

1 **Split-root systems applied to the study of the legume-rhizobial symbiosis: what**  
2 **have we learned?**

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33 **ABSTRACT**

34 Split-root system (SRS) approaches allow the differential treatment of separate and  
35 independent root systems, while sharing a common aerial part. As such, SRS is a useful  
36 tool for the discrimination of systemic (shoot origin) versus local (root/nodule origin)  
37 regulation mechanisms. This type of approach is particularly useful when studying the  
38 complex regulatory mechanisms governing the symbiosis established between legumes  
39 and *Rhizobium* bacteria. The current work provides an overview of the main insights  
40 gained from the application of SRS approaches to understand how nodule number  
41 (nodulation autoregulation) and nitrogen fixation are controlled both under non-  
42 stressful conditions and in response to a variety of stresses. Nodule number appears to  
43 be mainly controlled at the systemic level through a signal which is produced by  
44 nodule/root tissue, translocated to the shoot and transmitted back to the root system,  
45 involving shoot Leu-rich repeat receptor-like kinases. In contrast, both local and  
46 systemic mechanisms have been shown to operate for the regulation of nitrogenase  
47 activity in nodules. Under drought and heavy metal stress, the regulation is mostly local,  
48 whereas the application of exogenous nitrogen seems to exert a regulation of nitrogen  
49 fixation both at the local and systemic levels.

50

51 **INTRODUCTION**

52 The cultivation of legume crops offers a number of advantages from the  
53 environmental, economic and human perspective. The soil-enriching properties of  
54 legumes are known from ancient times and the high-protein content of their seeds  
55 makes them an invaluable source of protein for human consumption and animal fed  
56 worldwide. One of the reasons behind this high protein content is the ability of most  
57 legume plants to associate with soil bacteria, collectively named as “rhizobia”, which

58 provides them with a source of reduced nitrogen (N) to sustain growth, without the need  
59 for an additional N supply.

60 This symbiotic association is generally initiated by a molecular dialogue  
61 between both bacterial and plant partners and results in the formation of a new organ in  
62 the plant root called a “nodule”. One of the most widely studied systems is the one  
63 based both on crop and model legume plants, in which plant recognition of the  
64 symbiotic partner is initiated by the perception, at the plasma membrane, of rhizobial  
65 signaling molecules, “Nod factors” (NFs), required for the processes of root bacterial  
66 infection and nodule organogenesis. In this type of symbiosis, compatible rhizobium  
67 bacteria are able to penetrate into the root cortex through an infection thread and  
68 differentiate into a symbiotic form named “bacteroid” surrounded by a plant-derived  
69 membrane (Oldroyd et al. 2011). There is, however, a large diversity in the mechanisms  
70 underlying the establishment and operation of the legume-rhizobial symbiosis, and  
71 systems that do not require NFs (Giraud et al. 2007) or use alternative “crack entry”  
72 mechanisms (Sprent 2008; Madsen et al. 2010) have been described.

73 The reaction in which atmospheric dinitrogen ( $N_2$ ) is reduced into ammonium in  
74 a reaction catalyzed by the bacteroid nitrogenase complex (Nase) is called symbiotic  
75 nitrogen fixation (SNF). The legume-rhizobial mutualistic symbiosis is established  
76 through the exchange of nutrients between both partners: plants provide a carbon source  
77 to the rhizobium bacteria to fuel the energy-demanding reduction of  $N_2$  in a  
78 microaerobic environment, obtaining in return a N source to sustain plant growth.

79 As they represent such a sink of energy for the plant, the process of nodulation  
80 and SNF are subjected to tight control from the plant side. Whether this regulation  
81 occurs at the local (root/nodule tissue) or systemic level (shoot tissue) has been a matter  
82 of debate during the last decades. The use of systems in which the root is split into

83 different compartments has significantly contributed to answering these type of  
84 questions. The current review summarizes the main contributions of split-root system  
85 (SRS)-based studies towards understanding the regulation of the legume-rhizobia  
86 symbiotic interaction.

87

## 88 **SPLIT-ROOT SYSTEM: A TOOL TO STUDY LOCAL AND SYSTEMIC** 89 **REGULATION PROCESSES**

90         Depending on the plant being studied, SRS can be generated using different  
91 strategies. Generally, seeds are germinated and plantlets are first grown for some days  
92 until they develop a main root. Subsequently, the root is longitudinally cut and the two  
93 root sections are then grown in independent pots until they fully develop. Alternatively,  
94 the main root tip can be removed to induce secondary roots that will then be split in two  
95 separate environments (Kassaw and Frugoli, 2012; Marino et al. 2013). The great  
96 advantage of this system is that it allows the differential treatment of separate and  
97 independent root systems, while sharing a common aerial part. Thus, SRS is a useful  
98 tool for the discrimination of systemic (shoot origin) versus local (root/nodule origin)  
99 regulation mechanisms (Fig. 1).

