). Thiol-based redox  
815 signaling in the nitrogen-fixing symbiosis. *Frontiers in Plant Science*, 4, 376.

816 Fukudome, M., Watanabe, E., Osuki, K. I., Imaizumi, R., Aoki, T., Becana, M., & Uchiumi,  
817 T. (2019). Stably-Transformed *Lotus japonicus* Plants Overexpressing Phytoglobin  
818 LjGlb1-1 Show Decreased Nitric Oxide Levels in Roots and Nodules as well as  
819 Delayed Nodule Senescence. *Plant & Cell Physiology*, 60, 816-825.

820 Gil-Quintana, E., Lyon, D., Staudinger, C., Wienkoop, S., & Gonzalez, E. M. (2015).  
821 *Medicago truncatula* and *Glycine max*: Different Drought Tolerance and Similar



822 Local Response of the Root Nodule Proteome. *Journal of Proteome Research*, 14,  
823 5240-5251.

824 Gogorcena, Y., Gordon, A. J., Escuredo, P. R., Minchin, F. R., Witty, J. F., Moran, J. F., & Becana,  
825 M. (1997). N<sub>2</sub> Fixation, Carbon Metabolism, and Oxidative Damage in Nodules of Dark-  
826 Stressed Common Bean Plants. *Plant Physiology*, 113, 1193-1201.

827 Gonzalez, E. M., Galvez, L., & Arrese-Igor, C. (2001). Abscisic acid induces a decline in nitrogen  
828 fixation that involves leghaemoglobin, but is independent of sucrose synthase activity. .  
829 *Journal of Experimental Botany*, 52, 285-293.

830 Harrison, J., Jamet, A., Muglia, C.I., Van de Sype, G., Aguilar, O.M., Puppo, A., & Frendo, P. (2005).  
831 Glutathione plays a fundamental role in growth and symbiotic capacity of *Sinorhizobium*  
832 *meliloti*. *Journal of Bacteriology*, 187, 168-174.

833 Hernández-Jiménez, M. J., Mercedes Lucas, M., & de Felipe, M. R. (2002). Antioxidant defence and  
834 damage in senescing lupin nodules. *Plant Physiology and Biochemistry*, 40, 645-657.

835 Hichri I, Boscari A, Castella C, Rovere M, Puppo A, & Brouquisse R. (2015). Nitric oxide: a  
836 multifaceted regulator of the nitrogen-fixing symbiosis. *Journal of Experimental Botany*, 66,  
837 2877–2887.

838 Hichri, I., Meilhoc, E., Boscari, A., Bruand, C., Frendo, P., & Brouquisse, R. (2016). Nitric Oxide:  
839 Jack-of-All-Trades of the Nitrogen-Fixing Symbiosis? In "Advances in Botanical Research",  
840 Vol. 77, pp. 193-218.

841 Holzmeister, C., Gaupels, F., Geerlof, A., Sarioglu, H., Sattler, M., Durner, J., & Lindermayr, C.  
842 (2015). Differential inhibition of *Arabidopsis* superoxide dismutases by peroxynitrite-  
843 mediated tyrosine nitration. *Journal of Experimental Botany*, 66, 989-999.

844 Horchani, F., Prevot, M., Boscari, A., Evangelisti, E., Meilhoc, E., Bruand, C., et al. (2011). Both  
845 plant and bacterial nitrate reductases contribute to nitric oxide production in *Medicago*  
846 *truncatula* nitrogen-fixing nodules. *Plant Physiology*, 155, 1023-1036.

847 Kardailsky, I. V., & Brewin, N. J. (1996). Expression of cysteine protease genes in pea nodule  
848 development and senescence. *Molecular Plant-Microbe Interactions*, 9, 689-695.

849 Klucas, R. V. (1974). Studies on soybean nodule senescence. *Plant Physiology*, 54, 612-616.

850 Kondorosi, E., Mergaert, P., & Kereszt, A. (2013). A paradigm for endosymbiotic life: cell  
851 differentiation of *Rhizobium* bacteria provoked by host plant factors. *Annual Review of*  
852 *Microbiology*, 67, 611-628.

853 Kunert, K. J., van Wyk, S. G., Cullis, C. A., Vorster, B. J., & Foyer, C. H. (2015). Potential use of  
854 phytocystatins in crop improvement, with a particular focus on legumes. *Journal of*  
855 *Experimental Botany*, 66, 3559-3570.

856 Lahiri, K., Chattopadhyay, S., Chatterjee, S., & Ghosh, B. (1993). Biochemical changes in nodules of  
857 *Vigna mungo* (L.) during vegetative and reproductive stages of plant growth in the field.  
858 *Annals of Botany*, 71, 485-488.

859 Lamouche, F., Bonadé-Bottino, N., Peter Mergaert, P., Alunni, B. (2019). Symbiotic Efficiency of  
860 Spherical and Elongated Bacteroids in the *Aeschynomene-Bradyrhizobium* Symbiosis.  
861 *Frontiers in Plant Science*, 10, 377.

862 Lawn, R. J., & Brun, W. A. (1974). Symbiotic Nitrogen Fixation in Soybeans. I. Effect of  
863 Photosynthetic Source-Sink Manipulations1. *Crop Science*, 14, 11-16.

