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RESEARCH PAPER

Nodulation of *Crotalaria podocarpa* DC. by *Methylobacterium nodulans* displays very unusual features

Adeline Renier[1,4], Sergio Miana De Faria[2], Philippe Jourand[3], Eric Giraud[1], Bernard Dreyfus[1], Sylvie Rapior[4] and Yves Prin[5,*]

[1] IRD, UMR LSTM, F-34398 Montpellier Cedex 5, France
[2] EMBRAPA, Agrobiologia, Seropedica, 23851-970RJ, Brazil
[3] IRD, F-98848 Nouméa Cedex, Nouvelle Calédonie
[4] UMR CEFE, Faculté de Pharmacie, 34093 Montpellier Cedex 5, France
[5] CIRAD, UMR LSTM, 34398 Montpellier Cedex 5, France

* To whom correspondence should be addressed. E-mail: prin@cirad.fr

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Abstract

Crotalaria **are plants of the Fabaceae family whose nodulation characteristics have been little explored despite the recent discovery of their unexpected ability to be efficiently nodulated in symbiosis with bacteria of the genus** ***Methylobacterium*****. It has been shown that methylotrophy plays a key role in this unusual symbiotic system, as it is expressed within the nodule and as non-methylotroph mutants had a depleting effect on plant growth response. Within the nodule,** ***Methylobacterium*** **is thus able to obtain carbon both from host plant photosynthesis and from methylotrophy. In this context, the aim of the present study was to show the histological and cytological impacts of both symbiotic and methylotrophic metabolism within** ***Crotalaria podocarpa*** **nodules. It was established that if** ***Crotalaria*** **nodules are multilobed, each lobe has the morphology of indeterminate nodules but with a different anatomy; that is, without root hair infection or infection threads. In the fixation zone, bacteroids display a spherical shape and there is no uninfected cell.** ***Crotalaria*** **nodulation by** ***Methylobacterium*** **displayed some very unusual characteristics such as starch storage within bacteroid-filled cells of the fixation zone and also the complete lysis of apical nodular tissues (where bacteria have a free-living shape and express methylotrophy). This lysis could possibly reflect the bacterial degradation of plant wall pectins through bacterial pectin methyl esterases, thus producing methanol as a substrate, allowing bacterial multiplication before release from the nodule.**

Key words: *Crotalaria*, Fabaceae, *Methylobacterium*, methylotrophy, nitrogen fixation, nodulation, symbiosis.

Introduction

Among the Crotalariae tribe of the Papilionoideae subfamily, two genera were recently described as forming efficient nodulation with æ-Proteobacteriu belonging to the genus *Methylobacterium*: *Crotalaria* (Sy *et al.*, 2001*a*) and *Lotononis* (Jaftha *et al.*, 2002). Depending on their symbionts, two groups can be distinguished among *Crotalaria* species. The first group contains species which are efficiently nodulated by *Methylobacterium* strains, whereas the second group contains species which are efficiently nodulated by *Bradyrhizobium* strains (Sy *et al.*, 2001*b*).

Methylobacterium strains are characterized by the ability to utilize, as a sole source of carbon and energy, methanol and other C1 compounds as well as a variety of multicarbon substrates (Lidstrom, 2006). *Methylobacterium nodulans* was originally isolated from *Crotalaria podocarpa* (Sy *et al.*, 2001*b*). *Methylobacterium nodulans* was proved to possess a *mxa* genes cluster, coding for methanol dehydrogenase (MDH) (Sy *et al.*, 2001*a*). Inactivation of methylotrophy (through insertional mutagenesis in the *mxaF* gene) was found to drastically affect nodule number and plant growth

response (Jourand *et al.*, 2005). This is a major point of difference between *Crotalaria* and *Lotononis* as, in this last genus, *Methylobacterium* strains are unable to utilize methanol (Ardley *et al.*, 2009). The ability to use methanol, which is a plant by-product essentially resulting from pectin methyl esterase activity, was believed to give a competitive advantage to *Methylobacterium* for nodulation. Here the histological and cytological characteristics of the *C. podocarpa* nodulation by the *M. nodulans* ORS 2060^T strain are described.