100         Plants use both long- and short-distance transport mechanisms to communicate  
101 between shoot and root tissue. These transport routes also participate in the complex  
102 signal perception systems used by plants to respond to a constantly changing  
103 environment. Different molecules, including hormones, reactive oxygen species, RNA,  
104 proteins, peptides and lipids are implicated in the long-distance signaling mechanisms  
105 associated with developmental processes and biotic and abiotic stress responses  
106 (Turnbull and Lopez-Cobollo, 2012).

107         In the SRS studies discussed in this review, a stimulus is applied to one side of

108 the SRS and the response of the other side of the root not directly exposed to the  
109 stimulus is analyzed (Fig. 2). Similarly, treatments are applied to each root at different  
110 time points to check whether there is a systemic priming effect. When stimuli are  
111 related to a systemic regulation, the untreated part of the root system will show a  
112 response, suggesting that long-distance transport mechanisms are involved in this  
113 response (Fig. 1). On the other hand, when a certain stimulus induces a local response,  
114 the effects are only visible on the root side directly exposed to the stress (Fig. 1). It is  
115 often the case, however, that local and systemic responses coexist as part of the  
116 functional interaction between shoots and roots. Thus, it is crucial to clearly identify  
117 which is the target of the study and special care should be taken in order to use  
118 adequate references to compare control and treated plants.

119

## 120 **SPLIT-ROOT SYSTEM TO UNDERSTAND NODULATION REGULATION**

121 Nodule formation is energetically expensive and, therefore, nodulation is a  
122 strictly-controlled process. Both environmental conditions, such as soil N availability,  
123 and endogenous plant signals, including plant hormones, are some of the factors  
124 involved in this regulation. The number of nodules is tightly controlled by a complex  
125 root-to-shoot-to-root signaling loop named autoregulation of nodulation (AON). Several  
126 experiments based on SRS have provided evidence for a systemic regulation of AON.  
127 Pre-inoculation of one half of the split-root has been shown to dramatically inhibit  
128 subsequent nodule formation in the other half root in several legume species, including  
129 soybean (*Glycine max* L. Merr.; Kosslak and Bohlool, 1984), alfalfa (*Medicago sativa*  
130 L.; Caetano-Anollés et al. 1990), common bean (*Phaseolus vulgaris* L.; George et al.  
131 1992), pea (*Pisum sativum* L.; Li et al. 2009), common vetch (*Vicia sativa*; van Brussel  
132 et al. 2002), subterranean clover (*Trifolium subterraneum*; Sargent et al. 1987) and the

133 model legumes *Lotus japonicus* (Suzuki et al. 2008) and *Medicago truncatula* (Jeudy et  
134 al. 2010). NFs have also been shown to be essential for AON both in experiments in  
135 which the split roots were pre-treated directly with NFs (van Brussel et al. 2002;  
136 Catford et al. 2003) and upon the inoculation of bacterial mutant strains impaired in  
137 their ability to produce NFs or form nodules (Sargent et al. 1987; Caetano-Anollés et al.  
138 1990; Champion et al. 1992; George et al. 1992; van Brussel et al. 2002; Suzuki et al.  
139 2008). Overall, it appears that NF production, rhizobial infection and the presence of  
140 bacteria inside nodules are required for a fully functional AON.

141 Genetic approaches have allowed the identification of legume orthologs of  
142 *Arabidopsis thaliana* CLAVATA1-like Leu-rich repeat receptor-like kinases (LRR-  
143 RLKs), which have been found to be key players in the systemic regulation of AON.  
144 Mutants in several legume LRR-RLKs such as *L. japonicus* HAR1 (Nishimura et al.  
145 2002), soybean NARK (Olsson et al. 1989; Perigio and Harper, 1995; Lin et al. 2012)  
146 and *M. truncatula* SUNN (Jeudy et al. 2010) lack the typical nodulation inhibitory  
147 responses observed in wild type plants. Based on the evidence that *A. thaliana*  
148 CLAVATA1 is activated by the interaction with the CLE-family peptide CLV3, SRS-  
149 based approaches have identified two *GmCLE* genes (*GmRIC1* and 2) involved in AON  
150 in soybean (Lim et al. 2011). Although the exact nature of the signal controlling AON  
151 remains unknown, CLE peptides (CLAVATA3/Embryo-surrounding region protein-  
152 related peptides) are good candidates for this role (Mortier et al. 2012a; Mortier et al.  
153 2012b; Okamoto et al. 2013).

