Original Article

Secondary metabolite profiling of the model legume *Lotus japonicus* during its symbiotic interaction with *Mesorhizobium loti*

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ABSTRACT

Plant secondary metabolites, particularly flavonoids, are key components in the early stages of nitrogen-fixing symbiosis. Despite their importance, the endogenous secondary metabolites involved in symbiosis have not yet been identified in the model legume *Lotus japonicus*. We therefore determined changes in the secondary metabolic profile of *Lotus japonicus* roots in response to its symbiont. Analysis of the root secondary metabolite profiles 1 week after inoculation with *Mesorhizobium loti* revealed quantitative changes in the level of 14 phenolic peaks when compared with non-inoculated control plants. These changes affected compounds from most phenolic classes, possibly resulting from interconversion between classes since the total phenolic level remained constant. In addition, the use of 2 *M. loti* strains differing only in their capacity to synthesise Nod factor revealed that, although Nod factor signalling induced accumulation of a specific subset of 4 phenolic peaks, most changes were induced in response to both rhizobial strains.

KEYWORDS: Flavonoids, *Lotus japonicus*, *Mesorhizobium loti*, non-nodulating mutants, nodulation, Nod factor signalling, secondary metabolites, symbiosis.

INTRODUCTION

Legumes can interact symbiotically with the rhizobia, a sub-group of soil bacteria which improve legume nutrition through fixation of atmospheric nitrogen. This complex interaction requires specific genetic and metabolic communication between both partners (for reviews see Geurts et al., 2005 and Jones et al., 2007). Communication is mediated through the production of various signal molecules, by both rhizobia and plant host, leading to recognition and establishment of symbiosis. Rhizobia produce a range of signals: these include the crucial Nod factor and various surface polysaccharides that are important for both initiation of the symbiotic interaction and host specificity (for review see Jones et al., 2007 and references therein). The host plant synthesises and/or exudes specific secondary metabolites that are involved both in pre-infection events and as mediators of Nod factor signalling pathways (Cooper, 2007).

Among the secondary metabolites, flavonoids play a pivotal role at different stages of nodulation. Firstly, exuded flavonoids are important pre-infection signals leading to major alteration of rhizobia metabolism (Cooper, 2007). They induce expression of many rhizobial genes including the *nod* genes responsible for synthesis and secretion of the Nod factor. They may also attract specific rhizobial species and induce major changes in the protein and surface polysaccharide content of rhizobia (for review see Cooper, 2007 and references therein). Secondly, endogenous flavonoids could mediate Nod factor response and act as regulators of rhizobial colonisation (Grandmaison and Ibrahim, 1995; Mathesius et al., 1998b). Nod factor induces flavonoid accumulation and expression of flavonoid biosynthesis encoding genes, suggesting their implication during post-infection events (Estabrook and Senguptagopalan, 1991; Mathesius et al., 1998a). Flavonoids are natural regulators of auxin transport (Jacobs

1 and Rubery, 1988) and several lines of evidence in indeterminate nodulating species suggests
2 that some of them might be involved in the accumulation of auxin within cortical cells, which
3 is necessary for nodule formation (Mathesius et al., 1998b; Mathesius, 2001; Wasson et al.,
4 2006, Subramanian et al., 2007). Their anti-oxidant and anti-bacterial properties also
5 suggested that some endogenous flavonoids can act to protect the nitrogen-fixing rhizobium
6 from oxidative stresses while others may control the extent of rhizobial colonisation
7 (Grandmaison and Ibrahim, 1995).