864 Lee, I. C., Hong, S. W., Whang, S. S., Lim, P. O., Nam, H. G., Koo, J. C. (2011). Age-Dependent  
865 Action of an ABA-Inducible Receptor Kinase, RPK1, as a Positive Regulator of Senescence in  
866 *Arabidopsis* Leaves. *Plant & Cell Physiology*, 52, 651-662.

867 Lehtovaara, P., & Perttilä, U. (1978). Bile-pigment formation from different leghaemoglobins. Methine-bridge specificity of  
868 coupled oxidation. *Biochemical Journal*, 176, 359-364.

869 Li, Y., Zhou, L., Li, Y., Chen, D., Tan, X., Lei, L., & Zhou, J. (2008). A nodule- specific plant  
870 cysteine proteinase, AsNODF32, is involved in nodule senescence and nitrogen fixation  
871 activity of the green manure legume *Astragalus sinicus*. *New Phytologist*, 180, 185-192.

872 Liang, Y., Cao, Y., Tanaka, K., Thibivilliers, S., Wan, J., Choi, J., et al. (2013). Nonlegumes respond  
873 to rhizobial Nod factors by suppressing the innate immune response. *Science*, 341, 1384-1387.

874 Libault, M., Farmer, A., Brechenmacher, L., Drnevich, J., Langlely, R. J., Bilgin, D. D., et al.  
875 (2010). Complete transcriptome of the soybean root hair cell, a single-cell model, and

876 its alteration in response to Bradyrhizobium japonicum infection. *Plant Physiology*,  
877 152, 541-552.

878 Liese, R., Schulze, J., & Cabeza, R. A. (2017). Nitrate application or P deficiency induce a  
879 decline in *Medicago truncatula* N<sub>2</sub>-fixation by similar changes in the nodule  
880 transcriptome. *Scientific Reports*, 7, 46264.

881 Lipuma, J., Cinege, G., Bodogai, M., Oláh, B., Kiers, A., Endre, G., et al. (2014). A vapBC-  
882 type toxin-antitoxin module of *Sinorhizobium meliloti* influences symbiotic efficiency  
883 and nodule senescence of *Medicago sativa*. *Environmental Microbiology*, 16, 3714-  
884 3729.

885 Lohar, D. P., Sharopova, N., Endre, G., Penuela, S., Samac, D., Town, C., et al. (2006).  
886 Transcript analysis of early nodulation events in *Medicago truncatula*. *Plant*  
887 *Physiology*, 140, 221-234.

888 López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2013). The  
889 hallmarks of aging. *Cell*, 153, 1194-1217.

890 Loscos, J., Matamoros, M. A., & Becana, M. (2008). Ascorbate and homogluthathione  
891 metabolism in common bean nodules under stress conditions and during natural  
892 senescence. *Plant Physiology*, 146, 1282-1292.

893 Marino, D., Andrio, E., Danchin, E. G., Oger, E., Gucciardo, S., Lambert, A., et al. (2011). A  
894 *Medicago truncatula* NADPH oxidase is involved in symbiotic nodule functioning.  
895 *New Phytologist*, 189, 580-592.

896 Marquez-Garcia, B., Shaw, D., Cooper, J. W., Karpinska, B., Quain, M. D., Makgopa, E. M.,  
897 et al. (2015). Redox markers for drought-induced nodule senescence, a process  
898 occurring after drought-induced senescence of the lowest leaves in soybean (*Glycine*  
899 *max*). *Annals of Botany*, 116, 497-510.

900 Martínez, M., Cambra, I., Gonzalez-Melendi, P., Santamaria, M. E., & Diaz, I. (2012). C1A  
901 cysteine-proteases and their inhibitors in plants. *Physiologia Plantarum*, 145, 85-94.

902 Matamoros, M. A., Baird, L. M., Escuredo, P. R., Dalton, D. A., Minchin, F. R., Iturbe-  
903 Ormaetxe, I., et al. (1999). Stress-induced legume root nodule senescence.  
904 Physiological, biochemical, and structural alterations. *Plant Physiology*, 121, 97-112.

905 Matamoros, M. A., Fernandez-Garcia, N., Wienkoop, S., Loscos, J., Saiz, A., & Becana, M.  
906 (2013). Mitochondria are an early target of oxidative modifications in senescing  
907 legume nodules. *New Phytologist*, 197, 873-885.

908 Meakin, G. E., Bueno, E., Jepson, B., Bedmar, E. J., Richardson, D. J., & Delgado, M. J.  
909 (2007). The contribution of bacteroidal nitrate and nitrite reduction to the formation of  
910 nitrosylhaemoglobin complexes in soybean root nodules. *Microbiology*, 153, 411-  
911 419.

912 Meilhoc, E., Blanquet, P., Cam, Y., & Bruand, C. (2013). Control of NO level in rhizobium-  
913 legume root nodules: not only a plant globin story. *Plant Signaling & Behavior*, 8,  
914 e25923.

915 Meilhoc, E., Cam, Y., Skapski, A., & Bruand, C. (2010). The response to nitric oxide of the  
916 nitrogen-fixing symbiont *Sinorhizobium meliloti*. *Molecular Plant-Microbe*  
917 *Interactions*, 23, 748-759.

918 Melo, P. M., Silva, L. S., Ribeiro, I., Seabra, A. R., & Carvalho, H. G. (2011). Glutamine  
919 synthetase is a molecular target of nitric oxide in root nodules of *Medicago truncatula*  
920 and is regulated by tyrosine nitration. *Plant Physiology*, 157, 1505-1517.