154 The use of SRS-based approaches has also been useful to provide evidence for  
155 the crosstalk between AON and N signaling. In *M. truncatula*, inhibition of nodulation  
156 by the external application of a mineral N source was found to be suppressed in the  
157 AON mutant *sunn*, and the inhibitory effect of AON was partially reduced by the N

158 demand at the whole plant level (Jeudy et al. 2010). Indeed, long term N limitation in  
159 half of a split-root system induced compensatory responses in the other half through  
160 nodule expansion and new nodule formation (Jeudy et al. 2010; Laguerre et al. 2011).  
161 These results agree with previous studies in peanut (*Arachis hypogaea* L.; Daimon and  
162 Yoshioka, 2001) and soybean (Cho and Harper, 1991), in which the application of  
163 nitrate to one half of a split-root led to a partial or inhibition of nodule formation in the  
164 untreated side of the root, confirming the systemic nature of AON regulation by the  
165 plant N status. Besides mineral N availability in the soil, other environmental conditions  
166 can also regulate AON. For instance, alteration of soil acidic conditions in one side of  
167 the split-root systemically controlled AON and this control was suppressed in the  
168 soybean hypernodulating *NARK* mutant (Lin et al. 2012). Overall, a signal is locally  
169 produced in the nodules/roots, translocated to shoots and transmitted again to the roots  
170 to prevent excessive nodule formation (Fig. 2).

171         Although SRSs have been useful to study the systemic control of nodulation, it  
172 has also allowed identifying factors that locally affect nodule number, for instance  
173 abscisic acid (Biswas et al. 2009) and local Fe concentration (Tang et al. 1990). The rate  
174 of SNF itself also appears to regulate nodule development, since low SNF rates result in  
175 the arrest of nodule growth (Jeudy et al. 2010).

176         In summary, although much progress has been made in understanding the link  
177 between plant N status, NF perception, hormones, CLE peptides and AON, the nature of  
178 the signals involved and how they are perceived requires additional research efforts.  
179 SRS-based studies will surely contribute to shed further light on these unsolved  
180 questions in the future.

181

182 **SPLIT-ROOT SYSTEM STUDIES FOCUSED ON THE REGULATION OF**

183 **NITROGEN FIXATION**

184       Given the high-energy cost that nodule maintenance represents for nitrogen-fixing  
185 legumes, it is not surprising that the process of SNF is rapidly shut down upon  
186 environmental perturbations. Both abiotic and biotic stresses have been shown to  
187 provoke a rapid inhibition of SNF in legumes grown under symbiotic conditions.  
188 Drought stress has been one of the most studied abiotic factors affecting SNF in  
189 legumes, due to its significant impact on crop yield and plant productivity. Although  
190 several hypotheses have been proposed to explain the decline in SNF rates during  
191 drought, the origin of the inhibitory signal(s), the molecular mechanism(s) involved and  
192 the interaction among the factors responsible for the inhibition of SNF are not yet fully  
193 understood. The regulation of SNF under drought stress involves diverse factors: carbon  
194 (C) limitation, oxygen control and N-feedback regulation are some of the most studied  
195 (Arrese-Igor et al. 2011). Traditionally, SNF regulation has been thought to rely  
196 completely on shoot performance via the photosynthetic process. However, Durand *et*  
197 *al.* (1987) and references therein have shown that SNF inhibition under moderate  
198 drought occurs prior to any measurable drop in photosynthesis rates, highlighting the  
199 high sensitivity of this process.

