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The *Methylobacterium nodulans* / *Crotalaria podocarpa* symbiosis: a classic process for an original model

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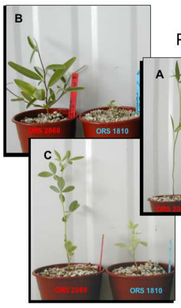


Plants belonging to the *Crotalaria* genus are tropical leguminous mainly making symbiosis with *Bradyrhizobium*. Nevertheless, some *Methylobacterium* spp. strains were isolated from root nodules of *Crotalaria* as well as *Lotononis* (*Fabaceae, Crotalariae*) (1).

Fig 1. *Crotalaria* plants inoculated with either *Methylobacterium nodulans* ORS 2060 or *Bradyrhizobium* sp. ORS 1810. A: *Crotalaria glaucoides*; B: *C. perrottetii*; C: *C. podocarpa*.

Methylobacterium strains were only isolated from three *Crotalaria* species, i.e., *C. glaucoides* (Fig 1A), *C. perrottetii* (Fig 1B) and *C. podocarpa* (Fig 1C), and were described as a single novel species: ***Methylobacterium nodulans***. The main feature of this original bacterial symbiotic partner is its ability to oxidize methanol, a methylotrophic property based on the presence of methanol dehydrogenase. During the symbiosis between *M. nodulans* and C. *podocarpa*, it has been shown that the bacterial methylotrophic property plays a major role in the symbiotic process (2).

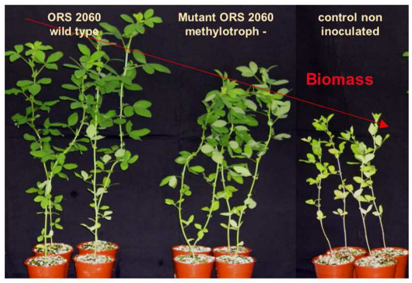
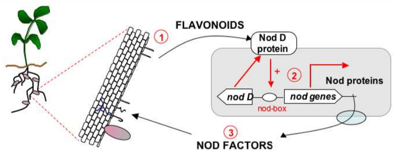


Fig 2. Comparison of growth (5 weeks after inoculation with *M. nodulans*) of *Crotalaria podocarpa*.

Nothing is known on the molecular dialogue occurring between *M. nodulans* and *C. podocarpa*. Commonly, the symbiosis between rhizobial soil bacteria and legumes was described as a multi-step process mediated by signal molecules produced from both two partners: exudation of phenolic compounds ① able to induce the transcription of bacterial *nod* genes ② leading to the biosynthesis of a bacterial signal, the nodulation factors ③.



Recently Giraud et al. (3) described photosynthetic *Bradyrhizobium* ORS 278 able to nodulate *Aeschynomene indica* L. (*Fabaceae*) in a Nod factor-independent pathway, opening the possibility that rhizobia could use alternative signals to nodulate legumes.

Which signal molecules are involved in the symbiosis between *Methylobacterium nodulans* and *Crotalaria podocarpa* ?

IDENTIFY

efficient flavonoid inducer of the *nod* gene expression

nodulation genes operon

structure of the Nod factor produced by *M. nodulans* ORS 2060

INDUCTION OF NOD GENES

To monitor the nodulation genes expression, six flavonoids were investigated for their ability to induce the *nodA* promoter of the recombinant strain *M. nodulans* ORS2060 *nodA-LacZ*.

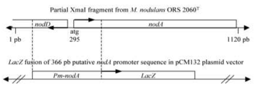
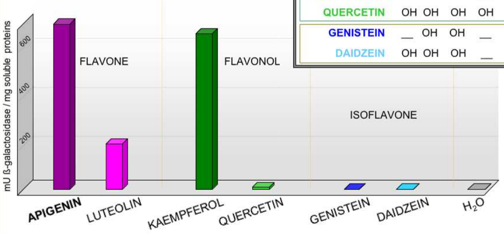


Fig 3: Construction of *M. nodulans* ORS 2060T recombinant strain containing *nodA-LacZ* transcriptional fusion promoter.

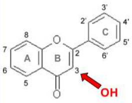
Hydroxylation pattern at the following carbons:

	3	5	7	3'	4'
APIGENIN	_	OH	OH	_	OH
LUTEOLIN	_	OH	OH	OH	OH
KAEMPFEROL	OH	OH	OH	_	OH
QUERCETIN	OH	OH	OH	OH	OH
GENISTEIN	_	OH	OH	_	OH
DAIDZEIN	OH	OH	OH	_	OH



♦ **Apigenin** and **kaempferol** act as strong *nod* gene inducers on strain ORS 2060

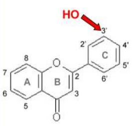
⇩



Additional C-3 hydroxylation does not affect the induction.