Materials and methods

Bacterial strains and growth conditions

Methylobacterium nodulans strain ORS 2060^T (Samba *et al.*, 1999; Sy *et al.*, 2001*a*; Jourand *et al.*, 2004) and the methylotrophic mutant A5:ORS 2060 *mTn5-GGm-mxaF* (Jourand *et al.*, 2005) were grown aerobically at 37 °C in YM medium (Vincent, 1970). To evaluate their growth capacity with pectins as the sole carbon and energy sources, both strains were cultivated on liquid minimum M72 medium (Green 1992) supplemented with 1% (w/v) of either 0% or 60% methylated polygalacturonic acid, and their $OD_{600\ nm}$ was measured after 170 h.

Plant tests

Crotalaria podocarpa seeds were obtained from IRD (Bel-Air, Dakar, Senegal). Germination and cultivation were conducted in Gibson tubes in plant culture rooms at 28 °C under a 16 h photoperiod and a light intensity of 60 microEinstein (μE) m^{-2} s^{-1} light as described by Le Roux *et al.* (2009). On day 15 after germination, plant roots were inoculated with 5 ml of bacterial culture, reaching an optical density at 600 nm of 1. Sterile distilled water was used for control.

Histological and cytological studies

Freshly harvested nodules were either directly sectioned to a thickness of 30 μm using a Vibratome (Leica France) or fixed for 2 h in 0.1 M sodium cacodylate-buffered 2.5% glutaraldehyde, pH 7.4 and washed three times in the same buffer. They were post-fixed for 45 min in 0.1 M sodium cacodylate-buffered 1% osmium tetroxide, pH 7.4, and washed three times for 20 min in the same buffer before dehydration in a graded series of ethanol. After impregnation in LR White resin (Newman *et al.*, 1982) and inclusion, polymerization was carried out at 60 °C for 24 h. Semi- and ultrathin sections were made with a Diatome (Leica, France) diamond knife. For transmission electronic microscopy, ultrathin sections were routinely treated with uranyl acetate and lead citrate and observed on a Jeol 1200EX transmission electron microscope. For light microscopy, semi-thin sections were stained according to standard procedures (Clarke, 1981) with 0.1% (w/v) toluidine blue in a 1% borax solution, 0.005% (w/v) ruthenium red, 0.01% (w/v) calcofluor white M2R, 0.1% (w/v) acridine orange, or standard periodic acid–Schiff (PAS), and observed on an Olympus Provis microscope.