8
9 Although the role of secondary metabolites, and, in particular, flavonoids, in
10 symbiosis has been extensively studied, these studies have been restricted either to species
11 accumulating isoflavonoids in their roots, such as soybean, or to species with indeterminate
12 nodules, such as alfalfa, *Medicago truncatula*, and clover. *Lotus japonicus* is a well-
13 established model species for studying the determinate nodulation process (Handberg and
14 Stougaard, 1992). This model species contributed greatly to resolving the genetic pathway of
15 nodulation (for reviews see Riely et al., 2004 and Oldroyd and Downie, 2008). Furthermore,
16 *L. japonicus* accumulates condensed tannins in its roots and nodules rather than isoflavonoids
17 so the symbiotic signals may be different (Stafford, 1997). Transcriptomic, proteomic and
18 metabolomic approaches have revealed drastic changes in *L. japonicus* root metabolism in
19 response to its symbiont *Mesorhizobium loti* (Wienkoop and Saalbach, 2003; Colebatch et al.,
20 2004; Desbrosses et al., 2005). However, the secondary metabolites were not targeted in these
21 studies, although some genes and proteins point to changes in the activity or expression of
22 their biosynthetic enzymes (Wienkoop and Saalbach, 2003; Colebatch et al., 2004). In
23 addition, apart from the condensed tannins and its cyanogenic glycosides, the secondary
24 metabolite content of *L. japonicus* roots has not been identified and its endogenous *nod* gene
25 inducers remain uncharacterised. Indeed, while the flavonoid content of roots, root exudates

1 and mature nodules have been monitored in other *Lotus* species, including *L. creticus* L.
2 (Yang et al., 1989; Mahmoud et al., 1990) and *L. pedunculatus* (Pankhurst and Jones, 1979;
3 Cooper and Rao, 1992; Steele et al., 1999), the secondary metabolite content of the model
4 legume *L. japonicus* is undetermined. Thus, the endogenous secondary metabolites that play a
5 role during the initial steps of nodule formation in this species remain unidentified.

6
7 In the present study, we undertook a metabolic profiling approach to determine the
8 secondary metabolite changes induced in *L. japonicus* roots in response to rhizobial symbiosis
9 before nodule formation.

10 11 MATERIAL AND METHODS

12 13 *Plant, bacteria and inoculations*

14 Seeds of *L. japonicus* (Regel) Larsen cv Gifu B129 were surface-sterilised with
15 sulphuric acid 99% for 20 min, rinsed three times with water and allowed to imbibe water for
16 5 h. Imbibed seeds were sown in washed and autoclaved medium-grade vermiculite in 800 ml
17 polypropylene pots at a density of 10 seeds per pot. Seedlings were grown in semi-sterile
18 conditions in a growth cabinet under $400 \mu\text{M m}^{-2} \text{sec}^{-1}$ photon flux density for 15 h
19 photoperiod at 22 °C (day) and 18 °C (night). Plants were watered every 2 days with sterile
20 tap water and weekly with a filter-sterilised nitrogen-free nutrient solution (Ryle et al., 1978).
21 Both water and nutrient solution were supplemented with 5 mM of potassium nitrate until 1
22 week before inoculation.

23
24 Seedlings were inoculated after 7 weeks of growth with 2 ml of an aqueous suspension
25 of either *M. loti* NZP 2037, a wild-type, nodulating strain, or *M. loti* PN 4047, a *NodC* mutant

obtained after *Tn5* mutagenesis of NZP 2037 (Scott et al., 1996), or with 2 ml of sterile water. At 1 week post-inoculation all plants were harvested. At harvest plants were separated into shoot and root samples, flash-frozen under liquid nitrogen and stored at -80°C before subsequent analysis. Each sample corresponded to the roots from 6 pots of 10 plants pooled together to limit environmental variation and ensure enough material for profiling analysis.

Assessment of plant-rhizobia interaction

A set of plants, grown as previously stated, was used to determine the infection stage and the infection level at the time of metabolic profiling. To observe the infection stages of roots challenged with *M. loti*, roots were excised, cut in small pieces and stored in 50% ethanol (v/v). Roots were cleared in glacial acetic acid and absolute ethanol (1:3, v/v) for 2 days. Cleared roots were thoroughly washed under constant tap water flux for 1 min. Roots were then fixed and stored in lactic acid: glycerol: water, (1:1:1, v/v/v), thereafter referred to as lactoglycerol, until microscopic observation.

