

INDUCTION OF A CRIPPLED PHENOTYPE IN *PSYCHOTRIA* (RUBIACEAE) UPON LOSS OF THE BACTERIAL ENDOPHYTE

S. Van Oevelen^{1,2*}, R. De Wachter², E. Robbrecht³, E. Prinsen¹

¹Laboratory of Plant Biochemistry and Physiology, Department of Biology, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium

²Laboratory of Molecular Biology, Department of Biochemistry, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium

³National Botanic Garden, Domein van Bouchout, B-1860 Meise, Belgium

Summary. Bacterial leaf nodulation is a model of exceptional collaboration of bacteria and higher plants. The presence of the endosymbiont is absolutely required to ascertain survival of the host. Abandonment of the symbiotic cycle leads to distorted leaf formation and stunted plant growth, eventually resulting in death of the host. Recently, the endophytes of several *Psychotria* have been identified as novel *Burkholderia* species. With our research we are trying to discover the strategies involved in this symbiosis, with *Psychotria* as the host and *Burkholderia* as the endosymbiont. To this purpose we thoroughly analyzed healthy nodulated plants and crippled bacteria-free plants.

Keywords: *Burkholderia* sp., nodulated *Psychotria*, obligate endosymbiosis, plant hormones

Introduction

Leaf symbiosis is by far the least studied form of known plant-bacterium interactions. Very little is known about these endosymbioses occurring in the leaves of higher plants. However, the curious nature of this intimate association makes it very interesting. Bacterial leaf nodulation is represented in several tropical genera belonging to the flowering plant families Rubiaceae, Myrsinaceae (dicotyledonous) and Dioscor-

* Corresponding author, e-mail: Sandravo@uia.ua.ac.be

eaceae (monocotyledonous). Nodulated Rubiaceae are limited to the genera *Psychotria*, *Pavetta* and *Sericanthe* (Robbrecht, 1988). Our research is mainly orientated towards the genus *Psychotria*, which contains 72 nodulated species.

The most visible aspect of bacterial leaf symbioses is the occurrence of galls or nodules on the leaves of the host plant, first described by Trimen (1894). Due to a great variation in size and location, the leaf galls proved to be of great taxonomic value. Ever since Zimmermann first discovered that these foliar structures contain bacteria (1902), other researchers have tried to reveal the true nature of these peculiar plant-microbe interactions. Very early it was discovered that the symbiotic cycle is closed (von Faber, 1914); i.e. bacteria are transferred from one plant generation to the next through the seeds, avoiding an external infection event. Later it was shown that a colony of the bacteria resides in the stem apical region, on a permanent basis, where it infects every new developing leaf and every ovary, and thus the seeds (Lersten & Horner, 1967; Whitmoyer and Horner, 1970, Miller et al., 1983). The bacteria are hence constant companions of the host throughout the plant's entire life cycle (for review see Miller, 1990). Bacterial leaf nodulation in Rubiaceae and Myrsinaceae was shown not only to be cyclic but also obligate; i.e. the presence of the bacterial partner is required for normal growth and development of the host. When the endophyte is lost, the plant is unable to undergo normal cell division and differentiation, resulting in morphological malformations, growth arrest and eventually death of the host (Gordon, 1963).

The role these bacteria play in the development of their host is crucial. Therefore their isolation, cultivation and identification was desirable. All previous attempts to identify the microbial symbiont were based on insufficient morphological descriptions, and assigned taxonomies had to be reviewed. The use of contemporary macromolecular methods allowed us to identify the symbiont of several *Psychotria* species. Based on SSU rDNA sequencing the endophytes could be assigned as novel species to the genus *Burkholderia* (Van Oevelen et al., 2002 a,b). Cultivation has not been successful as yet.

Changes occurring in the plant's phenotype upon loss of the bacteria suggest an altered hormone balance. This is currently being confirmed through extensive analysis of healthy nodulated plants, of natural occurring crippled dwarves and of experimentally induced crippled phenotypes in mature plants.

Materials and Methods

The plant species used in this study is *Psychotria kirkii* Hiern var. *tarambassica* (Bremek.) Verdc. (voucher 19536779), provided by the National Botanic Garden of Belgium. Plants are grown under optimal conditions (greenhouse: T = 21°C, humidity = 70–90%) and sub-optimal conditions (growth chamber: T = 28°C, day/night = 16/8 h, humidity = 58%).

Results

Cyclic symbiosis

Plants growing under optimal conditions are part of a closed symbiotic cycle. All developing leaves are inoculated with the endophyte residing in the stem apex and show galls on their lamina (Fig. 1, a, b, c). Leaf galls appear as small green islands retaining chlorophyll upon dehydration of the leaf. Seeds acquire the endophyte through inoculation of the ovary during floral organogenesis.