200       The existence of a systemic signal coming from shoots that controls SNF in nodules  
201 has been commonly accepted based on studies where exogenous N compounds were  
202 applied and these conclusions have often been extrapolated to other stresses.  
203 Nevertheless, the fact that similar regulatory mechanisms occur upon the exposure to  
204 other stress conditions needs to be demonstrated empirically. To our knowledge,  
205 Singleton (1983) used SRS to study nodule performance for the first time. Recent works  
206 using SRS-based approaches have addressed this question providing evidences for the  
207 operation of local regulatory mechanisms controlling SNF in pea (Marino et al. 2007),



208 *M. truncatula* (Gil-Quintana et al. 2013a) and soybean (Gil-Quintana et al. 2013b)  
209 under water deficit. Moreover, Marino *et al.* (2007) observed a differential  
210 accumulation of malate, the main C substrate used by bacteroids, in split-root pea plants  
211 subjected to water deficit. The content of malate locally declined in the un-watered  
212 split-root section concomitant with a reduction in nodule water potential, sucrose  
213 synthase and Nase activity. These results support the existence of a local C-based  
214 regulation of SNF in pea (Marino et al. 2007). More recently, Gil-Quintana *et al.*  
215 (2013a, b) also observed that the inhibition of Nase activity both in soybean and *M.*  
216 *truncatula* plants under drought conditions was locally regulated. Furthermore, results  
217 obtained after profiling the variations in amino acid and ureide content in different parts  
218 of the plant challenged the widely accepted N-based systemic regulation hypothesis, in  
219 which a putative signaling role was attributed to individual N compounds (King and  
220 Purcell, 2005; Sulieman et al. 2010; Vadez et al. 2000). The application of SRS for the  
221 study of other osmotic stresses remains to be explored.

222 Besides abiotic stresses, inorganic N supply is one of the most studied factors that  
223 inhibit SNF. Several hypotheses have tried to explain the mechanisms underlying Nase  
224 inhibition under high nitrate conditions, including an alteration in nodule oxygen  
225 permeability and nitric oxide (NO) binding to leghemoglobin (Minchin et al. 2008).  
226 Nevertheless, there are few studies in which SRS have been applied to address this  
227 question. For instance, Blumenthal et al. (1997) grew alfalfa plants using a SRS in  
228 which a gradient of nitrate concentrations was applied vertically. The authors concluded  
229 that it was the nitrate taken up by the plant that had a significant effect on specific Nase  
230 activity (systemic regulation) but not the local nitrate concentration of the nutrient  
231 solution surrounding the nodules. However, in peanut, a dual regulatory mechanism was  
232 observed upon plant exposure to high nitrate concentrations. In the short term (5 days

233 after nitrate exposure), the inhibition of SNF occurred at the local level, while longer  
234 term exposure to these inhibitory concentrations (30 days after nitrate exposure) was  
235 found to follow a systemic regulation pattern (Daimon and Yoshioka, 2001).

236 The regulation of Nase activity by contrasting N concentrations has also been an  
237 intense focus of study in the model plant *M. truncatula*. In a transcriptomic analysis,  
238 Ruffel et al. (2008) showed that the uptake of nitrate and ammonium, together with  
239 SNF, are subjected to systemic signals related to the plant N status. Interestingly, when  
240 plants grown in SRS were subjected to local N deprivation, only nitrate-fed plants were  
241 able to rapidly compensate for this deprivation and maintain the N status of the plant  
242 (Ruffel et al. 2008). Following this study, Jeudy *et al.* (2010) investigated short (4 days)  
243 vs. long-term (14 days) *M. truncatula* exposure to an N<sub>2</sub>-deprived atmosphere or to high  
244 N supply. Unlike nitrate-fed plants, plants grown under symbiotic conditions were not  
245 able to compensate the localized N limitation in the short-term treatment. However, in  
246 the long term there was an increase in nodule biomass and nodule numbers in the N<sub>2</sub>-  
247 fixing part of the root. These results, together with studies carried out with the *sun*  
248 hypernodulating mutant (Jeudy et al. 2010) and by the co-inoculation of fixing and non-  
249 fixing rhizobial strains (Laguerre et al. 2011), revealed that both local and systemic  
250 signaling mechanisms coexist in the regulation of SNF under N stress in *M. truncatula*.

251 External application of heavy metals also provokes stressful conditions leading to  
252 inhibition of SNF rates in legumes. The negative effects of aluminum (Al) on  
253 nodulation and nodule activity have been studied in soybean plants using a vertical  
254 SRS. Silva and Sodek (1997) concluded that acid soil pH had a detrimental effect on the  
255 number of infections initiated, while high Al concentrations primarily reduced nodule  
256 growth in a local manner. An SRS-based approach has also been employed to analyze  
257 the effects of cadmium (Cd) on nodulated *M. truncatula* plants (Marino et al. 2013).

258 The differential application of Cd to one part of the root was found to lead to a specific  
259 activation of the nodule antioxidant machinery and a concomitant inhibition of SNF,  
260 mostly likely under the control of local oxygen-based regulatory mechanisms.