The infection stage was assessed by observing 100 random ‘fields of view’ per sample at X20 magnification. One sample corresponded to 2 x 25 pieces of root on 1 mm thick glass slides (SuperFrost, BDH, VWR International, UK) in drops of lactoglycerol and covered with 22 x 50 mm coverslips (BDH, VWR International, UK). Microscopic observations were performed with a differential interference contrast microscope using X20 and X40 magnification objectives (Olympus BH-2, Olympus, Japan, equipped with a Normaski diaphragm). Images were taken using a digital camera (Optronics) coupled with the MagnaFireTM software (Optronics).

Broad secondary metabolite profiling

Secondary metabolite extraction

The extraction method used enables profiling of a broad range of secondary metabolite classes as previously described (Risipail et al., 2005b; patent EP 03706756.8). This method allows profiles including amino acids, amines, alkaloids, triterpenoids and fats. Most flavonoids are excluded by this method due to the ion exchange and HP-20 resins steps but are picked up in a methanol extract. The method is based on a sequential extraction of 2 g dry weight of plant tissue using 50% ethanol (v/v) to collect most polar and non-polar compounds followed by a dichloromethane extraction targeting the most non-polar components. The polar fraction was then further fractionated by cation-exchange chromatography using Dowex 50W-X8 resin (H^+ form). This polar fraction was divided into an amino acid/amine/alkaloid fraction which bound to the cation exchange resin and an unbound fraction containing non-ionic components. The cation exchange unbound fraction was further processed using HP-20 resin to remove most sugars and common flavonoids prior to analysis by reverse phase HPLC. The most non-polar dichloromethane fraction was also purified on reverse-phase HP-20 resin prior to further analyses.

GC-MS analysis

Before injection onto the GC-MS system (model Q-Mass 910, Perkin Elmer), the polar fractions were derivatised by trimethylsilylation as described previously (Risipail et al., 2005b) to enable the detection of sugars, amino acids, amines and some alkaloids. Separation was performed by a temperature gradient (180 °C for 5 min, then from 180 °C to 300 °C at 10 °C min⁻¹ and a further 5 min at 300 °C) on a capillary column coated with a non-polar liquid phase (25 m x 0.22 mm id x 0.25 µm BPX5 stationary phase, SGE Ltd.). Mass spectral data between 100 and 650 m/z were collected on a Perkin-Elmer TurboMass Gold quadrupole EI mass spectrometer.

HPLC-DAD/MS analysis

Non-polar fractions were freeze-dried and adjusted to 10 mg ml⁻¹ with 100% methanol. Then 3 µl of each fraction was injected onto an HPLC-DAD/MS (Water, UK). The separation was performed on a C₈ HPLC column (50 mm x 2.1 mm id x 3.5 µm, Waters) with a linear gradient, at a flow rate of 0.35 ml min⁻¹, starting at 90% water, 10% acetonitrile (with 0.01% trifluoroacetic acid), rising to 100% acetonitrile over 6 min, which was held for a further 6.5 min. Data of the eluted peak were collected from 200 to 600 nm and between 71 and 600 m/z by the photodiode array detector and the mass spectrometer respectively (Risipail et al., 2005b; patent EP03706757.6).

Analysis of soluble phenolics

Methanol extraction

Extraction of the soluble phenolics was performed on 100 mg of freeze-dried and ground plant tissue in 70% methanol as described previously (Risipail et al., 2005a). Soluble phenolics were concentrated and purified on a Sep Pack C₁₈ column (Sep-Pak[®] RC Cartridge, Water), eluted with 4 ml 100% ethanol and stored at 4 °C prior hydrolysis or analysis.