921 Mergaert P., Nikovics K., Kelemen Z., Maunoury N., Vaubert D., Kondorosi A., et al. (2003).  
922 A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific  
923 genes coding for small, secreted polypeptides with conserved cysteine motifs. *Plant*  
924 *Physiology*, 132, 161-173.

- 925 Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., et al. (2006).  
926 Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium–legume  
927 symbiosis. *Proceedings of the National Academy of Sciences*, 103, 5230-5235.
- 928 Mesa, S., Reutimann, L., Fischer, H. M., & Hennecke, H. (2009). Posttranslational control of  
929 transcription factor FixK2, a key regulator for the *Bradyrhizobium japonicum*-soybean  
930 symbiosis. *Proceedings of the National Academy of Sciences*, 106, 21860-21865.
- 931 Montiel, J., Arthikala, M. K., Cardenas, L., & Quinto, C. (2016). Legume NADPH Oxidases  
932 Have Crucial Roles at Different Stages of Nodulation. *International Journal of*  
933 *Molecular Sciences*, 17, 680.
- 934 Müller, A. N., Ziemann, S., Treitschke, S., Abmann, D., & Doehlemann, G. (2013).  
935 Compatibility in the *Ustilago maydis*–maize interaction requires inhibition of host  
936 cysteine proteases by the fungal effector Pit2. *PLoS pathogens*, 9, e1003177.
- 937 Muglia, C., Comai, G., Spegazzini, E., Riccillo, P.M., & Aguilar, O.M. (2008). Glutathione  
938 produced by *Rhizobium tropici* is important to prevent early senescence in common  
939 bean nodules. *FEMS Microbiology Letters*, 286, 191-198.
- 940 Müller, J., Boller, T., & Wiemken, A. (2001). Trehalose becomes the most abundant non-  
941 structural carbohydrate during senescence of soybean nodules. *Journal of*  
942 *Experimental Botany*, 52, 943-947.
- 943 Naito, Y., Fujie, M., Usami, S., Murooka, Y., & Yamada, T. (2000). The involvement of a  
944 cysteine proteinase in the nodule development in Chinese milk vetch infected with  
945 *Mesorhizobium huakuii* subsp. regei. *Plant Physiology*, 124, 1087-1096.
- 946 Navascués, J., Perez-Rontome, C., Gay, M., Marcos, M., Yang, F., Walker, F. A., et al.  
947 (2012). Leghemoglobin green derivatives with nitrated hemes evidence production of  
948 highly reactive nitrogen species during aging of legume nodules. *Proceedings of the*  
949 *National Academy of Sciences*, 109, 2660-2665.
- 950 Oda, K. (2012). New families of carboxyl peptidases: serine-carboxyl peptidases and glutamic  
951 peptidases. *Journal of Biochemistry*, 151, 13-25.
- 952 Oláh B1, Kiss E, Györgypál Z, Borzi J, Cinege G, Csanádi G, et al. (2001). Mutation in the  
953 ntrR gene, a member of the vap gene family, increases the symbiotic efficiency of  
954 *Sinorhizobium meliloti*. *Molecular Plant-Microbe Interactions*, 14, 887-894.
- 955 Oldroyd, G. E. (2013). Speak, friend, and enter: signalling systems that promote beneficial  
956 symbiotic associations in plants. *Nature Reviews Microbiology*, 11, 252.
- 957 Oono, R., Schmitt, I., Sprent, J. I., & Denison, R. F. (2010). Multiple evolutionary origins of  
958 legume traits leading to extreme rhizobial differentiation. *New Phytologist*, 187, 508-  
959 520.
- 960 Ott, T., van Dongen, J. T., Gu, C., Krusell, L., Desbrosses, G., Vigeolas, H., et al. (2005).  
961 Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but  
962 not for general plant growth and development. *Current biology*, 15, 531-535.
- 963 Palma, F., Lopez-Gomez, M., Tejera, N. A., & Lluch, C. (2014). Involvement of abscisic acid  
964 in the response of *Medicago sativa* plants in symbiosis with *Sinorhizobium meliloti* to  
965 salinity. *Plant Science*, 223, 16-24.
- 966 Pan, H., Stonoha-Arther, C., & Wang, D. (2018). *Medicago* plants control nodulation by  
967 regulating proteolysis of the receptor-like kinase DMI2. *Plant Physiology*, 177, 792-  
968 802.
- 969 Perez-Guerra, J. C., Coussens, G., De Keyser, A., De Rycke, R., De Bodt, S., Van De Velde,  
970 W., et al. (2010). Comparison of developmental and stress-induced nodule senescence  
971 in *Medicago truncatula*. *Plant Physiology*, 152, 1574-1584.
- 972 Peters, J. W., Fisher, K., & Dean, D. R. (1995). Nitrogenase structure and function: a  
973 biochemical-genetic perspective. *Annual Review of Microbiology*, 49, 335-366.

