

Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza

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Arbuscular mycorrhiza (AM) is a root endosymbiosis between plants and glomeromycete fungi. It is the most widespread terrestrial plant symbiosis, improving plant uptake of water and mineral nutrients. Yet, despite its crucial role in land ecosystems, molecular mechanisms leading to its formation are just beginning to be unravelled. Recent evidence suggests that AM fungi produce diffusible symbiotic signals. Here we show that *Glomus intraradices* secretes symbiotic signals that are a mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs), which stimulate formation of AM in plant species of diverse families (Fabaceae, Asteraceae and Umbelliferae). In the legume *Medicago truncatula* these signals stimulate root growth and branching by the symbiotic DMI signalling pathway. These findings provide a better understanding of the evolution of signalling mechanisms involved in plant root endosymbioses and will greatly facilitate their molecular dissection. They also open the way to using these natural and very active molecules in agriculture.

Arbuscular mycorrhiza (AM) is a root endosymbiosis between fungi of the ancient phylum Glomeromycota and terrestrial plants. AM is formed by 70–90% of land plant species, improving the uptake of water and mineral nutrients¹. Despite its crucial ecological importance, the mechanisms underlying the formation of this symbiosis are poorly understood. This is essentially because of the obligate biotrophy and multinucleate nature of AM fungi². In contrast, the root endosymbiosis associating nitrogen-fixing rhizobia and legumes involves genetically amenable bacterial partners, which has led to a better understanding of the mechanisms involved in the development of this association^{3–5}. Nod factors were shown to be LCO signals produced by most rhizobia and required for early steps of legume infection and root nodule organogenesis⁶ (Supplementary Note 1). Legumes can establish symbioses both with rhizobia and with AM fungi, and the use of model legumes has facilitated the identification of plant genes essential for these associations^{3,7,8}. In *M. truncatula* three of the genes that control the major Nod-factor signal-transduction pathway, *DMI1*, *DMI2* and *DMI3*, are required both for nodulation and for AM formation⁹. Thus it appears that these two different symbioses share central components of the signalling pathways used to trigger their symbiotic programmes^{10–12}. A model was proposed in which rhizobial Nod factors and putative AM fungal signals, the so-called ‘Myc factors’, would activate signalling pathways having common components, the DMI proteins^{7,9,13}. Recent findings indicate that Myc factors are diffusible compounds; spores separated by a membrane from roots of the host plant, or exudates of germinating spores, elicit plant symbiotic responses^{14–17}.

The AM symbiosis is extremely ancient and appeared more than 400 million years ago^{7,18} whereas the rhizobium–legume symbiosis is estimated to have appeared about 60 million years ago¹⁹. It is thus probable that symbiotic signalling mechanisms evolved first in the AM symbiosis and were then recruited and adapted for the rhizobium–legume association⁷. We thus made the working hypothesis that Myc signals, which are produced by fungi able to synthesize chitin, are the

ancestors of the more recent Nod factors and are thus likely to be LCOs. Our strategy to purify them was based on the use of two bioassays that are very sensitive to a broad variety of LCOs: (1) the *Vicia sativa* root-hair branching assay (VsHab), which can detect several different non-sulphated LCOs²⁰; (2) a transgenic line of *M. truncatula* carrying a fusion between the promoter region of the early nodulin gene *MtENOD11* and the *GUS* reporter gene (*ENOD11* assay²¹), which is induced by a variety of sulphated LCOs (F. Maillet, unpublished observations). In addition, we also used root branching of *M. truncatula* as a test for Myc signals¹⁵.

G. intraradices secretes LCOs

Sterile exudates of carrot roots mycorrhized by *G. intraradices* were extracted with butanol and ethyl acetate. The butanol phase, being the most active on both *ENOD11* and VsHab bioassays, was further separated using reverse solid-phase extraction. The most active fraction, eluting with 50% acetonitrile (ACN), was then purified on semi-preparative reverse-phase high-performance liquid chromatography (HPLC). Two fractions with biological activity could be detected: fraction A (eluting at around 40% ACN) was active on *ENOD11*, and fraction B (eluting at around 60% ACN) was active on VsHab (Fig. 1a). Both fractions stimulated root branching in *M. truncatula*, as already shown for diffusible factors secreted by AM fungi¹⁵ (Supplementary Fig. 1a). These fractions were submitted to ultraperformance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/Q-ToF MS). Ion currents with mass to charge ratios (*m/z*) corresponding to hypothetical LCOs ranging from glucosamine dimers to hexamers with a large variety of possible N-substitutions (acyl and methyl) and O-substitutions (methyl, carbamoyl, acetyl, fucosyl, sulphate and so on), including those identified in rhizobial LCOs^{6,22}, were searched for in the chromatograms. Fraction A (F4 and F5; Fig. 1a) showed, in the negative mode electrospray ionization/mass spectrometry, 12 molecular ions that had *m/z* values of sulphated simple LCOs. Six molecular ions (*m/z* values of 1,105.5,

