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SHORT COMMUNICATION

Medicago-Sinorhizobium-Ralstonia: A Model System to Investigate Pathogen-Triggered Inhibition of Nodulation

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How plants deal with beneficial and pathogenic microorganisms and how they can tolerate beneficial ones and face pathogens at the same time are questions that remain puzzling to plant biologists. Legume plants are good models to explore those issues, as their interactions with nitrogen-fixing bacteria called rhizobia results in a drastic and easy-to-follow phenotype of nodulation. Intriguingly, despite massive and chronic infection, legume defense reactions are essentially suppressed during the whole symbiotic process, raising a question about a potential negative effect of plant immune responses on the establishment of nodulation. In the present study, we used the model legume, *Medicago truncatula*, coinoculated with mutualistic and phytopathogenic bacteria, *Sinorhizobium medicae* and *Ralstonia solanacearum*, respectively. We show that the presence of *R. solanacearum* drastically inhibits the nodulation process. The type III secretion system of *R. solanacearum*, which is important for the inhibition of pathogen-associated molecular pattern-triggered immunity (PTI), strongly contributes to inhibit nodulation. Thus, our results question the negative effect of PTI on nodulation. By including a pathogenic bacterium in the interaction system, our study provides a new angle to address the influence of the biotic environment on the nodulation process.

Keywords: coinoculation, legume, nitrogen-fixing bacteria, pathogen, symbiosis, tripartite system

Legumes can interact with nitrogen-fixing bacteria called rhizobia, which provide them nitrogen. These bacteria are hosted in root nodules. At the early stages of the interaction, defense reactions that are normally induced by microbes through the plant perception of microbial-associated molecular patterns (MAMPs) or effectors are suppressed or weak and transient. For instance, in the model legume *Medicago truncatula*, defense-related genes are transiently upregulated upon interaction with *Sinorhizobium* spp. (Lohar et al. 2006), but then, there are no typical defense reactions all along the interaction (Benezech et al. 2020b). Interestingly,

these transient defense responses are strongly increased when *Medicago* spp. interact with a bacterial mutant altered in production of exopolysaccharide (EPS), a determinant known to suppress MAMP-triggered immunity (MTI) (Aslam et al. 2008; Jones et al. 2008). These observations raise a question about the influence of the biotic environment, including pathogens, on nodulation. In the past, this question was mostly addressed using systems devoid of pathogens, in which the presence of the aggressor was mimicked by treatment with MAMP (Lopez-Gomez et al. 2012) or in which the plant defense signaling pathways were artificially switched off (Stacey et al. 2006). Those molecular studies showed that plant defenses have an antagonistic effect on nodulation. However, other studies indicate an overlap of the plant response to MAMP and to nodulation signals (Serna-Sanz et al. 2011). Furthermore, boosting MTI through the constitutive expression of EFR, the receptor of the elf26 MAMP shared by bacterial pathogens and rhizobia (Berrabah et al. 2019), has no strong impact on nodulation (Pfeilmeier et al. 2019).

To evaluate whether the pathogen impacts the nodulation process, an in-vitro tripartite system involving *M. truncatula* (Pecrix et al. 2018), its symbiotic partner *Sinorhizobium medicae* WSM419 (Reeve et al. 2010), and the phytopathogenic bacterium *Ralstonia solanacearum* GMI1000 (Salanoubat et al. 2002) was set up. *R. solanacearum* has been known to trigger wilting symptoms on a non-nodulated *M. truncatula* A17 ecotype that is susceptible to *R. solanacearum* GMI1000 (Turner et al. 2009; Vailleau et al. 2007). *R. solanacearum* GMI1000 is a model soil pathogen that colonizes the xylem of its hosts. It displays a T3SS that allows the translocation of effector proteins (T3SEs) into the cells of its host. A total of 24 *Ralstonia* T3SEs were shown to inhibit MTI in *Arabidopsis thaliana* and in *Nicotiana benthamiana* (Landry et al. 2020), although this role has not yet been formally described in genus *Medicago*, in which GMI1000 effectors are crucial for pathogenicity (Vailleau et al. 2007). When efficient, this suppression of immunity is referred to as effector-triggered susceptibility (ETS) (Jones and Dangl 2006). On *M. truncatula* A17, despite development of plant defenses, *R. solanacearum* colonizes the nodules and roots (Benezech et al. 2020a), but the effect of this pathogen on *M. truncatula* nodulation has so far not been described.