- 974 Pierre, O., Engler, G., Hopkins, J., Brau, F., Boncompagni, E., & Herouart, D. (2013).  
975 Peribacteroid space acidification: a marker of mature bacteroid functioning in  
976 *Medicago truncatula* nodules. *Plant, Cell & Environment*, 36, 2059-2070.
- 977 Pierre, O., Hopkins, J., Combiér, M., Baldacci, F., Engler, G., Brouquisse, R., et al. (2014).  
978 Involvement of papain and legumain proteinase in the senescence process of  
979 *Medicago truncatula* nodules. *New Phytologist*, 202, 849-863.
- 980 Pladys D., Dimitrijevic L., Rigaud J. (1991). Localization of a protease in protoplast  
981 preparations of infected cells of French bean nodules. *Plant Physiology*, 97: 1174–  
982 1180.
- 983 Pladys D, Vance CP. (1993). Proteolysis during development and senescence of effective and  
984 plant gene-controlled ineffective alfalfa nodules. *Plant Physiology*, 103, 379–384.
- 985 Preisig, O., Zufferey, R., Thony-Meyer, L., Appleby, C. A., & Hennecke, H. (1996). A high-  
986 affinity cbb3-type cytochrome oxidase terminates the symbiosis-specific respiratory  
987 chain of *Bradyrhizobium japonicum*. *Journal of Bacteriology*, 178, 1532-1538.
- 988 Pucciariello C, Innocenti G, Van de Velde W, Lambert A, Hopkins J, Clément M, et al.  
989 (2009). (Homo)glutathione depletion modulates host gene expression during the  
990 symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti*. *Plant*  
991 *Physiology*, 151 1186-1196.
- 992 Puppo, A., Groten, K., Bastian, F., Carzaniga, R., Soussi, M., Lucas, M. M., et al. (2005).  
993 Legume nodule senescence: roles for redox and hormone signalling in the  
994 orchestration of the natural aging process. *New Phytologist*, 165, 683-701.
- 995 Puppo, A., Pauly, N., Boscari, A., Mandon, K., & Brouquisse, R. (2013). Hydrogen peroxide  
996 and nitric oxide: key regulators of the Legume-Rhizobium and mycorrhizal symbioses.  
997 *Antioxidants & Redox Signaling*, 18, 2202-2219.
- 998 Quain, M. D., Makgopa, M. E., Cooper, J. W., Kunert, K. J., & Foyer, C. H. (2015). Ectopic  
999 phytocystatin expression increases nodule numbers and influences the responses of  
1000 soybean (*Glycine max*) to nitrogen deficiency. *Phytochemistry*, 112, 179-187.
- 1001 Rawlings, N. D., Barrett, A. J., & Bateman, A. (2010). MEROPS: the peptidase database.  
1002 *Nucleic Acids Research*, 38, D227-D233.
- 1003 Redondo, F. J., Coba de la Pena, T., Lucas, M. M., & Pueyo, J. J. (2012). Alfalfa nodules  
1004 elicited by a flavodoxin-overexpressing *Ensifer meliloti* strain display nitrogen-fixing  
1005 activity with enhanced tolerance to salinity stress. *Planta*, 236, 1687-1700.
- 1006 Redondo, F. J., de la Peña, T. C., Morcillo, C. N., Lucas, M. M., & Pueyo, J. J. (2009).  
1007 Overexpression of flavodoxin in bacteroids induces changes in antioxidant metabolism  
1008 leading to delayed senescence and starch accumulation in alfalfa root nodules. *Plant*  
1009 *Physiology*, 149, 1166-1178.
- 1010 Reid, D. E., Heckmann, A. B., Novak, O., Kelly, S., & Stougaard, J. (2016). CYTOKININ  
1011 OXIDASE/DEHYDROGENASE3 Maintains Cytokinin Homeostasis during Root and  
1012 Nodule Development in *Lotus japonicus*. *Plant Physiology*, 170, 1060-1074.
- 1013 Rodrigues, A. C., Silveira, J. A. G., Bonifacio, A., & Figueiredo, M. D. V. B. (2013).  
1014 Metabolism of nitrogen and carbon: Optimization of biological nitrogen fixation and  
1015 cowpea development. *Soil Biology and Biochemistry*, 67, 226–234.
- 1016 Roux, B., Rodde, N., Jardinaud, M. F., Timmers, T., Sauviac, L., Cottret, L., et al. (2014). An  
1017 integrated analysis of plant and bacterial gene expression in symbiotic root nodules  
1018 using laser-capture microdissection coupled to RNA sequencing. *The Plant Journal*,  
1019 77, 817-837.
- 1020 Saez, I., & Vilchez, D. (2014). The Mechanistic Links Between Proteasome Activity, Aging  
1021 and Age-related Diseases. *Current Genomics*, 15, 38-51.
- 1022 Sainz, M., Calvo-Begueria, L., Perez-Rontome, C., Wienkoop, S., Abian, J., Staudinger, C., et  
1023 al. (2015). Leghemoglobin is nitrated in functional legume nodules in a tyrosine

1024 residue within the heme cavity by a nitrite/peroxide-dependent mechanism. *The Plant*  
1025 *Journal*, 81, 723-735.

1026 Sánchez, C., Gates, A. J., Meakin, G. E., Uchiumi, T., Girard, L., Richardson, D. J., et al.  
1027 (2010). Production of nitric oxide and nitrosylhemoglobin complexes in soybean  
1028 nodules in response to flooding. *Molecular Plant-Microbe Interactions*, 23, 702-711.

1029 Seabra, A. R., Pereira, P. A., Becker, J. D., & Carvalho, H. G. (2012). Inhibition of glutamine  
1030 synthetase by phosphinothricin leads to transcriptome reprogramming in root nodules of  
1031 *Medicago truncatula*. *Molecular Plant-Microbe Interactions*, 25, 976-992.