Acid hydrolysis of methanol extract

Acid hydrolysis was performed on 2 ml of the eluted sample obtained after purification on Sep Pack of the methanol extracts. Before hydrolysis, samples were air-dried at 50 °C using a SC-3 sample concentrator (Techne, Scientific Laboratory Supplies Ltd, UK) in the presence of 400 µl ultra-pure water. When only the water remained, 1 ml of 1 M HCl was added and the mixture obtained was incubated for 1 h at 100 °C. Samples were then left to cool to room temperature for approximately 30 min before being adjusted to pH 4.5-5.0,

1 purified on Sep Pack C₁₈ reverse-phase column and eluted with 4 ml of 100% methanol.
2 Samples were stored at 4 °C for further analysis.

4 *HPLC-PDA analysis*

5 Before analysis of the methanol extracts by HPLC on a Waters Millennium 32 system,
6 samples were dried and resuspended in 30 µl of 70% methanol (v/v) as described previously
7 (Risipail et al., 2005a). Soluble phenolics were separated by gradient HPLC onto a µ-Nova-
8 Pak reverse-phase C₁₈ (8 x 10) RCM column (Waters Ass.) with a linear methanol:acetic acid
9 (5%, v/v) gradient from 0 to 100% methanol in 50 min at a flow rate of 2 ml min⁻¹ (Robbins
10 et al., 1998). Eluting peaks were monitored with a photodiode array detector (Waters 996
11 PDA detector, Waters Ass.) at 280, 340 and 380 nm, and spectra were recorded between 240
12 and 400 nm. Data analysis were processed using an adapted processing method of Millennium
13 32 software (Robbins et al., 1998) and the UV/visible spectra of eluted peaks were matched
14 with those contained in our own libraries set up with standard and unknown compounds
15 previously detected.

17 *Quantification method and statistical tests*

18 Quantification of the phenolic peaks was carried out by estimating the area of each
19 detected peak from all wavelengths or by their relative proportion compared to the estimated
20 total phenolic level. In total, approximately 80 peaks were integrated, annotated with an
21 arbitrary number and monitored quantitatively. A few peaks were at the lowest detection
22 limit. A manual integration method was thus applied as it permits a more consistent
23 monitoring of all detected peaks including the minor ones. In addition, a few peaks were
24 completely distinct in some replicates but co-eluted in others, due to slight variations in the
25 HPLC separation. To simplify and avoid potential bias, such peaks were separately integrated

1 but subsequently quantified together, leading to a total of 48 peaks actually being monitored.
2 The quantification method did not take into account the specific response factor (R_f) of the
3 column for each compound but instead assumed an identical R_f for all ($R_f = 1$). Thus, it only
4 provided an indication of the level of each peak rather than their absolute amount. Although
5 assignment of a specific R_f for each class of compounds would have been possible, the
6 method described here was chosen due to the presence of many co-eluting peaks: the use of
7 specific R_f required purity of each peak, which could not be guaranteed using this separation
8 method. Also, it proved more accurate to quantify co-eluting peaks as a complete entity rather
9 than attempting to separate them artificially, which could have introduced error or
10 approximation in the quantification.

11
12 To analyse the significance of the quantitative changes of phenolic compounds upon
13 rhizobial inoculation, different statistical tests were performed. All statistical analyses were
14 performed with Genstat 7.1 software. Firstly, a Canonical Variate Analysis (CVA) was
15 performed to analyse the overall treatment effect. The significance of the potential differences
16 was checked by analysis of variance. Whenever the ANOVA were statistically significant (p
17 ≤ 0.05), a Student-Newman-Keuls (SNK) test was performed. Nine replicates were used for
18 each treatment in a randomised design.