Crippled seedlings

When the transfer of bacteria to the embryo is inadequate, e.g. due to misplacement, natural cripples may occur. These crippled seedlings lack nodules on their leaves, they show stunted growth and their development normally ceases after formation of the second leaf pair. Dwarfed plants can survive in this inactive state for several years, after which they will eventually die. On rare occasions the transfer of bacteria to the seed happens in an appropriate manner, however, in too small numbers to ensure effective nodulation. These dwarves can revert to the nodulated phenotype when the small number of bacteria, residing in the apex, reaches the threshold for effective inoculation of leaves and seeds.

Induction of the crippled phenotype in mature plants

When mature nodulated plants are transferred from optimal conditions to sub-optimal conditions, the crippled phenotype will appear. The first sign indicating the loss of the bacterial partner is the absence of galls on newly formed leaves. Once the bacterial colony inhabiting the stem apex is killed, leaves are no longer inoculated and leaf development becomes anomalous; leaves are dark colored, wrinkled, and the leaf laminae are not fully formed (Fig. 1, d, e, f). In a later stage, usually after development of two or three deformed leaf pairs, the stem apex is reduced to callus and apical dominance is lost. Lateral branches may be formed and develop leaves; however, all formed leaves show the malformations described previously. These new stem apices will soon also decay to callus and lose their apical dominance. Another remarkable trait these gall-free plants show, is the deposit of xylem on veins and young internodes. Plants can endure this crippled condition for several years, until death ensues.

The crippled phenotype is reversible

After replacing a crippled plant back under optimal growth condition, the crippled phenotype may be suppressed. Nodulation may be restored and newly formed leaves acquire their normal appearance. This reversion to nodulation does not happen in pre-

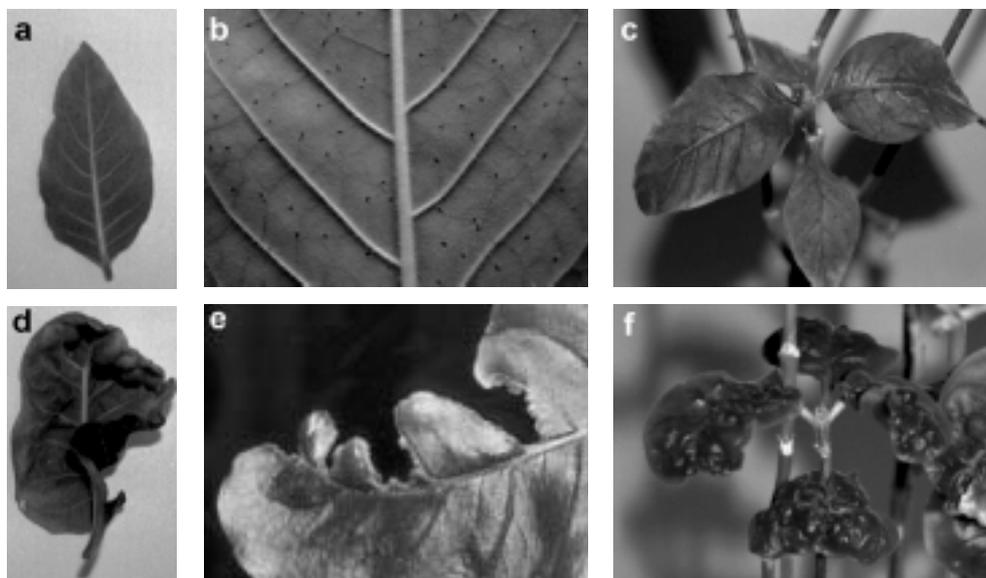


Fig 1. The nodulated and crippled phenotype in *Psychotria kirkii*. Panels a to c represent a nodulated plant; leaves are smooth (a, c) and show nodules on the laminae (b). Panels d to f show different aspects of the crippled phenotype in mature plants; leaves are dark colored and wrinkled (d, f), they lack nodules on the laminae and leaf discs are not fully formed (e).

existing branches. Only new lateral branches, formed after replacement of the plants to optimum conditions, may be restored to the nodulated phenotype. The reappearance of leaf galls indicates the presence of a living bacterial colony in the apex. As there is no external infection, this return of a bacterial colony in the apex must originate from a small number bacteria stored in the dormant axillary buds.

Discussion

Under normal circumstances, *Psychotria kirkii* and its endosymbiotic *Burkholderia* live closely associated. The bacteria travel as constant companions of the host throughout its entire life cycle, as well as through the life cycle of every descendant. As long as the plant maintains a viable bacterial colony in its stem apex, all leaves and seeds will be inoculated, and plant development remains normal (Fig. 2, grey arrows). However, as soon as the plant leaves the symbiotic cycle, i.e. when it loses the bacterial colony in its stem apex or when the number of bacteria drops too low, its survival becomes uncertain (Fig. 2, black arrows). Simply looking at the morphological changes that occur in the plant, upon loss of the bacteria, leaves no doubt about the importance of the endophyte for the host's survival. Transferring healthy plants to a growth chamber with high temperature and low humidity can easily induce the crippled pheno-