In our laboratory, the routine conditions used to study *R. solanacearum*–*M. truncatula* pathogenic interaction and nodulation differ only by temperature (Fahraeus solid medium [Fahraeus 1957] at 28°C for interactions with *R. solanacearum* and at 25°C for nodulation). To determine whether temperatures adapted for pathogenic interaction might be used

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to evaluate the impact of *R. solanacearum* on nodulation, we first verified whether *M. truncatula* can nodulate at 28°C (Fig. 1). At both 14 and 21 days postinoculation (dpi), plants cultivated at 28°C display an average of one nodule per plant, while 25°C controls harbor two times as many nodules. These results indicate that, despite a negative impact of high temperature on the symbiotic process, 28°C is a temperature suitable to study the effect of *R. solanacearum* on *Medicago* nodulation.

Thus, *M. truncatula* seedlings were then coinoculated with both the pathogenic and the mutualistic bacteria at equal densities onto plants (optical density at 600 nm [OD₆₀₀] = 0.1 in sterile distilled water), and the development of nodules was monitored for 28 days. *S. medicae* and *R. solanacearum* cells were cultivated overnight in, respectively, tryptone yeast (Beringer 1974) and Phi medium (Poueymiro et al. 2009). While on control plants, inoculated only with the rhizobium, nodules were observable from day 14 and continued to develop continuously until the last point analyzed (28 dpi) (Fig. 2A), we were not able to detect any nodules on coinoculated plants during the whole experiment (Fig. 2B). Plant age and nutritional status can affect, respectively, plant susceptibility to pathogens and interaction with rhizobia (Campisi and Robert 2014). For this reason, we also analyzed the influence of *R. solanacearum* on nodulation with older plants in which the nitrogen storage in cotyledons was already reduced (11-day-old seedlings). No nodules were formed in those coinoculated plants (Fig. 2D). In contrast, in seedlings inoculated only with *S. medicae*, the nodule number increased over the time (Fig. 2D). At 28 dpi, for plants that were inoculated when they were 0 and 11 days old, two nodules per plant were present in average on 34 control plants, but none were detected on 34 coinoculated plants (Fig. 2D). These results indicate that the presence of the phytopathogenic bacterium interferes drastically and early with nodulation (independently of the nitrogen need of the plants).

To evaluate whether *R. solanacearum*-mediated inhibition of nodulation is dose-dependent and potentially due to direct antagonism with *S. medicae* or to root congestion by

R. solanacearum, the same experiment was performed with ten times fewer cells of *R. solanacearum*. In such a condition, as when the two microorganisms were inoculated at equal densities, no nodules developed on coinoculated plants (Fig. 2D), suggesting that nodulation inhibition is not due to competition for the access to the infection site.

Because of its key role in the subversion of plant cell physiology, we evaluated the importance of *R. solanacearum* T3SS in the inhibition of nodulation. Seedlings were coinoculated with *S. medicae* and the *hrcV* mutant of *R. solanacearum*, deficient for the T3SS (Cunnac et al. 2004). Root tip browning was observed on seedlings coinoculated with *S. medicae* and the *R. solanacearum* wild-type strain (WT), which can be due to the appearance of disease (ETS) or the development of defense responses (effector-triggered immunity-like) (Fig. 2E). In contrast, no such a symptom was detectable when plants were coinoculated with *S. medicae* and the *hrcV* mutant (Fig. 2F). On those plants, nodules were frequently observed (Fig. 2C). However, the *Ralstonia* T3SS mutant still has the ability to partially inhibit nodulation by *S. medicae* (Fig. 2D). The effect was not significantly different when the disarmed pathogen was inoculated at reduced density and a similar tendency was observed for plants inoculated immediately after transfer on Fahreus medium. Indeed, from 28 dpi, regardless the age of seedlings and the densities of *R. solanacearum* inoculum (OD₆₀₀ = 0.1 or 0.01), on average, less than one nodule per plant was present on 34 coinoculated plants (Fig. 2D). Thus, our results indicate that *R. solanacearum* T3SS strongly contributes to the inhibition of nodulation.