261 Many abiotic stresses, including Cd and drought, are known to generate reactive  
262 oxygen species (ROS) over-production and cell redox imbalance. SNF is known to be  
263 extremely sensitive to these redox changes. Interestingly, it has been shown that drought  
264 effects on nodule metabolism can be mimicked by methyl viologen, a compound that  
265 exacerbates ROS production and induces alterations in the redox status (Marino et al.  
266 2006). Water deprivation in one side of a split-root also generated a redox imbalance  
267 only in this fraction of the root, suggesting a localized control of nodule redox status  
268 (Marino et al. 2007). Similarly, Cd application to one side of a *M. truncatula* split-root  
269 generated a local induction of the nodule antioxidant machinery (Marino et al. 2013).  
270 However, some antioxidants were also partially induced in the non-treated half, a  
271 response which could be due to a low level translocation of Cd, a very mobile element,  
272 to the untreated half (Marino et al. 2013). Taken together, these results suggest that  
273 ROS are involved not only in the signal transduction pathway connecting abiotic stress  
274 perception and SNF inhibition, but also in causing direct oxidative damage to nodule  
275 components.

276 Alternatively, although the hypothesis of a long-distance movement of ROS  
277 remains controversial, ROS waves have been involved in the propagation of systemic  
278 signals in *A. thaliana* (Suzuki et al. 2013). Indeed, nitrate-induced nodule senescence  
279 has been shown to cause ROS over-production in pea (Escuredo et al. 1996).  
280 Furthermore, in recent years the involvement of Reactive Nitrogen Species (RNS) and,  
281 especially NO, in nodule establishment and functioning has become clear (Minchin et  
282 al. 2008; Arrese-Igor et al. 2011; Puppo et al. 2013). In this regard, SRS-based studies

283 will greatly contribute to decipher the role of ROS/RNS in the regulation of symbiotic  
284 nitrogen fixation.

285

## 286 **CONCLUSIONS**

287         The current review illustrates the usefulness of SRS to better understand the  
288 regulation of complex biological systems such as the legume-rhizobial symbiosis (Fig.  
289 2). Overall, SRS-based approaches have been fundamental in demonstrating the  
290 systemic long-distance suppression of nodulation, as well as the systemic control of  
291 SNF by the plant N status. Although much progress has been made in understanding the  
292 link between plant N status, NF perception, hormones, CLE peptides and AON,  
293 defining the specific signals involved and how they are perceived requires additional  
294 research efforts.

295         Short-term abiotic stress (e.g. drought, Cd and Al) exposure leads to a local  
296 inhibition of SNF. However, longer-term treatments may lead to a generalized response  
297 affecting the whole plant, depending on the nature and intensity of the stress.  
298 Additionally, SRS approaches have confirmed a close interconnection between SNF  
299 inhibition, C limitation, nodule redox alterations and accumulation of nitrogenous  
300 compounds. However, the molecular mechanisms behind these responses and the time  
301 sequence of the events that finally lead to down-regulation of SNF are barely known.

302         In summary, SRS studies have greatly contributed to a better understanding of the  
303 legume-rhizobial symbiosis and it is more than likely that this simple experimental  
304 setup will continue being a reference tool for plant physiologists interested in analyzing  
305 the local and systemic nature of plant signaling processes.