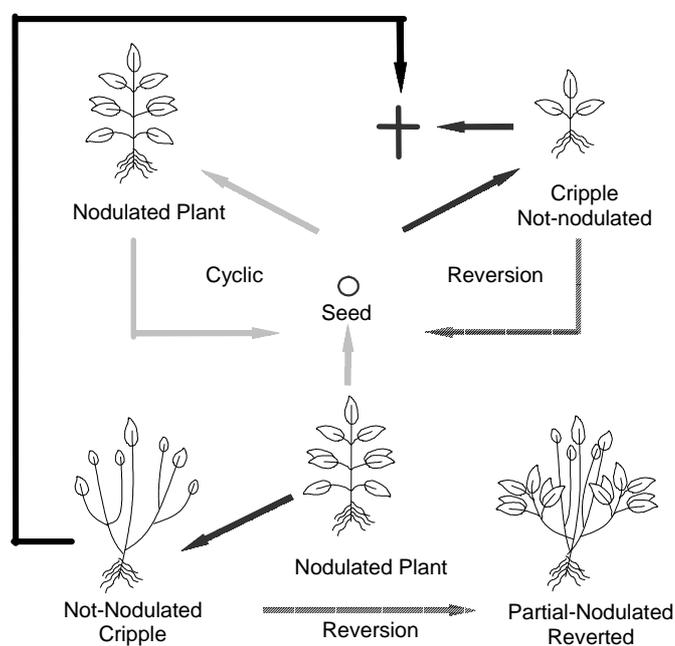


Fig 2. Schematic overview of leaf nodulation in *Psychotria*. Arrows indicate the closed symbiotic cycle (grey), the loss of bacteria in the apex (black), and the regain of bacteria (striped).

type. The bacterial colony in the apex resides in a protein/carbohydrate-based mucilage, secreted by dendroid colleters, which fills the entire shoot tip and thus surrounds developing leaves. This mucilage probably serves as nourishment for the bacteria and is in the mean time a mode of transportation enabling infection of leaves and seeds. High temperature and low humidity causes the mucilage to dehydrate, rendering the bacteria unable to actively grow and prohibiting further inoculation. Whether the crippled phenotype appears in seedlings or in mature plants, if the plant does not succeed in restoring leaf nodulation it will eventually die (Fig. 2, black arrows). This degree of dependence has been rarely seen before. Previous research revealed several possible factors which might be involved in leaf nodulation, including nitrogen fixation or production of plant growth promoting substances by the bacteria. With further research we will try to fully understand to which extend these two organisms mutually interact.

References

- Gordon, J. F., 1963. "The nature and distribution within the plant of the bacteria associated with certain leaf-nodulated species of the families Myrsinaceae and Rubiaceae". PhD Thesis, Imperial College, University of London, England.

- Lersten, N. R., H. T. Jr. Horner, 1967. Development and structure of bacterial leaf nodules in *Psychotria bacteriophila* Val. (Rubiaceae), *J. Bacteriol.*, 94, 2027–2036.
- Miller, I. M., 1990. Bacterial leaf nodule symbiosis, *Adv. Bot. Research*, 17, 163–243.
- Miller, M. I., A. Scott, I. C. Gardner, 1983. Leaf nodule development in *Psychotria kirkii* Hiern (Rubiaceae), *Ann. Bot.*, 52, 791–802.
- Robbrecht, E., 1988. Tropical woody Rubiaceae. Characteristic features and Progressions. Contributions to a new subfamilial classification, *Opera Bot. Belg.*, 1, 1–272.
- Trimen, H., 1894. A Handbook to the flora of Ceylon Vol. 2. Dulau, London, UK.
- Van Oevelen, S., E. Prinsen, R. De Wachter, E. Robbrecht, 2002a. The plant taxonomic value of bacterial identification in African *Psychotria* (Rubiaceae), *Syst. Geogr. Pl.*, 71, 557–563
- Van Oevelen S., R. De Wachter, P. Vandamme, E. Robbrecht, E. Prinsen, 2002b. Identification of the bacterial endosymbionts in leaf galls of *Psychotria* (Rubiaceae, angiosperms) and proposal of ‘*Candidatus Burkholderia kirkii*’, *Int. J. Syst. Evol. Microbiol.*, in Press.
- von Faber, F. C., 1912. Das erbliche Zusammenleben von Bakterien und tropischen Pflanzen, *Jahrb. Wiss. Bot.*, 54, 243.
- Whitmoyer, R. E., H. T. Horner, 1970. Developmental aspects of bacterial leaf nodules in *Psychotria bacteriophila* Val. (Rubiaceae), *Bot. Gaz.*, 131, 193–200.
- Zimmermann, A., 1902. Über Bakterienknoten in den Blättern einiger *Rubiaceen*, *Jahrb. Wiss. Bot.*, 37, 1–11.