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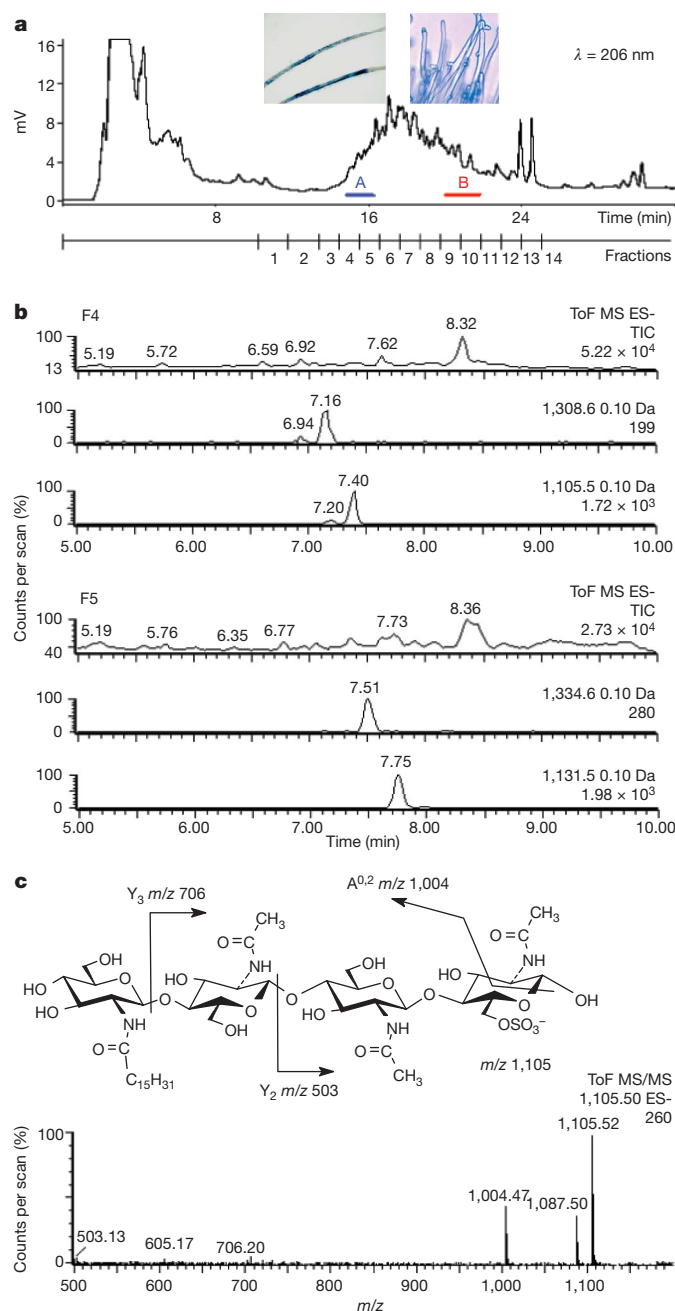


Figure 1 | Detection and characterization of LCOs in mycorrhizal carrot root exudates. **a**, Reverse-phase (C18) HPLC of exudate butanol extract separated two active fractions (test responses are given as inserts): A (fractions F4 and F5) and B (F9 and F10). **b**, UPLC/Q-ToF chromatograms of F4 and F5. Ion current m/z values of 1,308.6 and 1,105.5 \pm 0.1 Da recorded for F4 are characteristic of LCO-V(C16:0, S) and LCO-IV(C16:0, S) respectively, whereas m/z values of 1,334.6 and 1,131.5 \pm 0.1 Da for F5 are characteristic of LCO-V(C18:1, S) and LCO-IV(C18:1, S). **c**, Top, structure of LCO-IV(C16:0, S) showing Y and A fragmentations. Bottom, tandem mass spectrometry (MS/MS) spectrum of m/z value of 1,105.5. The presence of Y_3 , Y_2 and $A^{0.2}$ fragments (in the correct ratios) and the parity change of successive fragments demonstrate the LCO nature of this compound.

1,103.5, 1,101.5, 1,133.5, 1,131.5, 1,129.5) could correspond to sulphated tetrameric LCOs (LCO-IV, S) N-acylated with a C16 or a C18 fatty acid with no, one or two unsaturations. Six other molecular ions (m/z values of 1,308.6, 1,306.6, 1,304.6, 1,336.6, 1,334.6 and 1,332.6) could correspond to sulphated pentameric LCOs (LCO-V, S) N-acylated with the same acyl chains (Fig. 1b and Supplementary Figs 2 and 3). Potential tetrameric LCOs were about tenfold more abundant than pentameric

ones. To confirm the structure of these molecular ions, UPLC/Q-ToF tandem MS was performed and the typical fragmentation of LCOs was observed (Fig. 1c, Supplementary Fig. 4 and Supplementary Note 2). Exact masses and isotopic profiles corresponded to the calculated ones (Supplementary Fig. 5). Fraction B (F9 and F10; Fig. 1a) was also analysed in UPLC/Q-ToF MS and tandem MS in the positive mode, and characteristic ion currents and B fragmentation, corresponding to non-sulphated LCO-IV(C16:0) and LCO-IV(C18:1), were observed (Supplementary Fig. 6). In fractions F1 to F3, we could not detect any ion currents corresponding to more hydrophilic LCOs. In fractions F6 to F8, we could not detect more hydrophobic LCOs. Thus a mixture of sulphated and non-sulphated simple LCOs is present in exudates of mycorrhizal roots. In exudates of non-mycorrhizal roots, grown in the same conditions but without AM fungi, no LCOs could be detected by very sensitive LC/Q-Trap analyses, supporting the fungal origin of these LCOs (Supplementary Fig. 7). To confirm this, sterile germinating spore exudates (GSE) of *G. intraradices* were analysed. A GSE butanol extract elicited *M. truncatula* root branching (Supplementary Fig. 1b). Extracts were analysed by LC/Q-Trap in the positive multiple reaction monitoring (MRM) mode. The MRM traces, characteristic of LCO-IV(C18:1) (1,053 > 426, 1,053 > 629, 1,053 > 832; Fig. 2c) and LCO-IV(C16:0) (1,027 > 400, 1,027 > 603, 1,027 > 806, data not shown) were observed in fraction B (Fig. 2a). HPLC retention times and MRM traces observed in GSE were identical to those of corresponding synthetic Myc-LCOs (see Supplementary Note 3 and Fig. 3c), demonstrating the presence of LCOs in fungal exudates. Biological activity of spore exudates increased during the 9-day germination period as well as quantities of LCOs detected by MRM mass spectrometry showing that bioactive LCOs are secreted *de novo* by germinating spores (data not shown). Similar analyses of fraction A showed traces of sulphated LCOs (Fig. 2d). The presence of sulphated LCOs in GSE was confirmed by mild methanolysis of the HPLC fraction A. This mild methanolysis, which affects the sulphate group but not the saccharidic bonds of LCOs²³, resulted in a clear shift in the biological activity of fraction A, with a strong decrease in the *ENOD11* response (characteristic of sulphated LCOs) to an increase in the VsHab response (characteristic of non-sulphated LCOs) (Supplementary Fig. 8).

Palmitic acid (C16:0) and a C18:1 fatty acid are the major N-acyl substitutions of AM fungal LCOs. To determine the structure of the C18:1 substituent, fatty acids resulting from hydrolysis of the HPLC fraction B from GSE were analysed using gas chromatography/positive electron impact mass spectrometry. Comparison of retention times and spectra with those of commercial standards demonstrated that the major C18:1 fatty acid present in the fraction was oleic acid (C18:1 Δ^9Z) (Supplementary Note 2). The spectrum exhibited 97% quality compared with the standard (Fig. 2e). We can conclude that the AM fungus *G. intraradices* secretes a mixture of sulphated and non-sulphated simple LCOs. The proportion of sulphated LCOs was quite variable among samples but was in general higher in mycorrhizal root exudates than in germinating spore exudates. The proposed structures are represented in Fig. 3.