306

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314

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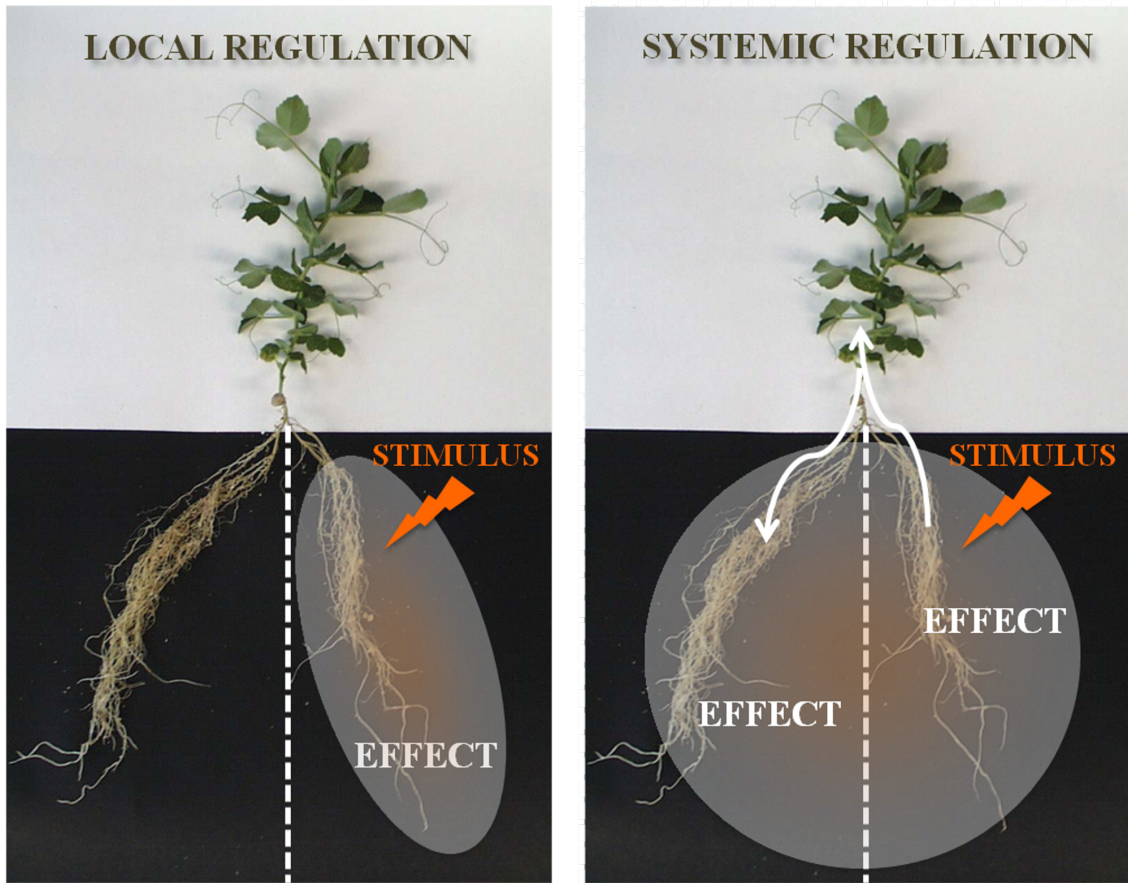
#### 454 **FIGURE LEGENDS**

455 **Figure 1:** Split-root system set up as a tool to study the local or systemic nature of plant  
456 signaling processes.

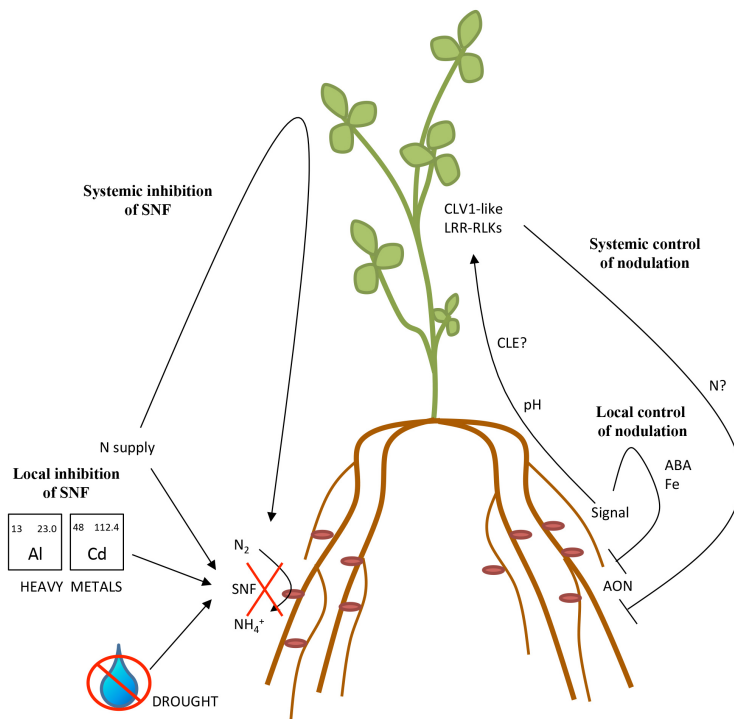
457 **Figure 2:** Schematic diagram of the main legume-rhizobium symbiotic plant processes

458 for which SRS has been useful to determine whether there is a local or systemic  
459 regulation. LRR-RLK: Leu-rich repeat receptor-like kinase; ABA: abscisic acid; AON:  
460 autoregulation of nodulation.

461



462  
463 Figure 1



464  
465 Figure 2