Our data reinforces the idea that the biotic environment can impact the nodulation process. Legume-rhizobia interactions are most often studied with experimental systems restricted to the two symbiotic partners. However, such a reductionist approach is far from the complex reality of natural environments and it is important to develop experimental systems including one or more additional microbial partners to understand the complexity of the interactions. We took a first step in this direction that allows evaluating how other interactions can influence nodulation efficiency. We observed that WT *R. solanacearum* totally inhibits nodulation, perhaps through defense response elicitation (although A17 is susceptible to GMI1000). In contrast, the *hrcV* mutant does not totally block nodulation (Fig. 3B). This partial inhibition of nodulation might be due to induction of defense responses that are not counteracted by an effector normally translocated through *R. solanacearum* T3SS. Among the factors potentially triggering defense responses are, notably, surface components acting as MAMPs and ethylene produced by *Ralstonia* spp. Indeed, ethylene is both a defense response elicitor and an inhibitor of nodulation in *Medicago* spp. (Oldroyd et al. 2001; Peters and Crist-Estes 1989; Valls et al. 2006). Other *R. solanacearum* factors as well as rhizobial determinants, such as the nod factors, EPS, and 1-aminocyclopropane-1-carboxylate deaminase (that might reduce ethylene production by degrading the ethylene precursor), could contribute to the suppression of defense responses induced by the disarmed pathogen. In addition, WSM419 strain possesses a T4SS that might also play such a role (Fig. 3C). Whether the reduced inhibitory effect of the *hrcV* mutant on nodulation reflects an inability of PTI to prevent nodule development in our system or whether the rhizobium or the *hrcV* mutant are able to suppress PTI induced by the *R. solanacearum* mutant remains to be determined (Fig. 3).

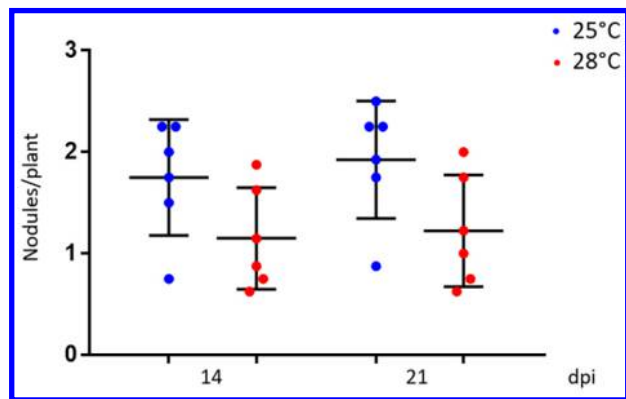


Fig. 1. Temperature impacts *Medicago truncatula* nodule development. *Medicago truncatula* A17 inoculated with *Sinorhizobium medicae* WSM419 cultivated under the standard nodulation condition (25°C) or a condition adapted to study *Medicago truncatula*-*Ralstonia solanacearum* interaction (28°C). The number of nodules is lower when plants were grown at 28°C. A Kruskal-Wallis test was performed, followed by a Wilcoxon test at 14 days postinoculation (dpi) and 21 dpi (P value > 0.05). Experiments were replicated two times on 40 plants per condition and for each biological replication.