20 RESULTS

21
22 Assessment of plant-rhizobial interaction at 1 week after inoculation showed that only
23 those roots inoculated with *M. loti* NZP 2037, the nodulating wild-type strain, had distinct and
24 numerous symbiotic root hair deformations (Table 1). All control samples were characterised
25 by straight root hairs with rare deformations observed in only 5.7% of the total fields of view

1 monitored (Table 1). In these samples, no complete curling was observed but rather a hook or
2 a re-direction of the root hair tip that is unlikely to be related to a symbiotic response. By
3 contrast, *L. japonicus* roots inoculated with the wild-type *M. loti* strain, NZP 2037, showed
4 numerous curled root hairs, with 30.3% of the monitored fields of view showing root hair
5 curling. In parallel to these curled hairs, more root hair swelling/branching was detected in
6 response to this strain compared to non-inoculated roots or roots inoculated with the *NodC*⁻
7 mutant strain, PN4047 (Table 1). In addition, 2 nodule primordia were detected in the root
8 sections sampled (data not shown). The appearance of *L. japonicus* roots inoculated with the
9 non-nodulating *NodC*⁻ mutant PN 4047 resembled more closely the non-inoculated roots than
10 those inoculated with the wild-type strain (Table 1). Indeed, root hair deformations were only
11 occasionally detected with 9.9% of total monitored fields of view containing a deformed root
12 hair. In addition, no completely curled root hairs were observed in these samples.

13
14 Analysis of chromatograms of the different fractions generated by broad secondary
15 metabolite profiling yielded a total of 300-500 molecules. There were no qualitative
16 differences between treatments, but several novel alkaloids and nitrogenous compounds were
17 detected, some of which are being identified. Similarly, no qualitative changes were detected
18 in the more targeted analysis of the soluble phenolics (Fig. 1), even after hydrolysis of the
19 glycosidic bond (data not shown). However, canonical variate analysis of the whole dataset
20 separated the secondary metabolite content of plants inoculated with the wild-type *M. loti*
21 strain from those plants either non-inoculated or inoculated with the *NodC*⁻ mutant (data not
22 shown), suggesting interesting differences between these treatments.

23
24 Before examining each peak individually, the quantitative changes induced by the
25 presence of *M. loti* close to or within *L. japonicus* roots were assessed on the basis of the total

phenolic content. Surprisingly, the total phenolic content was not significantly affected by inoculation (data not shown), which indicated that the quantitative changes induced by the rhizobium involved redistribution of the phenolic components within a pre-existing pool. To test this hypothesis, all detected peaks were grouped according to their phenolic class based on their UV spectra characteristics. In this way, most peaks were identified as flavonol glycosides, flavanones, coumestans, chalcones, pterocarpenes or phenolic precursors. A few peaks could not be assigned in any of these classes, either because they were composed of co-eluting peaks (referred to as ‘mixture of compounds’ in Table 2) or because their UV spectra did not correspond to any known group (referred to as ‘undetermined class’ in Table 2), and were re-grouped within another class called ‘Others’. Comparison of each phenolic class according to inoculation treatment revealed that most classes were not quantitatively changed by the presence of either rhizobial strain (Fig. 2). However, a slight, but highly significant ($p < 0.001$), increase in the flavanone content was observed after 1 week inoculation with the wild-type *M. loti* strain, NZP 2037 (Fig. 2).

The one-way ANOVA indicated that 14 out of the 80 detected peaks or groups of peaks were significantly affected ($p \leq 0.05$) at 1 week post-inoculation with either rhizobial strain compared to non-inoculated plants and, thus, were likely to play a role during the early symbiosis events. The affected compounds were evenly distributed across the complete phenolic profile at both quantitative and structural levels (Fig. 3; Table 2). Although many peaks either co-eluted or were composed of a mixture of molecules with diverse natures, observation of their typical UV spectra characteristics, shown in Fig. 3, indicated that components from all classes of phenolics were represented, with the exception of pterocarpenes. For instance, according to their UV spectra, the co-eluting peaks P12/14 and P15/17 corresponded to various flavonol glycosides deriving from kaempferol whereas P4/7

1 was composed of a phenolic precursor and one or two flavonol glycosides deriving from
2 quercetin (Fig. 3). Table 2 summarises the characteristics of the phenolic peaks or group of
3 co-eluting peaks showing significant differences between non-inoculated control plants and
4 plants inoculated with the *NodC*⁻ mutant or the wild-type strain of *M. loti*.