1032 Serova, T., & Tsyganov, V. (2014). Symbiotic nodule senescence in legumes: molecular-  
1033 genetic and cellular aspects (review). *Agricultural Biology*, 5, 3-15.

1034 Serova, T. A., Tikhonovich, I. A., & Tsyganov, V. E. (2017). Analysis of nodule senescence  
1035 in pea (*Pisum sativum* L.) using laser microdissection, real-time PCR, and ACC  
1036 immunolocalization. *Journal of Plant Physiology*, 212, 29-44.

1037 Shindo, T., Misas-Villamil, J. C., Hörger, A. C., Song, J., & van der Hoorn, R. A. (2012). A  
1038 role in immunity for Arabidopsis cysteine protease RD21, the ortholog of the tomato  
1039 immune protease C14. *PLoS one*, 7, e29317.

1040 Smalle, J., & Vierstra, R. D. (2004). The ubiquitin 26S proteasome proteolytic pathway.  
1041 *Annual Review of Plant Biology*, 55, 555-590.

1042 Smirnov, N., & Arnaud, D. (2019) Hydrogen peroxide metabolism and functions in plants.  
1043 *New Phytologist*, 221, 1197-1214.

1044 Smith, S. E., & Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and  
1045 growth: new paradigms from cellular to ecosystem scales. *Annual Review of Plant*  
1046 *Biology*, 62, 227-250.

1047 Soupène, E., Foussard, M., Boistard, P., Truchet, G., & Batut, J. (1995). Oxygen as a key  
1048 developmental regulator of *Rhizobium meliloti* N<sub>2</sub>-fixation gene expression within the  
1049 alfalfa root nodule. *Proceedings of the National Academy of Sciences*, 92, 3759-3763.

1050 Streeter, J. G. (1981). Seasonal Distribution of Carbohydrates in Nodules and Stem Exudate  
1051 from Field-grown Soya Bean Plants. *Annals of Botany*, 48, 441-450.

1052 Swaraj, K., & Bishnoi, N. R. (1996). Physiological and biochemical basis of nodule  
1053 senescence in legumes: a review. *Plant Physiology and Biochemistry*, 23, 105-116.

1054 Tang, G., Li, N., Liu, Y., Yu, L., Yan, J., & Luo, L. (2018) *Sinorhizobium meliloti* glutathione  
1055 reductase is required for both redox homeostasis and symbiosis. *Applied and*  
1056 *Environmental Microbiology*, 84, e01937-17

1057 Tang, G., Xing, S., Wang, S., Yu, L., Li, X., Staehelin, C., et al. (2017) Regulation of cysteine  
1058 residues in LsrB proteins from *Sinorhizobium meliloti* under free-living conditions and  
1059 symbiotic oxidative stress. *Environmental Microbiology*, 19, 5130-5145.

1060 Timmers, A. C. J., Auriac, M. C. & Truchet, G. (1999). Refined analysis of early symbiotic  
1061 steps of the *Rhizobium-Medicago* interaction in relationship with microtubular  
1062 cytoskeleton rearrangements. *Development*, 126, 3617-3628.

1063 Tittabutr, P., Sripakdi, S., Boonkerd, N., Tanthanuch, W., Minamisawa, K., & Teamroong,  
1064 N. (2015). Possible Role of 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase  
1065 Activity of *Sinorhizobium* sp. BL3 on Symbiosis with Mung Bean and Determinate  
1066 Nodule Senescence. *Microbes and Environments*, 30, 310-320.

1067 Tjepkema, J. D., & Yocum, C. S. (1974). Measurement of oxygen partial pressure within  
1068 soybean nodules by oxygen microelectrodes. *Planta*, 119, 351-360.

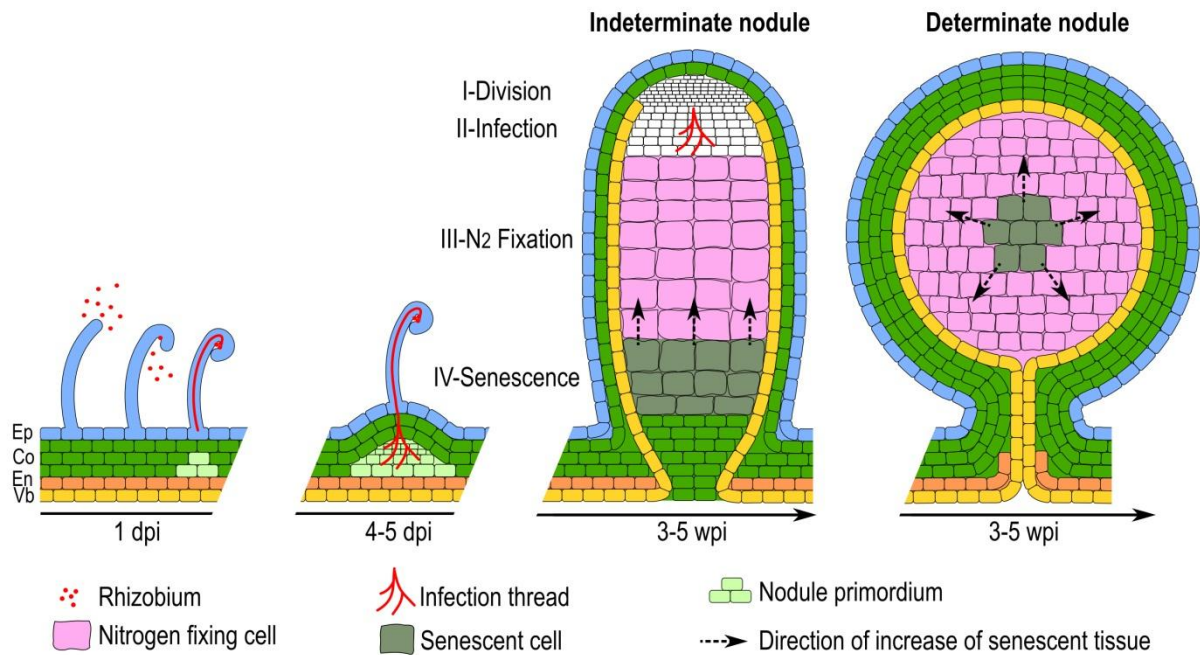
1069 Trinchant, J. C., & Rigaud, J. (1982). Nitrite and nitric oxide as inhibitors of nitrogenase from  
1070 soybean bacteroids. *Applied and Environmental Microbiology*, 44, 1385-1388.