Purification of AM fungal LCOs resulted in extremely low yields (picogram quantities). To facilitate the study of their biological activities, milligram quantities of Myc-LCO molecules were synthesized by bacterial genetic engineering (Supplementary Note 3), either by using appropriate *nod* gene mutants of *Sinorhizobium meliloti* and *Rhizobium leguminosarum*^{24–26} (LCOs termed (Rhi)Myc-LCOs in the following text) or by the ‘*E. coli* cell factory’ procedure^{27–29} (LCOs called (Syn)Myc-LCOs). For biological assays the two types of sulphated compound (with C16:0 or C18:1 N-acyl chains) were mixed (1/1) as sulphated LCOs, and the same was done with the non-sulphated LCOs.

Myc-LCOs stimulate AM formation

AM fungal LCOs, produced by these procedures, were used to study their influence on formation of mycorrhiza. *M. truncatula* seedlings were grown in test tubes on gellified slants¹⁵ in which a 1/1 mixture of

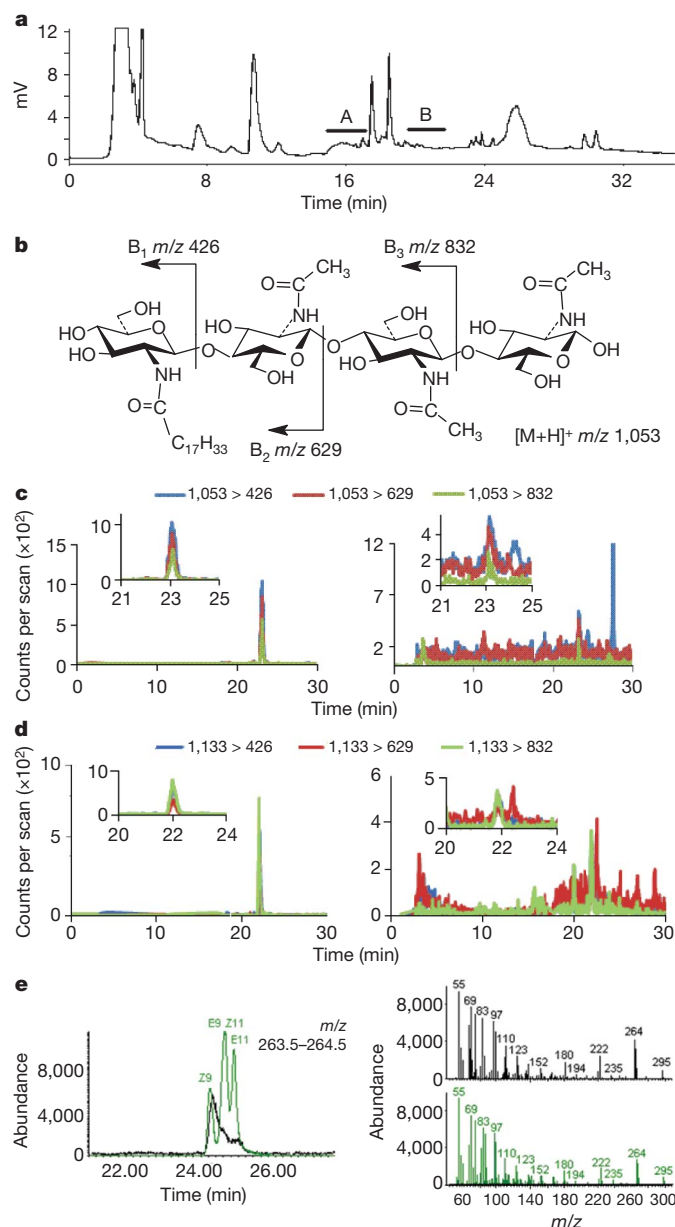


Figure 2 | Detection and characterization of LCOs in germinating *G. intraradices* spore exudates. **a**, Reverse-phase (C18) HPLC of butanol extract of exudates from 3 million germinating spores (GSE), showing two active fractions (A and B). **b**, LCO-IV(C18:1) molecule and B ions observed in LC/Q-Trap MS analysis in the positive mode. **c**, **d**, LC-QTRAP analysis in the MRM mode. **c**, Non-sulphated LCO-IV(C18:1) (transitions: blue, 1,053 > 426; red, 1,053 > 629; green, 1,053 > 832). Left, synthetic standard; right, GSE fraction B. **d**, Sulphated LCO-IV(C18:1) (transitions: blue, 1,133 > 426; red, 1,133 > 629; green, 1,133 > 832). Left, synthetic standard; right, GSE fraction A. The relevant part of the chromatograms is enlarged in the insets. **e**, Characterization of the fatty acyl C18:1 N-substitution (C18:1Δ9Z = oleic acid) of fraction B by positive electron impact mass spectrometry/gas chromatography. Left, chromatogram of fraction B hydrolysate (black) and four different standard C18:1 fatty acids (green). Right, electron impact mass spectrum of the fraction B hydrolysate, characteristic of oleic acid (top) and the corresponding standard (bottom).

sulphated and non-sulphated (Syn)Myc-LCOs (10 nM) was incorporated, and they were inoculated with sterile spores of *G. intraradices*. The number of infection units per plant (separate zones containing arbuscules and internal hyphal networks; Fig. 4a), as well as the density of infection sites per centimetre of root length, were greatly increased by the Myc-LCO treatment (Fig. 4a). We checked whether Myc-LCOs could induce *M. truncatula* genes involved at early steps of the