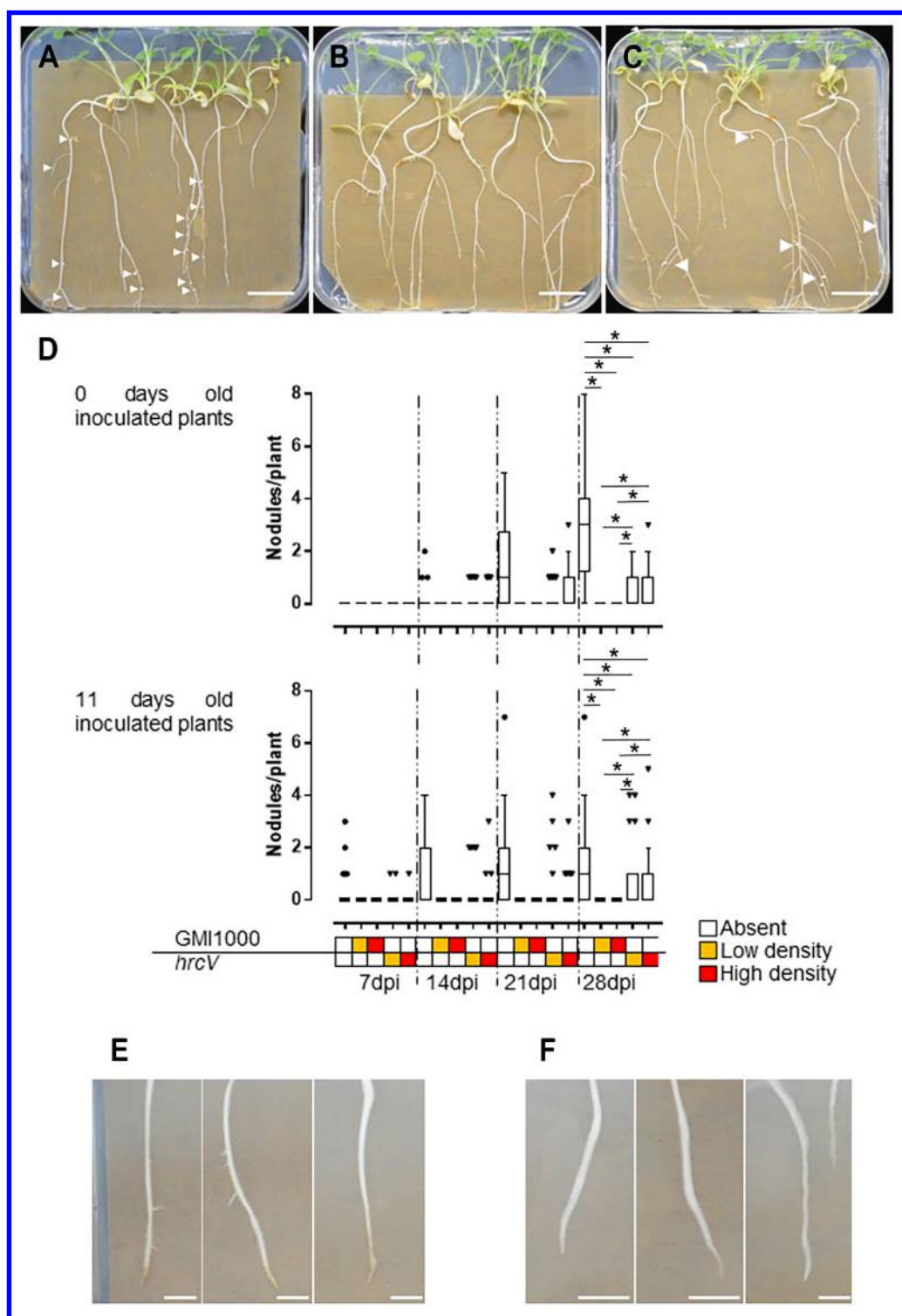


Fig. 2. Inhibition of *Medicago truncatula* nodulation by *Ralstonia solanacearum* relies on the pathogen type III secretion system (T3SS). **A**, *Medicago truncatula* A17 inoculated with *Sinorhizobium medicae* WSM419 at 28 days postinoculation (dpi). **B**, *M. truncatula* coinoculated with *S. medicae* WSM419 and *Ralstonia solanacearum* GMI1000 wild-type (WT) strain at 28 dpi. **C**, *M. truncatula* coinoculated with strain WSM419 and the *hrcV* mutant of *R. solanacearum* altered in the T3SS at 28 dpi. *M. truncatula* A17 developed nodules upon inoculation with *S. medicae* WSM419 (A) and upon coinoculation with the *hrcV* mutant of *R. solanacearum* (C). No nodules were observed when *M. truncatula* was coinoculated with *S. medicae* WSM419 and *R. solanacearum* GMI1000 WT strains (B). Bars = 2 cm. White arrows indicate nodules. **D**, Monitoring of nodule numbers by plants inoculated or coinoculated as described above when they are 0 and 11 days old according to the pathogen density during a kinetic experiment. A Kruskal-Wallis test was performed followed by a Wilcoxon test at 28 dpi (asterisks (*) indicate P value < 0.05). Experiments were replicated three times on 32 plants per condition and for each biological replication. **E**, Roots of *M. truncatula* coinoculated with *S. medicae* WSM419 WT strain and *R. solanacearum* GMI1000 WT strain at 7 dpi. **F**, *M. truncatula* coinoculated with *S. medicae* WSM419 WT strain and the mutant *hrcV* of *R. solanacearum* GMI1000 strain, at 7 dpi. Browning root tips were observed when plants were inoculated with *R. solanacearum* GMI1000 WT strain but are not observed upon inoculation with the mutant *hrcV* of *R. solanacearum* GMI1000 strain. Bars = 5 mm.