1071 Van de Velde, W., Guerra, J. C., De Keyser, A., De Rycke, R., Rombauts, S., Maunoury, N.,  
1072 et al. (2006). Aging in legume symbiosis. A molecular view on nodule senescence in  
1073 *Medicago truncatula*. *Plant Physiology*, 141, 711-720.

- 1074 Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., et al.  
1075 (2010). Plant peptides govern terminal differentiation of bacteria in symbiosis.  
1076 *Science*, 327, 1122-1126.
- 1077 Van Der Heijden, M. G., De Bruin, S., Luckerhoff, L., Van Logtestijn, R. S., & Schlaeppi, K.  
1078 (2016). A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity,  
1079 plant nutrition and seedling recruitment. *The ISME journal*, 10, 389.
- 1080 Van der Hoorn, R. A. (2008). Plant proteases: from phenotypes to molecular mechanisms.  
1081 *Annual Review of Plant Biology*, 59, 191-223.
- 1082 Van der Linde, K., Hemetsberger, C., Kastner, C., Kaschani, F., van der Hoorn, R. A.,  
1083 Kumlehn, J., & Doehlemann, G. (2012). A maize cystatin suppresses host immunity  
1084 by inhibiting apoplastic cysteine proteases. *The Plant Cell*, 24, 1285-1300.
- 1085 Van Wyk, S. G., Du Plessis, M., Cullis, C. A., Kunert, K. J., & Vorster, B. J. (2014). Cysteine  
1086 protease and cystatin expression and activity during soybean nodule development and  
1087 senescence. *BMC Plant Biology*, 14, 294.
- 1088 Vance, C. P., Heichel, G. H., Barnes, D. K., Bryan, J. W., & Johnson, L. E. (1979). Nitrogen  
1089 Fixation, Nodule Development, and Vegetative Regrowth of Alfalfa (*Medicago sativa*  
1090 L.) following Harvest. *Plant Physiology*, 64, 1-8.
- 1091 Vasse, J., de Billy, F., Camut, S., & Truchet, G. (1990). Correlation between ultrastructural  
1092 differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *Journal of*  
1093 *Bacteriology*, 172, 4295-4306.
- 1094 Venkateshwaran, M., Volkening, J. D., Sussman, M. R., & Ané, J. M. (2013). Symbiosis and  
1095 the social network of higher plants. *Current Opinion in Plant Biology*, 16, 118-127.
- 1096 Wang, W., Shi, J., Xie, Q., Jiang, Y., Yu, N., & Wang, E. (2017). Nutrient exchange and  
1097 regulation in arbuscular mycorrhizal symbiosis. *Molecular plant*, 10, 1147-1158.
- 1098 Xu J, Li S-L, Luo L. (2012). Effects of engineered *Sinorhizobium meliloti* on cytokinin  
1099 synthesis and tolerance of alfalfa to extreme drought stress. *Applied and*  
1100 *Environmental Microbiology*, 78, 8056–8061.
- 1101 Yu, H., Xiao, A., Dong, R., Fan, Y., Zhang, X., Liu, C., et al. (2018). Suppression of innate  
1102 immunity mediated by the CDPK-Rboh complex is required for rhizobial  
1103 colonization in *Medicago truncatula* nodules. *New Phytologist*, 220, 425-434.
- 1104 Yuan, S. L., Li, R., Chen, H. F., Zhang, C. J., Chen, L. M., Hao, Q. N., et al. (2017). RNA-Seq  
1105 analysis of nodule development at five different developmental stages of soybean  
1106 (*Glycine max*) inoculated with *Bradyrhizobium japonicum* strain 113-2. *Scientific*  
1107 *Reports*, 7, 42248.
- 1108 Yun, B. W., Feechan, A., Yin, M., Saidi, N. B., Le Bihan, T., Yu, M., et al. (2011). S-  
1109 nitrosation of NADPH oxidase regulates cell death in plant immunity. *Nature*, 478,  
1110 264-268.
- 1111 Zwack, P. J., & Rashotte, A. M. (2013). Cytokinin inhibition of leaf senescence. *Plant*  
1112 *Signaling & Behavior* 8, e24737.
- 1113 Zwack, P. J., Robinson, B. R., Risley, M. G., & Rashotte, A. M. (2013). Cytokinin response  
1114 factor 6 negatively regulates leaf senescence and is induced in response to cytokinin  
1115 and numerous abiotic stresses. *Plant and Cell Physiology*, 54, 971-981.