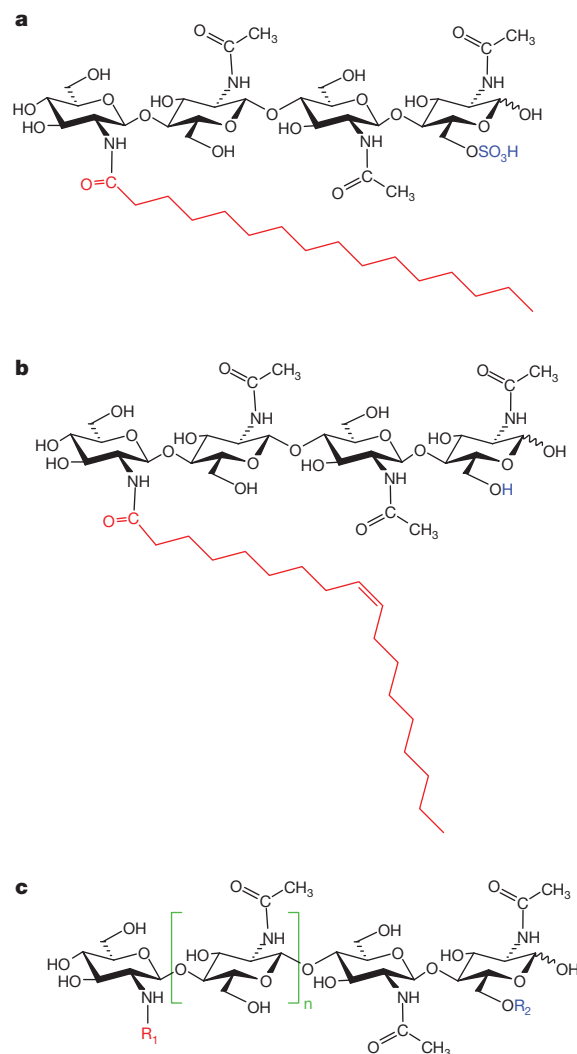


Figure 3 | Chemical structures of natural and synthetic Myc-LCOs. **a**, **b**, Proposed chemical structures of two major Myc-LCOs, (a) LCO-IV(C16:0, S) and (b) LCO-IV(C18:1Δ9Z). **c**, General Myc-LCO structure. For both natural and synthetic Myc-LCOs $n = 1$ or 2 , $R_2 = \text{H}$ or SO_3H . For natural Myc-LCOs, $R_1 = \text{C16}$, C16:1, C16:2, C18:0 or C18:1Δ9Z (oleic acid); for synthetic Myc-LCOs, $R_1 = \text{C16:0}$ or C18:1Δ9Z (oleic acid); and for rhizobial Myc-LCO analogues, $R_1 = \text{C16:0}$ or C18:1 Δ11Z (*cis*-vaccenic acid).

interaction. Transcriptomic analysis of roots of model legumes has identified hundreds of genes upregulated by AM fungal infection or fungal diffusible factors^{17,30}. Among these genes, ten were selected thanks to an *M. truncatula* transcriptome analysis using (Syn)Myc-LCOs (J. Becker and A. Niebel, personal communication). Quantitative PCR with reverse transcription showed that among the ten genes tested, four were upregulated in a *DMI3*-dependent manner by the (Syn)Myc-LCO treatment (Supplementary Note 4 and Supplementary Fig. 9), providing additional evidence that these molecules act as signals during the AM fungal symbiosis.

We then tested Myc-LCOs on the non-legume species *Tagetes patula* (Asteraceae) and *Daucus carota* (Umbelliferae). For *T. patula*, mycorrhization assays were performed on charred clay granules. Plantlets treated with a mixture (1/1) of sulphated and non-sulphated (Syn)Myc-LCOs showed a highly significant increase in the number of infection units per plant (Fig. 4b). Then, inoculated plants were treated with either pure sulphated or non-sulphated (Syn)Myc-LCOs, or with a 1/1 mixture of both. Treatment with the mixture elicited a doubling of the root colonization level (+104%), whereas pure non-sulphated and pure sulphated Myc-LCOs resulted in 75% and 42% increases, respectively (Fig. 4b).

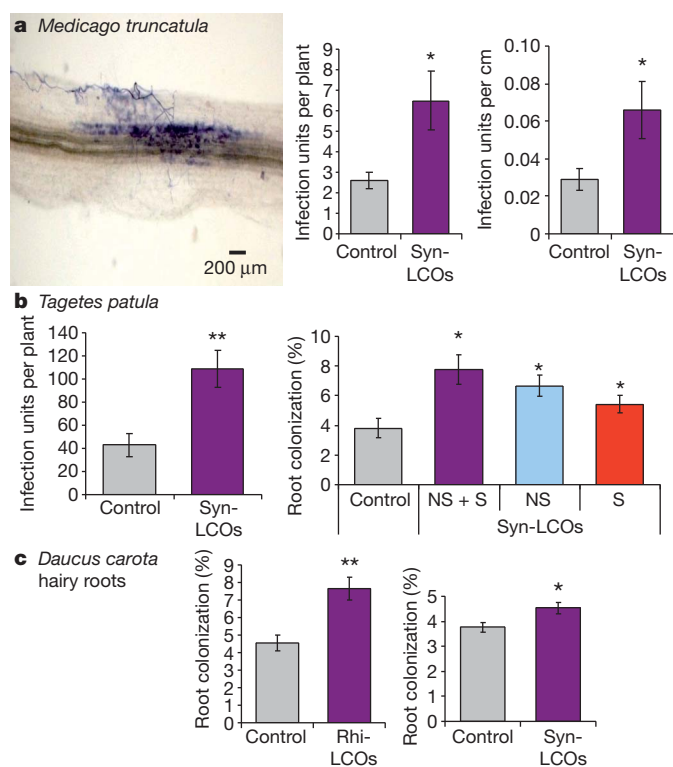


Figure 4 | Effect of Myc-LCOs on mycorrhization by *G. intraradices*. **a**, *M. truncatula*. Left, AM infection unit. Right, number of infection units per plant and density of infection in seedlings treated by sulphated and non-sulphated mixture of (Syn)Myc-LCOs (6 weeks, $n = 15$). **b**, *T. patula*. Left, effect of a sulphated and non-sulphated mixture of (Syn)Myc-LCOs on the number of infection units per plant (4 weeks, $n = 12$); right, percentage root colonization of plants treated by non-sulphated (NS), sulphated (S) and a 1/1 non-sulphated plus sulphated (NS + S) (Syn)Myc-LCO mixture (4 weeks, $n = 40$). **c**, Carrot excised transformed roots. Left, percentage colonization of roots treated by a non-sulphated plus sulphated mixture of (Rhi)Myc-LCO rhizobial analogues (8 weeks, $n = 10$). Right, percentage colonization of roots treated by a non-sulphated plus sulphated mixture of (Syn)Myc-LCOs (8 weeks, $n = 15$). In all experiments Myc-LCOs were used at 10 nM. Data are mean \pm s.e.m. Statistics: **a**, Wilcoxon test; **b** (left) and **c**, t -test; **b** (right), Kruskal–Wallis; ** $0.001 < P < 0.01$; * $0.01 < P < 0.05$.