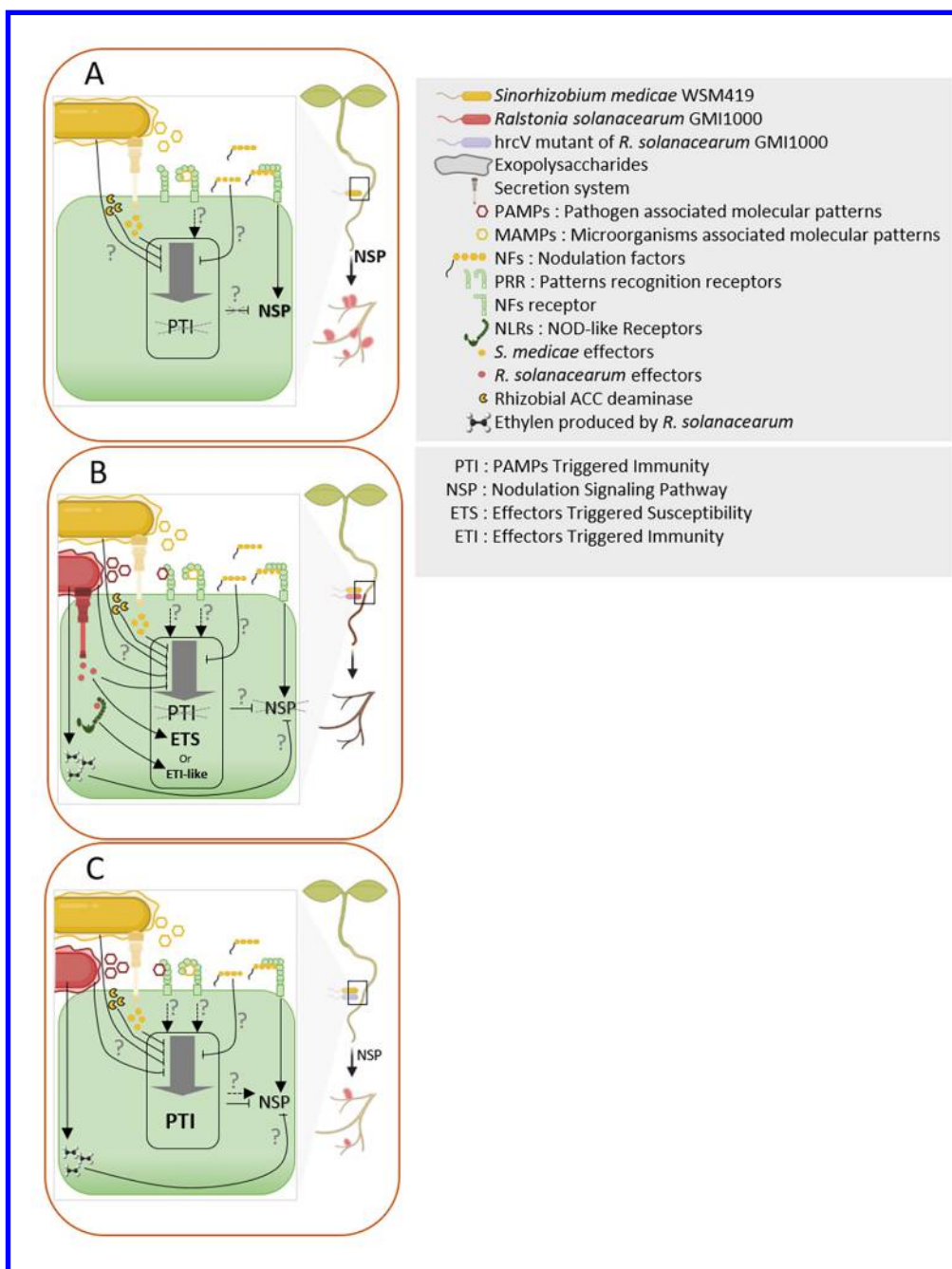


Fig. 3. Hypothetical model of *Ralstonia*-mediated inhibition of *Medicago* nodulation. **A**, Absence of pathogen. Pathogen-associated molecular pattern-triggered immunity (PTI) is presumed to be transiently and moderately activated (Jones et al. 2008; Lohar et al. 2006), which allows nodulation. **B**, *R. solanacearum* represses PTI through effector-triggered susceptibility (ETS) or *R. solanacearum* effectors are recognized by Nod-like receptors (NLR), which induces defense responses (effector-triggered immunity-like) and no nodulation is observed. **C**, The *hrcV* disabled mutant of *R. solanacearum* is not able to translocate the effector that would inhibit PTI. Nevertheless, nodulation is not totally abolished, giving rise to two hypotheses: i) PTI does not have the potential to inhibit nodulation or ii) one or more rhizobial or *R. solanacearum* determinants, such as Nod factors or effectors, suppress PTI and allow nodulation.

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