1116

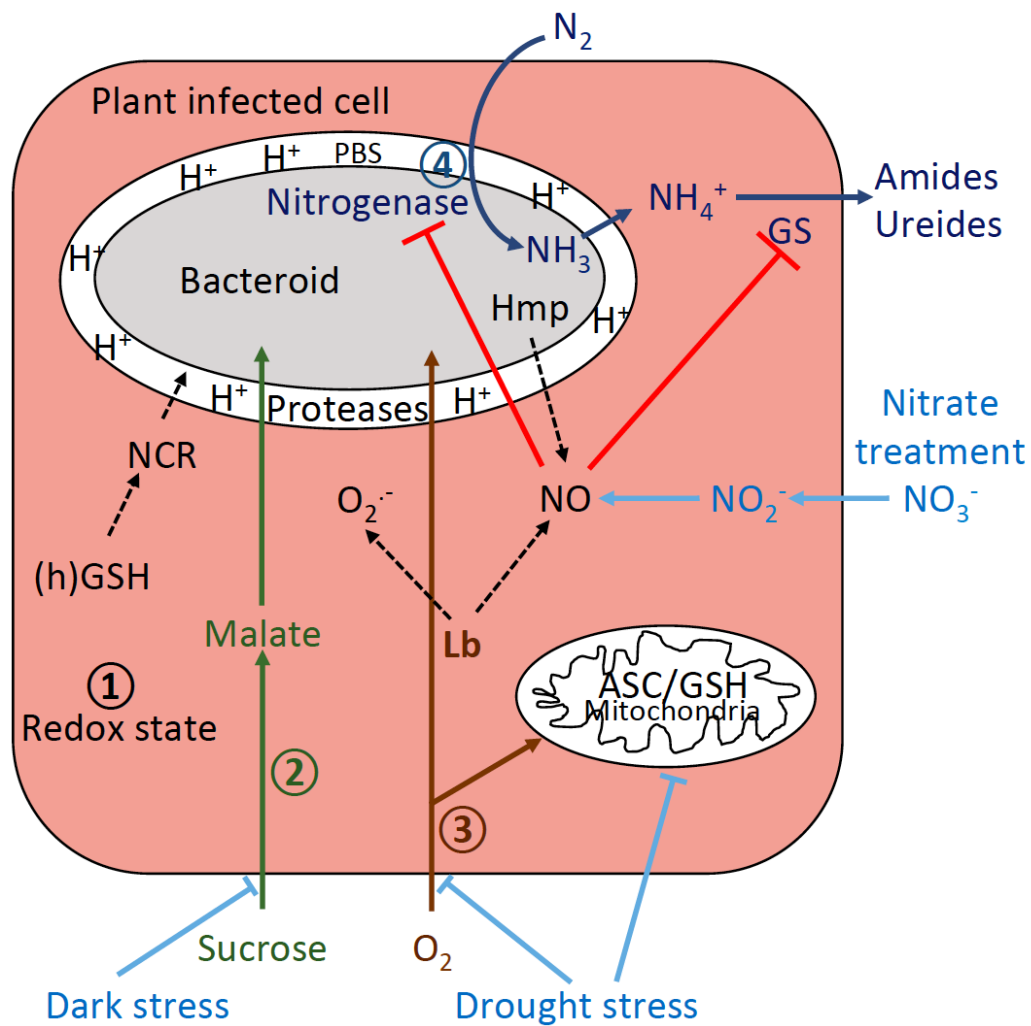
1117 **Figures & Legends**



1118

1119 **Figure 1 : Schematic representation of the establishment and of the development of the**  
 1120 **legume-rhizobium symbiosis, and of the structure of mature indeterminate and**  
 1121 **determinate nodules.**

1122 En, endodermis; Co, cortex; dpi, day post-inoculation; Ep, epidermis; Vb, vascular bundle;  
 1123 wpi, week post-inoculation.



1124

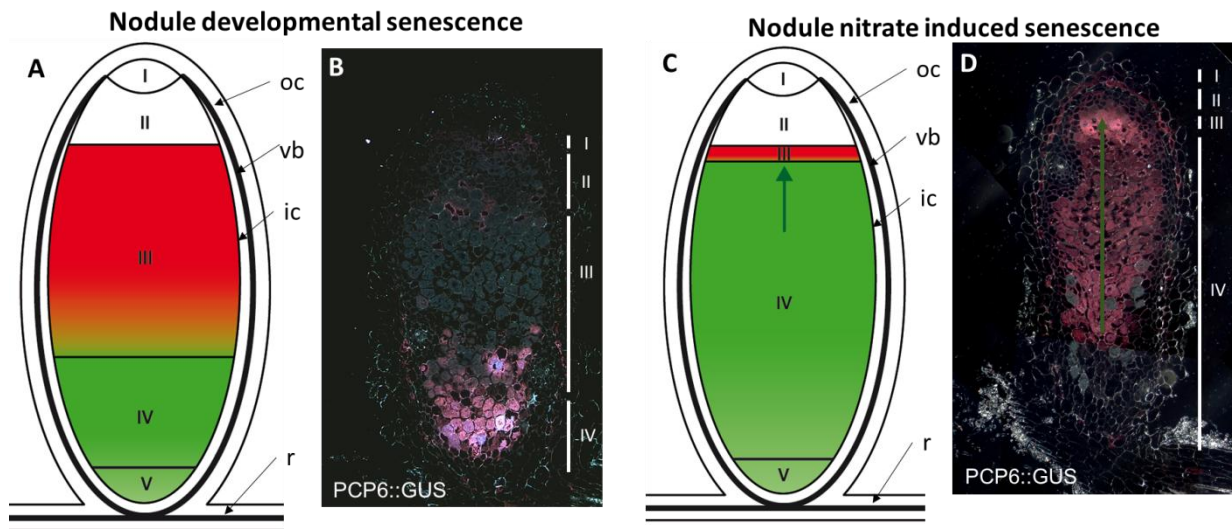
1125 **Figure 2: Major processes triggering nodule senescence in response to environmental**  
 1126 **constraints.**