AM fungi can form mycorrhiza on transformed root organ cultures³¹. To test whether Myc-LCOs could stimulate mycorrhization of such excised carrot roots, we used a 1/1 mixture of sulphated and non-sulphated (Rhi)Myc-LCO analogues, produced by rhizobial mutants (Supplementary Note 3 and Fig. 3). The incorporation of these compounds into the growth medium resulted in a very strong increase in colonization (+68%) (Fig. 4c). Similarly a significant increase of 20% was observed when a 1/1 mixture of synthetic sulphated and non-sulphated (Syn)Myc-LCOs was used. Therefore the response of roots to these Myc signals did not require the presence of aerial parts of the plant. On the three plant species, including legumes and non-legumes, synthetic Myc-LCOs enhance AM formation, probably by stimulating both root branching and infection density. This is strong evidence that the Myc-LCOs we have identified are genuine mycorrhizal signals.

Myc-LCOs stimulate root branching

AM fungi secrete diffusible compounds which stimulate root branching in *M. truncatula*, and this response can be genetically dissected¹⁵. We observed that HPLC fractions A and B, containing *G. intraradices* sulphated and non-sulphated LCOs respectively, elicited root-branching stimulation (RBS) (Supplementary Fig. 1b). To determine whether this was really due to LCOs and not to contaminating fungal compounds, we tested sulphated and non-sulphated (Syn)Myc-LCOs, separately or as a 1/1 mixture, at concentrations ranging from 10 nM to 10 pM on *M.*

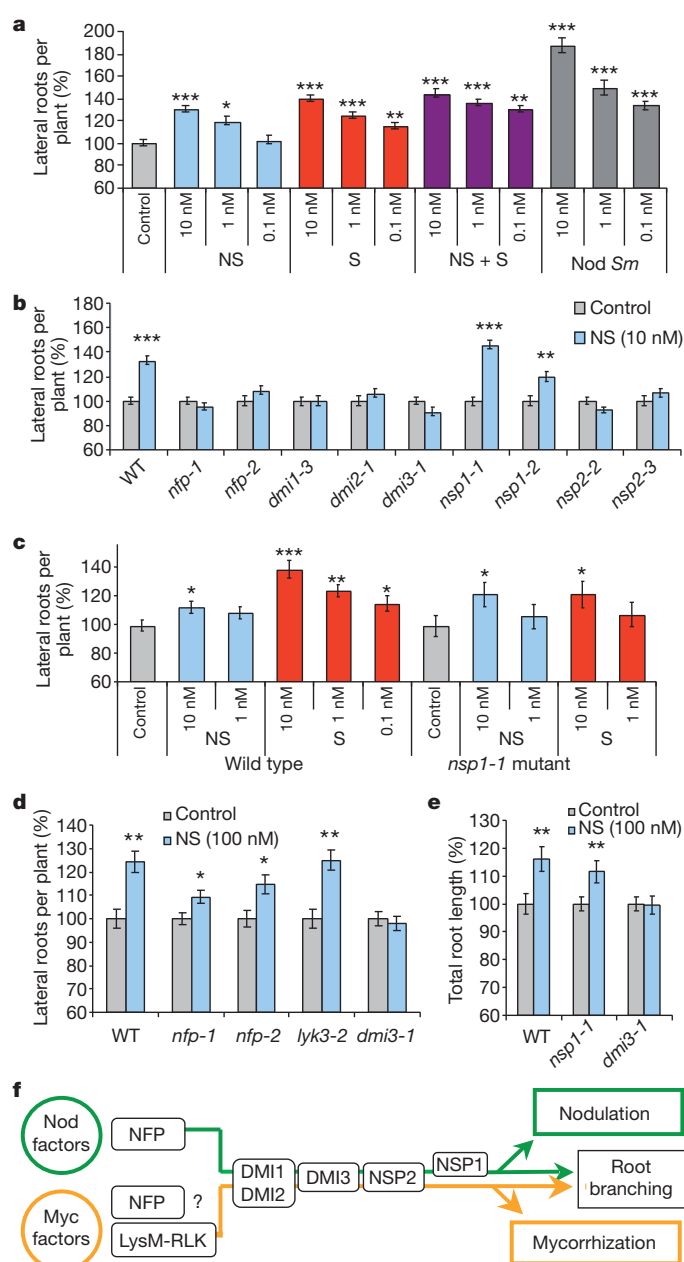


Figure 5 | Synthetic Myc-LCOs stimulate *M. truncatula* root branching by the DMI pathway. **a**, Effect of non-sulphated, sulphated, non-sulphated plus sulphated (Syn)Myc-LCOs and *S. meliloti* Nod factors (from 10 nM to 0.1 nM) on RBS in wild-type plants ($n = 120$). **b**, Effect of (Syn)Myc-LCOs in wild type and symbiotic signalling pathway mutants, with 10 nM NS (Syn)Myc-LCOs ($n = 120$). **c**, Effect of the *nsp1-1* mutation with non-sulphated or sulphated (Syn)Myc-LCOs ($n = 80$). **d**, Effect of 100 nM non-sulphated (Syn)Myc-LCOs in wild type and symbiotic mutants ($n = 120$). **e**, Effect of 100 nM non-sulphated (Syn)Myc-LCOs on total root length of wild type, *nsp1* and *dmi3* mutants ($n = 80$). **f**, Model for the genetic control of Nod- and Myc-factor-activated signal transduction pathways leading to RBS, nodulation and mycorrhization. Data are mean \pm s.e.m. Statistics: **a**, **b**, analysis of variance; **c**–**e**, t -test; *** $P < 0.001$; ** $0.001 < P < 0.01$; * $0.01 < P < 0.05$. For root branching histograms, means of measures for days 5, 6, 7 and 8, are presented as percentages of the control value.

truncatula seedlings (Fig. 5a and Supplementary Fig. 10). Non-sulphated (Syn)Myc-LCOs elicited RBS at concentrations down to 10 nM/1 nM. Sulphated (Syn)Myc-LCOs and the mixture of the two types elicited RBS at concentrations down to 0.1 nM, and in some experiments down to 0.01 nM. Both sulphated and non-sulphated

Myc-LCOs were thus extremely active, but sulphated molecules were about 100-fold more active.