♦ For **luteolin**, a 3 times decrease of activity is observed when compared to **apigenin**. More drastically, **quercetin** has no induction effect when compared to **kaempferol**.

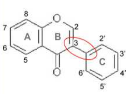
⇩



The gain of the C-3' hydroxyl group results in a dramatic decrease of the promoter-inducing ability.

♦ **Genistein** and **daidzein** are not able to induce nod gene in *M. nodulans*

⇩



The attachment of the B ring to C-3 leads to no induction.

NODULATION GENES OPERON

A 7,13-kb region in the inserted DNA fragment, showing a positive hybridization signal to the *nodA* probe from *M. nodulans* ORS 2060 was fully sequenced (Fig 4). The analysis of the nucleotide sequence revealed the existence of six entire open reading frames (ORFs).

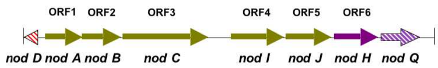


Fig 4. Organization nodulation genes in *Methylobacterium nodulans* ORS 2060 into the 7,13-kb XmaI DNA fragment. Common nodulation genes are show in brown, specific nod genes in violet, and the *nod D* regulator gene in red. Partially sequence of others nod genes are indicated with hatchings.

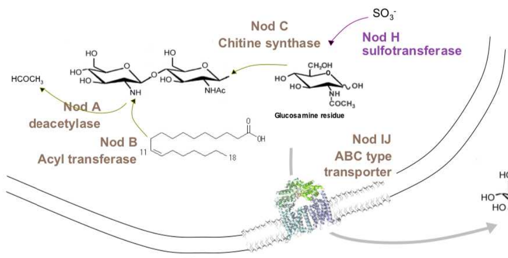


Fig 5. Functions of the different Nod proteins encoded by the genes belonging to the XmaI fragment.

NODULATION FACTORS

In order to prepare Nod factors, *M. nodulans* ORS 2060 was grown in the presence of apigenin. After induction, Nod factors were extracted from the culture supernatant and purified. HPLC analyses revealed the presence of only two fractions (Fig 6), then analysed by LC/MS.

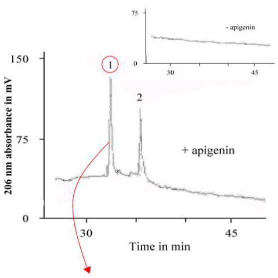


Fig 6. HPLC profile of a Nod factor extract from a apigenin-inducing *M. nodulans* ORS 2060 culture supernatant.

The positive ion spectra of compounds identified in fraction 1 show two DP5 glucosamine sulfated forms as $C_{18:1}$ (vaccenic acid) and $C_{16:0}$ (palmitic acid) in proportion 10/1 (Fig 7). No Nod factor was found in fraction 2.

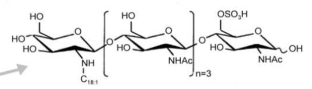


Fig 7. Structure of the major lipo-chito-oligosaccharide identified produced by *M. nodulans* ORS 2060.

Methylobacterium nodulans is able to distinguish between flavonoids (flavone and flavonol) and isoflavonoids (isoflavone and isoflavonol). Thus, the attachment of the B ring to C-2 is of crucial importance for induction.
Commonly apigenin and surprisingly kaempferol, usually found as suppressor, are highlighted as to be the most powerful flavonoid inducers of the *nodA* promoter of *M. nodulans*.

The presence of nod genes is checked and a DNA fragment containing 8 nodulation genes *nod DABCIJHQ* have been identified.

M. nodulans produces one major Nod factor structure identified as to be Nod Mn-V($C_{18:1}$,S), suggesting a classic symbiosis model for *M. nodulans / C. podocarpa* association.

(1) Sy A, Giraud E, Jourand P, et al. (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *Journal of Bacteriology* **183**, 214-220.
(2) Jourand P, Renier A, Rapior S, et al. (2005) Role of methylotrophy during symbiosis between *Methylobacterium nodulans* and *Crotalaria podocarpa*. *Molecular Plant-Microbe Interactions* **18**, 1061-1068.
(3) Giraud E, Moulin L, Vallenet D, et al. (2007) Legumes symbioses: absence of *nod* genes in photosynthetic bradyrhizobia. *Science* **316**, 1307-1312.