1127 (1), Modification of the redox balance; (2), alteration of the bacteroid carbon nutrition; (3),  
 1128 alteration of O<sub>2</sub> homeostasis; (4), alteration of N<sub>2</sub> fixation (nitrogenase). Red lines indicate  
 1129 down-regulation. Black dotted lines indicate scavenging of NO or O<sub>2</sub> by Lb or Hmp proteins.  
 1130 The pink color of the plant cell cytoplasm illustrates the Lb content. ASC: Ascorbate; Hmp:  
 1131 Flavoheemoglobin; GS: Glutamine; (h)GSH: (homo)Glutathione; synthetase; Lb:  
 1132 Leghemoglobin; PBS: Peribacteroid space; Proteases: Cysteine proteases; NCR: Nodule  
 1133 cysteine-rich peptides.

1134

1135





1136

1137 **Figure 3: Developmental and induced senescence in the *M. truncatula* nodule.**

1138 **A)** Schematic representation of developmental/aging senescence in indeterminate nodules. I,  
 1139 meristematic zone ; II, rhizobial infection zone ; III, nitrogen fixing zone (in red); IV,  
 1140 senescence zone (in green); V, saprophytic zone (in green). oc, outer cortex; ic, inner cortex;  
 1141 vb, vascular bundle; r, root. **B)** Section of a nodule expressing the reporter gene *uidA* (GUS)  
 1142 under control of the *MtCP6* promoter 6 weeks post-inoculation. **C)** Schematic representation  
 1143 of nitrate induced-senescence in indeterminate nodules. The arrow shows the extension of the  
 1144 senescent tissues revealed by the *pMtCP6*:GUS reporter. **D)** Section of a nodule expressing  
 1145 the reporter gene *uidA* (GUS) under control of the *MtCP6* promoter 3 days after the nitrate  
 1146 treatment.

1147

1148

1149

1150

1151

1152

1153

1154

1155

1156

1157

1158

1159

1160

1161

Gene (mutant)	Species	Gene characteristics	Reference
<i>LjIPT3</i>	<i>Lotus japonicus</i>	Reduced nitrogen fixation observed in RNAi plants. Coding for an Isopentenyl-Transferase, a rate limiting step in cytokinin biosynthesis	Chen <i>et al.</i> , 2014
<i>MtCP6</i> and <i>MtVPE</i>	<i>Medicago truncatula</i>	RNAi plants are delayed in the apparition of senescence in nodule	Pierre <i>et al.</i> , 2014
<i>MtNAC969</i>	<i>Medicago truncatula</i>	Accumulation of senescence marker transcripts in RNAi plants	de Zélicourt <i>et al.</i> , 2012
<i>Hmp</i> ( <i>hmp</i> <sup>++</sup> )	<i>Sinorhizobium meliloti</i>	Hmp overexpression delays nodule senescence. Hmp encodes a flavohemoglobin involved in NO detoxication	Cam <i>et al.</i> , 2012
<i>nnrS1</i> <i>nnrS2</i>	<i>Sinorhizobium meliloti</i>	<i>nnrS1/2</i> mutants induce early nodule senescence. <i>nnrS1/2</i> encode for cooper-containing membrane proteins involved in NO detoxication	Blanquet <i>et al.</i> , 2015
<i>norB</i>	<i>Sinorhizobium meliloti</i>	<i>norB</i> mutants induce early nodule senescence. <i>norB</i> encodes for a NO reductase in the denitrification pathway	Meilhoc <i>et al.</i> , 2013
<i>LjGlb1-1</i>	<i>Lotus japonicus</i>	<i>Glb1-1</i> overexpression delays nodule senescence. <i>Glb1-1</i> encodes a Phytoglobin involved in NO detoxification	Fukudome <i>et al.</i> , 2019
<i>vapBC-5</i>	<i>Sinorhizobium meliloti</i>	Delayed senescent phenotype. Coding an active bacteria toxin	Lipuma <i>et al.</i> , 2014

1162

1163 **Table 1: Genes involved in symbiotic nodule senescence.**1164 *IPT* : IsoPentenyl Transferase, *CP* : Cystein Protease, *VPE*: Vacuolar Processing Enzyme,1165 *NAC* : NAM ATAF CUC transcription factor, *Hmp* : flavohemoglobin, *nnrS*: nitrite and nitric1166 oxide reductase S, *norB*: NO reductase B, *Glb1* : Class I Phytoglobulin, *vapBC* : toxin-

1167 antitoxin module.

1168

1169

1170