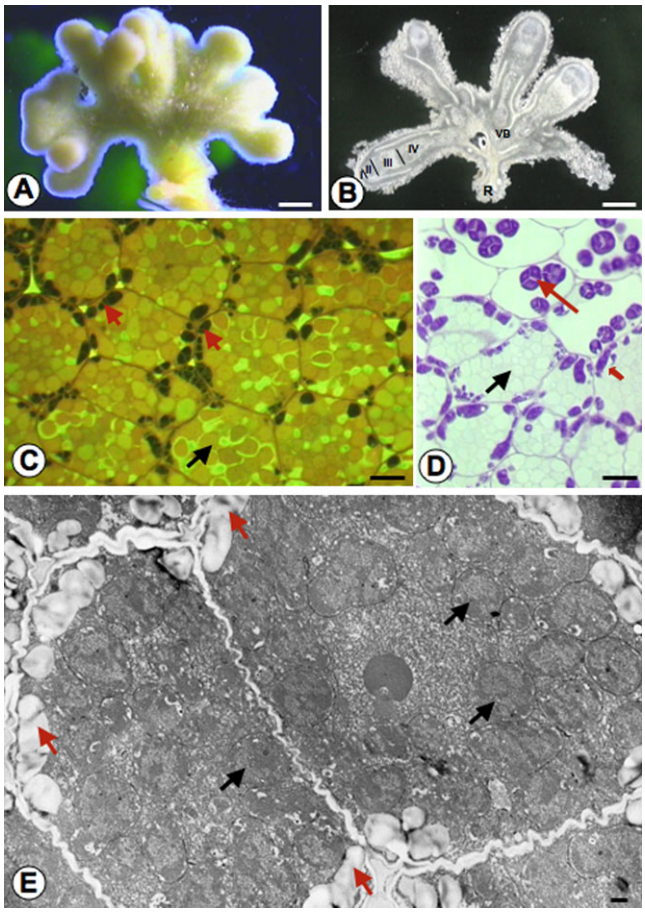

Fig. 1. Histological analysis of a *Crotalaria podocarpa–Methylobacterium* strain ORS 2060^T root nodule. (A) General view of a nodule with its typical crotalarioid shape, i.e. lobed and flattened. Bar=1 mm. (B) Longitudinal section through a multilobular nodule showing the anchoring of the vascular bundles (VB) on the root system (R), and the different nodule zones (numbered I–IV according to Vasse *et al.*, 1990). Bar=1 mm. (C and D) Histological staining of resin-embedded sections through fixation zone III. (C) Acridine orange staining observed under fluorescence microscopy showing the general occurrence of both bacteroids (black arrow) and amyloplasts (red arrows) within infected cells. Bar=8 μm. (D) Periodic acid–Schiff stain, observed under a light microscope, showing the different shape of amyloplasts (red arrows) between cortical uninfected cells (round shape, large arrow) and central infected cells (flattened shape, in the peripheral position, small arrow). Black arrow: bacteroids. Bar=8 μm. (E) Transmission electron microscope images of sections through infected cells in fixation zone III of a nodule, illustrating amyloplasts (red arrows) and spherical bacteroids (black arrows) co-existing within active cells of fixation zone III. Bar=2 μm.

Results

Mature (i.e. 2–4 weeks old) *C. podocarpa* nodules are multilobed (Fig. 1A), anchored to the root at a single point from which vascularization ramifies to all the lobes (Fig. 1B). Within one mature lobe (Fig. 1B) the morphology is apparently that of an indeterminate nodule as in alfalfa (Vasse *et al*, 1990), with an apical meristematic zone (I), an invasion zone (II), a fixation zone (III), and a senescence zone (IV).

Within zone II, bacteria are present as classical rods both in the intercellular spaces and intracellularly (not illustrated). No infection threads delivering bacteria to young plant cells were observed in this zone. Within fixation zone III, bacteroids are globally spherical to ovoid (Fig. 1C–E), with a mean diameter of ~4 μm. All the plant cells are

infected, lacking uninfected interstitial cells. Amyloplasts are abundant in the peripheral (cortical) uninfected cells of the nodules, clearly stained by PAS (Fig. 1D). More surprisingly, they also are frequently observed with a more densely packed and flattened shape, within the bacteroid-filled cells of fixation zone III (Fig. 1C–E). In nodules induced by the methylotrophy-defective mutant A5 (Supplementary Fig. S1A available at *JXB* online), spherical bacteroids also co-exist with amyloplasts in fixation zone III.

In older nodules (i.e. 5–6 weeks old), the invasion zone II and the whole apex has an unusual shape, with a progressive loss of plant cell wall integrity, clearly revealed by cell wall stains such as Calcofluor White (Fig. 2A, B), toluidine blue (Fig. 2C), and ruthenium red (Supplementary Fig. S1B at *JXB* online). The whole zone is totally disorganized, with no more plant cell wall, but only longitudinally oriented cell remnants that seem, from histological stainings, to be of polysaccharidic nature (Fig. 2A, B, Supplementary Fig. S1B). In this zone, bacteria are often surrounded by a white halo (Fig. 2C, D), suggesting a lytic activity. In older, 6-week-old nodules, this zone sometimes is completely empty, with images looking like dental decay (Fig. 2E). Interestingly, nodules induced by mutant A5 did not present these apical degradations (Fig, 2F).

Bacterial growth of strains ORS 2060^T and A5 with pectins as the sole carbon and energy sources is presented in Supplementary Fig. S2 at *JXB* online. On polygalacturonic acid, neither of the two strains was able to grow. On 60% methylated pectin, only ORS 2060^T was able to grow.