The symbiotic signalling pathway identified in *M. truncatula* includes genes coding for Nod factor perception (*NFP* and *LYK3*)^{32,33}, calcium signalling (*DMI1*, *DMI2* and *DMI3*)^{34–36} and nodulation-specific transcription factors (*NSP1* and *NSP2*)^{9,37,38}. All of these genes are required for nodulation whereas *DMI1*, *DMI2* and *DMI3* are also required for mycorrhization. They are all required, except *LYK3*, for RBS by *S. meliloti* Nod factors (ref. 15 and F. Maillet, personal communication). Sulphated Myc-LCOs, having structural similarities with *S. meliloti* Nod factors, might induce RBS by the Nod signalling pathway: to avoid possible cross talk between Nod and Myc pathways, we first tested non-sulphated (Syn)Myc-LCOs (at 10 nM) (Fig. 5b). The RBS response was dependent on the three *DMI* genes, which confirmed that Myc-LCOs are symbiotic signals. Downstream of *DMI3*, the response was independent of the *NSP1* gene, showing that non-sulphated Myc-LCOs do not trigger RBS by the Nod pathway. Surprisingly, the response was dependent on *NSP2* whereas *nsp2* mutants were previously reported to be Myc⁺. We re-assessed the Myc phenotype by quantitatively measuring mycorrhiza formation at early stages: the *nsp2-2* mutant exhibited a highly significant 41% lower colonization level than wild-type plants, showing that *NSP2* is involved in Myc signalling (Supplementary Fig. 11). In addition, a mutation in *RAM1*, a gene specifically involved in Myc signalling, suppressed RBS (F. Maillet and G. Oldroyd, personal communication). All these data are consistent with non-sulphated Myc-LCOs stimulating root branching by a Myc pathway. Mutants of the two putative Nod factor receptor genes *LYK3* and *NFP*, acting upstream of *DMI* genes, are Myc⁺. Mutation in *LYK3*, a gene involved in rhizobial recognition during infection, did not affect the RBS response to Myc-LCOs (Fig. 5d). The RBS response of *nfp* mutants varied with Myc-LCO concentration. At 10 nM, *nfp* mutants did not show a significant response (Fig. 5b). At 100 nM, whereas the *dmi3* mutant did not respond and the *lyk3* mutant exhibited a full RBS response as the wild type, *nfp* mutants showed a significant but intermediate stimulation (Fig. 5d).

With sulphated Myc-LCOs, the signalling leading to RBS seems to be by the Nod pathway (requirement for *NSP1*) (Fig. 5c and Supplementary Figs 12 and 13). The stimulating effect of sulphated Myc-LCOs at low concentrations, such as 0.1 nM (Fig. 5a and Supplementary Fig. 13), could be due to activation of the Nod pathway. Using the *nsp1-1* mutant to block Nod signalling, we observed that sulphated Myc-LCOs elicit RBS at concentrations similar to the non-sulphated ones, about 100-fold higher than the concentrations required to trigger RBS in wild-type plants (Fig. 5c). Thus, to elicit RBS by the Myc pathway, the required sulphated or non-sulphated Myc-LCO concentrations are about 100-fold higher than those required to elicit this response by the Nod pathway.

From an agronomical point of view, it was important to investigate the possible influence of Myc-LCOs on whole root development. To avoid a possible bias due to cross talk with the Nod pathway, non-sulphated compounds were used, as well as a mutant in the *NSP1* gene. At 100 nM, non-sulphated Myc-LCOs resulted in a highly significant 16% increase in total root length. This response was dependent on the *DMI3* gene but did not require the *NSP1* gene, specific to the Nod pathway (Fig. 5e).

Discussion

The discovery of the structure of diffusible Myc signals is an important milestone in our understanding of the evolution of plant root endosymbioses, and opens many possibilities for the molecular and cellular dissection of these interactions. The AM fungus *G. intraradices* secretes a mixture of sulphated and non-sulphated LCOs that have structural similarities with rhizobial Nod factors. In both Nod and Myc symbiotic signals, the chitin oligosaccharidic backbone is composed of four or five glucosamine residues, N-acylated on the glucosamine residue at the non-reducing end. *G. intraradices* Myc signals

have simpler structures than the Nod factors described so far^{6,22} (Supplementary Note 1), and it is worth noting that rhizobial mutants that produce simple LCOs similar to Myc-LCOs are unable to penetrate into their legume hosts and to form nodules^{24,25}. Interestingly, broad host-range rhizobia produce a mixture of sulphated and non-sulphated Nod factors^{6,20,22}.

The Myc signals that we have identified correspond to the definition of Myc factors that was proposed earlier^{7–9,13}: (1) they elicit plant responses by the DMI pathway; (2) in a legume, downstream of *DMI3* they activate a pathway that is *NSP1* independent (a transcription activator specific to the nodulation pathway), and *RAM1* dependent (a gene specific to the Myc signalling pathway; F. Maillet and G. Oldroyd, personal communication). In addition they stimulate mycorrhiza formation in legumes and non-legumes. Other AM fungal signals inducing symbiotic gene expression or influencing root architecture have been reported, but the root responses did not require the *DMI* genes^{39,40}.

Genetic analysis of Myc signalling in a legume has revealed that the intricacy with Nod signalling is even more complex than previously thought (Fig. 5f). The *NSP2* transcription activator now appears to be a component common to the Nod and Myc pathways. Mutants in the putative Nod factor receptor genes *NFP* and *LYK3*, not being altered for mycorrhiza formation, were not thought to be involved in Myc factor perception. This is the case for *LYK3*, but our data suggest that *NFP* is partly involved in the Myc-signal-elicited RBS response. Legumes have to discriminate the two types of symbiotic signal, despite their similarities, to activate the appropriate symbiotic programme. Nod factor and Myc factor receptors could discriminate their respective ligands by a combination of qualitative (structural) and quantitative differences (Supplementary Note 5).

The AM symbiosis is much more ancient than the rhizobium–legume association^{18,19}. Bacterial *nod* genes required for LCO synthesis could have been acquired by lateral transfer from AM fungi about 60 million years ago and subsequently spread to a variety of soil bacteria^{5,6,22}. Several data support the hypothesis that the Nod-factor-activated signalling pathway leading to legume nodulation is largely derived from a pre-existing signalling pathway leading to endomycorrhization^{7,8,41–43}. Myc factors are not only symbiotic signals that stimulate mycorrhiza formation but also plant growth regulators. This suggests that the AM symbiosis, in the course of evolution, has selected fungal signals that can modify root development to facilitate further symbiotic infection.

The RBS assay, much simpler than the quantitative study of mycorrhization, could be used to facilitate genetic analysis of plant responsiveness to mycorrhizal signals. We have developed biotechnological procedures to produce Myc factors in large quantities. This opens the way to exploring the use of these very active natural molecules in agriculture.

METHODS SUMMARY

To detect fungal symbiotic signals, two bioassays were used: vetch root hair branching²⁰ and induction of a *pENOD11::GUS* construct in *M. truncatula* roots²¹. Three hundred litres of sterile exudates from mycorrhized carrot roots⁴⁴, and exudates from 40 million germinating spores of *G. intraradices*, were extracted with butanol, followed by solid-phase extraction on a C18 reverse-phase column eluted with different proportions of water/acetonitrile. The 50% acetonitrile fraction was purified by HPLC on a C18 reverse-phase column. Active fractions from mycorrhized root exudates were analysed by a UPLC-QToF mass spectrometer to determine accurate molecular masses. Exudates from germinating spores were analysed with the multiple-reaction monitoring device on a 4000 Q-Trap mass spectrometer. Milligram quantities of Myc-LCO analogues were synthesized by bacterial genetic engineering, either by using appropriate *nod* gene mutants of *S. meliloti* and *R. leguminosarum*^{24–26} or by the 'E. coli cell factory' procedure^{27–29}. Mycorrhization experiments were performed using sterile *G. intraradices* spores with seedlings of *M. truncatula*^{15,45}, *T. patula* and transformed root cultures of *D. carota* (carrot)⁴⁴. Root colonization was estimated with a binocular microscope by counting the number of infection units per plant¹⁵ or by the gridline intersect method⁴⁶. The developmental activity of Myc-LCOs was studied using the *M.*

truncatula root branching bioassay¹⁵. Root system length was measured by image analysis.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions F.M. coordinated experiments for all bioassays, designed and performed the VsHab assay, and designed and performed *M. truncatula* bioassays with O.A. and V.P. purified fungal Myc-LCOs. M.G. and A.H. prepared germinating spores. L.C. and A.H. performed the *ENOD11* bioassay. A.H. designed and performed mycorrhization tests on carrots and *Tagetes*. O.A. performed statistical analyses. F.M. and D.G. extracted Myc-LCO analogues from rhizobial mutant cultures, and E.A.M. and H.D. synthesized Myc-LCOs by the cell factory technique. For mass spectrometry, UPLC/QToF was performed by V.P. and V.P.-P., and QTRAP by V.P.-P. Quantitative PCR with reverse transcription experiments were designed and analysed by A.N. and realized by D.F. and O.A. G.B. supervised devising, planning and interpreting experiments with AM fungal material (material production, spore germination and mycorrhization tests). J.D. conceived and directed the project, and wrote the manuscript with the help of F.M., V.P. and G.B.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.D. (jean.denarie@toulouse.inra.fr).

METHODS

Exudates from mycorrhized roots and from germinating spores. Exudates of carrot roots mycorrhized by *G. intraradices* were purchased from Premier Tech Biotechnologies. Purified sterile spores of *G. intraradices* were purchased from Premier Tech Biotechnologies and from Agronutrition. Spores were germinated at 30 °C in a 2% CO₂ incubator for 10 days.

Plant material. Seeds of *M. truncatula* var. Jemalong A17 wild type⁹ and symbiotically defective mutants derived from this line^{9,32}, *nfp-1* (C31), *nfp-2*, *lyk3-2* (W1), *dmi1-3* (Y6), *dmi2-1* (TR25), *dmi3-1* (TRV25) and *nsp1-1* (B85) were provided by C. Gough. Seeds of the *nsp2-2* and *nsp2-3* mutants¹⁷ were provided by G. Oldroyd. Seeds of *V. sativa* subsp. *nigra* were multiplied by SCJ INRA Dijon. Seeds of the *M. truncatula* line carrying the *pENOD11::GUS* fusion⁴⁸ were provided by E. Journet. Seeds of *T. patula* (French marigold) var. Légion d'honneur were from Caillaud. Sterile cultures of carrot hairy root were obtained as previously described⁴⁴.

Bioassays for purification of AM fungal symbiotic signals. The root hair branching assay of vetch²⁰ was used with the following modifications. Young seedlings were left 3 days in Petri dishes, on Fahraeus agar, in a vertical position in a growth chamber (22 °C). Observations of root hairs were performed in a 0.02% methylene blue solution under a light microscope, 30 h after treatment (40 µl per seedling). The *MeENOD11* gene induction assay²¹ was used with no paper on the agar surface and 40-µl treatment per seedling root.

Myc-LCO purification. Active LCO compounds were extracted as previously described⁴⁹ with the following modifications. No ammonium acetate was added to the eluents and a pre-purification step was used with a solid-phase extraction on a Discovery DSC C18 5-µm (Supelco) column, with three elution steps: 20%, 50% and 100% ACN in water. Samples were collected every minute along the HPLC gradient.

UPLC/Q-ToF MS analyses. The UPLC column was a C18 Acquity (2.1 mm × 100 mm, 1.7 µm, Waters) and the flow rate was 0.45 ml min⁻¹. For the more hydrophilic compounds (semi-preparative HPLC fractions 1–9), the programme was a linear gradient ranging from 10% ACN in 1% acetic acid/water to 100% ACN within 7 min, followed by an isocratic step at 100% ACN for 2 min, 2 min return to the initial conditions and 1 min stabilization. The more hydrophobic compounds (HPLC fractions 6–11) were separated using a linear gradient from 25% ACN in 0.1% acetic acid/water to 100% ACN within 7 min, followed by similar reconditioning. Ten microlitres were injected.