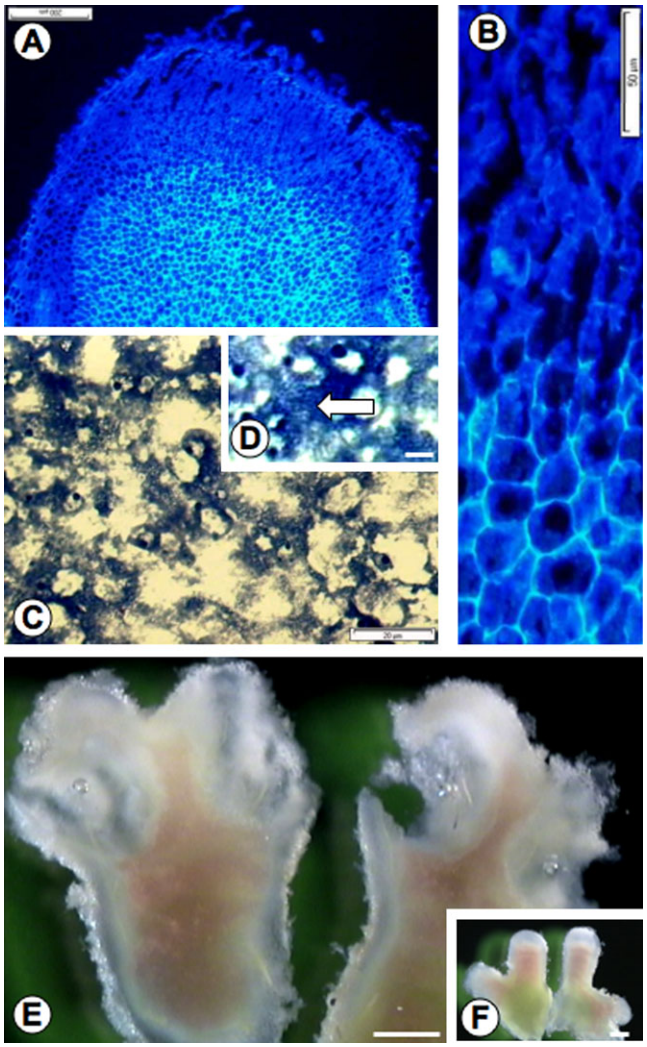

Fig. 2. Histological analysis of *Crotalaria podocarpa* root nodules after inoculation with either *Methylobacterium* strain ORS 2060^T or the methylotrophy-defective mutant A5. (A) Longitudinal Vibratome section through a mature nodule stained with Calcofluor White, under fluorescence microscopy, showing the progressive disorganization of the plant cell wall between fixation zone II and the nodule apex. Bar=200 μm. (B) Magnification of a section through the apical zone of a mature nodule stained with Calcofluor White showing the loss of plant cell wall integrity in this zone. Bar=50 μm. (C and D) Longitudinal Vibratome section through the apical part of a mature nodule stained with toluidine blue illustrating the total disorganization of the nodule tissue, and the presence of numerous unstained rod-shaped bacteria (white arrow) in the densely stained polysaccharidic deposits. Bar=20 μm. (E) Thick hand-cut longitudinal sections of 6-week-old nodules with their emptied apical zones, opened to the outside. Bar=1 mm. (F) Thick hand-cut longitudinal sections of 6-week-old nodules obtained in the same conditions as in E but with the non-methylotrophic mutant strain A5. No apical decay is observed. Bar=1 mm.

Discussion

Crotalaria are tropical legumes with poorly explored nodulation properties. The early steps of the nodulation process of *C. podocarpa* by *M. nodulans* have not been described in detail. Root hairs are present, sometimes being deformed following inoculation, but infection threads were never observed. Together with the lack of interstitial cells, this would justify more studies to depict the penetration and internalization of bacteria clearly, and the induction of the nodule primordium.

Corby (1988), in his extensive morphotyping of legume nodules, attributed to *Crotalaria* nodules a particular type: crotalarioid. In *Lotononis*, also a member of the Crotalariae and nodulated by *Methylobacterium*, nodules are quite different, being lupinoid or coralloid and often completely enveloping or girdling the root (Yates *et al.*, 2007). With *C. podocarpa*, it was observed that each lobe has the morphology of an indeterminate nodule with a zonation as described by Vasse *et al.* (1990), but not with all the features described in *Medicago*, such as the presence of infection threads. These nodules have a limited growth, relayed by lateral branching and emergence of new lobes.

Within mature nodules, the dual shape of bacteria (i.e. rods and spherical bacteroids) is observed, in zone II and III, respectively. In *Lotononis*, symbiosomes appear spherical in sections but they are 'normally oblong in wet mounts' (Yates *et al.*, 2007). Large spherical bacteroids were described in *Arachis* spp. (Sutton, 1983), among the Dalbergioids, a tribe phylogenetically distant from the Crotalarioids.

The occurrence of amyloplasts in nitrogen fixation zone III, co-existing with the spherical, fully differentiated bacteroids is a striking characteristic of *Methylobacterium* nodules. This association was proved to be symbiotically effective, as it was shown to induce higher plant growth responses than the association with the methylotrophy-defective mutant A5 (Jourand *et al.*, 2005). In *Medicago sativa*, it is assumed that, in central zone III, the metabolism of bacteroids is largely devoted to symbiotic functions such as nitrogen fixation and nutrient exchanges with the plant. Using *nif* mutants, Vasse *et al.* (1990) showed that the abundance of amyloplasts within zone III can be indicative of a non-efficient association. Amyloplasts were reported as particularly abundant in interzone II–III—a transition zone limited to 1–3 cell layers—and absent from fixation zone III in nitrogen-fixing alfalfa nodules (Vasse *et al.*, 1990). However, in efficient alfalfa nodules, amyloplasts seem relatively rare in the nodule cortex (Vasse *et al.*, 1990). Noticeably, amyloplasts also co-exist with bacteroids within nodules of the *mxaF* mutant strain A5, suggesting that starch storage has no direct link to methylotrophy. In *C. podocarpa*, Jourand *et al.* (2005), using *mxaF:LacZ* expression mutants, showed that methylotrophy would be restricted to free-living bacteria in zone II at the nodule apex, suggesting that after their differentiation bacteroids are no longer able to carry out methylotrophy.

Degeneration of the apical nodule tissue is another striking feature of *Methylobacterium–C. podocarpa* symbioses. In this zone, where the bacterial cells had their free-living shape, an intense lytic activity affects zones I and II in 6-week-old nodules. Despite efforts in the present study, it was not possible to fix and preserve this degenerated zone: it systematically emptied during passage through the different fixation, dehydratation, and substitution baths required for inclusion in paraffin or plastic resins. This zone is likely to be a kind of soft 'jelly' whose aspect after cutting with a Vibratome (i.e. fresh slicing at 30 μm thickness, without any fixation treatment) is apparently always out of focus as if this putative 'jelly' was limiting the resolution of microscopic observation and photography (e.g. Fig, 2C, D, Supplementary Fig. S1B at *JXB* online). The spontaneous emptying of these zones was observed in hand-cut sections of older, 6-week-old nodules (Fig. 2A). In this apical tissue, methylotrophy was intense at younger developmental stages, suggesting that methanol is present inside the nodule and subsequently used by the bacteria (Jourand *et al.*, 2005). These authors showed that methanol was detected at a substantially higher rate in the nodule than in the root and other plant tissues. In plants, methanol can be produced from different sources, among which is cell wall pectin demethylation (Downie *et al.*, 2004). During symbiosis between *M. nodulans* and *Crotalaria* spp., it is hypothesized that methanol could be produced from pectin cell wall demethylation. It was shown (Supplementary Fig. S2 at *JXB* online) that strain 2060^T was able to use methylated pectins—but not polygalacturonic acid—in pure culture, thus potentially being able to degrade plant cell walls progressively, to produce and use methanol. The non-methylotrophic mutant strain A5 (Supplementary Fig. S2) was not able to use methylated pectins. Nodules formed by this mutant A5 do not present apical degradation (Fig. 2B), indicating a link between methylotrophy and this unusual characteristic. Strain ORS 2060^T would thus degrade plant cell walls in apical zones—where it is in a free-living shape—of the nodule, leading to the production of methanol from pectin methyl esterase activity, and allowing methylotrophy. The findings of Jourand *et al.* (2005) that methylotrophy and nitrogen fixation exist in two distinct and contiguous zones of the mature nodule, the apical zone II and the fixation zone III, respectively, were confirmed here. Since a loss of methylotrophy significantly affected nitrogen fixation efficiency (Jourand *et al.*, 2005), functional relationships should exist between these two zones, which remain to be uncovered.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. (A) Section through a nodule fixation zone of *Methylobacterium nodulans* non-methylotrophic mutant strain A5 after periodic acid–Schiff stain. There is no visible impact of the loss of methylotrophic ability in this zone. Bar=20 μm. (B) Longitudinal section through a 6-week-old *M. nodulans strain* ORS 2060^T nodule, after staining with ruthenium red. Plant cell walls are totally disorganized in the nodule apex. Bar=200 μm.

Figure S2. Growth response (optical density at 600 nm) of *Methylobacterium nodulans* strain ORS 2060^T (open lozenges) and its mutant A5 (open triangles) on minimum M72 medium (Green 1992) supplemented with 1% (w/v) of either 0% or 60% methylated polygalacturonic acid.

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