The mass spectrometer was a Q-ToF Premier (Waters). The capillary was set to 3.2 kV and the cone to 10 V. Internal lock mass was performed by continuous introduction of leucine-enkephalin. Analyses were performed in the negative and positive modes. For fragmentation experiments, a collision energy of 15 V was used.

LC/Q-Trap analyses in the MRM mode. The binary HPLC pump (Agilent) was equipped with a C18 Acclaim 120 column (2.1 mm × 250 mm, 5 µm, Dionex). Separation started at 50% ACN in water for 5 min, followed by a 25-min gradient to 100% ACN maintained for 5 min, at a constant flow rate of 200 µl min⁻¹. Ten-microlitre samples were injected. The mass spectrometer was a 4000 Q-Trap (Applied Biosystems) with a Turbo V (MDS Sciex) source. Samples were monitored in the positive MRM mode as reported previously⁵⁰. The capillary voltage was set to 4,000 V. Collision energy was between 25 and 65 V and declustering potential was between 60 and 75 V, optimized for each molecule. The MRM channels were set according to the transitions of molecular ion [M + H]⁺ to the fragment ions corresponding to the loss of one, two or three GlcNAc at the reducing end (sulphated or not): LCO-IV(C16:0), 1,027 > 806, 1,027 > 603, 1,027 > 400; LCO-IV(C16:0, S), 1,107 > 806, 1,107 > 603, 1,107 > 400; LCO-IV(C18:1), 1,053 > 832, 1,053 > 629, 1,053 > 426; LCO-IV(C18:1, S), 1,133 > 832, 1,133 > 629, 1,133 > 426.

Biochemical characterization of Myc-LCOs. To remove the sulphate group, mild hydrolysis was performed as previously described²³. For the fatty-acid analysis of Myc-LCOs, HPLC fractions (F8–F11) of butanol extracts of germinating spore (33 million spores) exudates, were dissolved in 4 M KOH and heated at 100 °C for 3 h, neutralized and extracted with chloroform. Methylation of the fatty acids was done by addition of diazomethane. Analysis was on a GC/MS system (5973N, Hewlett Packard). GC: He = 1 ml min⁻¹; programme, 70 °C (3 min), up to 100 °C at 5 °C min⁻¹, then to 240 °C at 3 °C min⁻¹ and finally at 10 °C min⁻¹ to 300 °C maintained for 3 min. MS: positive electron impact 70 eV; source, 230 °C; quadrupole, 150 °C.

Production of synthetic Myc-LCOs and Myc-LCO analogues. Rhizobial *nod* mutants and the 'E. coli cell factory' method were used to produce milligram quantities of Myc-LCO analogues. Sulphated Myc-LCO analogues were purified from liquid cultures of the *S. meliloti nodFL* mutant (strain GMI 6629 containing

the pMH682 plasmid), as previously reported^{24,51}. For the production of non-sulphated Myc-LCO analogues, the *R. leguminosarum* strain LPR5045 (pMP247) was used²⁵. LCOs were purified as previously reported²⁵. Synthesis of Myc-LCOs by the 'E. coli cell factory' was as described in the literature^{28,29} using the *S. meliloti nodBC* and *nodH* genes. LCO purity control and structural determination were performed by mass spectrometry (UPLC/Q-ToF, LC/Q-Trap) and NMR⁵² spectroscopy (Supplementary Fig. 14). Their biological activity was checked with the *ENOD11::GUS* and vetch root hair deformation assays (Supplementary Fig. 15).

Mycorrhization. The effect of exogenous LCO addition on AM formation was tested in non-optimal conditions, to avoid the possible risk that the fungal endogenous synthesis of LCOs might be sufficient to ensure an optimal AM formation. In these conditions, mycorrhization percentages were expected to be low. For mycorrhization of *M. truncatula* in test-tubes, 50 sterile spores were put at the bottom of each slope of medium containing Myc-LCOs or control solution. For mycorrhization of *T. patula* on charred clay granules (Oil Dry US Special, Brenntag) hydrated with Long Ashton low phosphate solution⁵³, 50-ml Falcon tubes, pierced with three small holes at the bottom, were filled with sterilized substrate and individually placed in 120-ml plastic boxes (5.5 cm diameter, 7 cm height), closed with an opaque pierced cap, filled with 80 ml water and wrapped in aluminium foil. For inoculation of *T. patula*, spores were suspended in 1 ml of 100 nM Myc-LCOs (to obtain a 10 nM final concentration) or control solution and 100 spores were dropped around the seed. Each plant received 1 ml of treatment solution, twice a week for 3 weeks. Pots were placed in a growth chamber (25 °C, 16 h photoperiod, 180 µE m⁻² s⁻¹) for 4 weeks. For mycorrhization of excised carrot roots, root fragments were laid on Petri dishes (diameter = 90 mm) containing a first layer of 20 ml M medium (0.3% Phytagel) and a second layer of the same medium containing 20 or 200 spores per millilitre and Myc-LCOs at the appropriate concentration. Dishes were incubated in the dark (24 °C, 50% humidity). Myc-LCOs were added once a week on the plate surface during the first 3 or 4 weeks. Root colonization was estimated under a binocular microscope, after staining root fragments with Schaeffer black ink⁵⁴, by (1) counting infection units (discrete zones containing arbuscules) per plant¹⁵ or (2) estimating the percentage of root length colonized by the fungus by the gridline intersection method⁴⁶.

Root branching assay. This was performed with *M. truncatula* seedlings as previously reported¹⁵ but vitamins were not added to the M medium and plates were incubated at 20 °C. For measurement of root length we used an Epson scanner 10000 XL and the image analysis system of Winrhizo Scientific Software (Instruments Regent).

Statistics. *M. truncatula* mycorrhization data were analysed by the non-parametric Wilcoxon test. Statistical software was from the R system⁵⁵. *M. truncatula* root length, carrot root and *T. patula* mycorrhization data were analysed by Student's *t*-test. For root branching data, statistical analyses were performed with Statgraphics Centurion (version XV) software (Statpoint Technologies). Data were tested by analysis of variance with a general linear model procedure of data from several (mostly three) independent experiments. To analyse repeated measure experiments, the statistical general linear model was built to take into account the impact of four factors on variability: experiments, treatments, plants and days (5–8). The normality of residues was verified by the Kolmogorov–Smirnov test.

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