5
6 The levels of several compounds, or groups of peaks, did not significantly differ
7 between plants inoculated with either of the strains but were significantly altered compared
8 with non-inoculated plants. Thus, the phenolic precursors P43 and P54/56, the unknown
9 compound P79 and the chalcone P81 significantly increased in plants inoculated with both
10 strains compared with the non-inoculated plants, whereas the flavonol glycosides P4/7,
11 P12/14 and P15/17, the unknown compound P34 and the phenolic precursor P51 decreased in
12 inoculated plants. The modulation of these compounds by both *M. loti* strains suggests they
13 are involved in a symbiosis-independent recognition of *M. loti* by the plants.

14
15 Four compounds significantly increased their concentration in response to the wild-
16 type *M. loti* strain compared to the *NodC*⁻ mutant or the non-inoculated plants. These were
17 P32, which appears to be a coumestan, presumably deriving from coumestrol, P59, which was
18 composed by a mixture of molecules and could not be assigned to any particular class, and the
19 flavanones P60 and P62. The exclusive modulation of these compounds by the wild-type
20 strain suggests a possible role in nodulation.

21
22 Finally the level of P2 was significantly increased only in those plants inoculated with
23 the *NodC*⁻ mutant strain, compared with non-inoculated plants. Close examination of P2
24 during the integration process indicated that, among the different molecules present in this
25 peak, one flavonol glycoside and a coumarin were present, relating this peak to defence

processes. On the other hand, no pterocarpene components were affected by the inoculation. Detection of pterocarpenes was highly inconsistent between replicates of the same treatment. It appeared, therefore, unlikely that this class, generally considered to consist of defence molecules, was linked with nodulation. It was also noteworthy that the isoflavans vestitol and sativan, the 2 *Lotus* phytoalexins, were not consistently observed and, if detected, appeared in higher amounts in non-inoculated control roots rather than in inoculated samples (data not shown).

DISCUSSION

Endogenous secondary metabolites appear to be crucial signal molecules during the earliest stages of the rhizobia-legume interaction, when they participate in nodule inception. Thus, analysis of secondary metabolite changes in *L. japonicus* roots following inoculation with *M. loti* focussed on the earliest stages of nodule initiation. Two different strains of *M. loti* with similar genetic backgrounds but differing in their ability to produce Nod factor were used to differentiate between the plant response to the presence of rhizobia and specific changes induced during nodule initiation. This offered an opportunity to detect putative metabolic changes due to rhizobia recognition and/or Nod factor signalling.

This study showed that *L. japonicus* responded to rhizobia by quantitative changes in pre-existing phenolic components rather than by synthesis of new compounds. Our observations confirmed the limited effect of compatible rhizobia on endogenous flavonoid content previously reported in *Vicia sativa* (Recourt et al., 1991), *Medicago sativa* (Tiller et al., 1994), *Trifolium subterraneum* (Lawson et al., 1996) and *T. pratense* (Edwards et al., 1997). However, while these reports showed only quantitative changes in a very limited

number of phenolic components (Tiller et al., 1994; Lawson et al., 1996; Edwards et al., 1997), the present investigation revealed 14 peaks that were significantly and specifically affected by *M. loti* regardless of the production of Nod factor. In contrast to many studies that showed an increase in total flavonoid content associated with a local increase in both flavonoid enzyme activity and gene expression (Estabrook and Senguptagopalan, 1991; Recourt et al., 1991; Lawson et al., 1996; McKhann et al., 1997; Lohar et al., 2006), we did not find any significant changes in total phenolics. This surprising result led us to speculate that the changes we detected may be the result of inter-conversions of phenolic components between pre-existing phenolic pools rather than induction of *de novo* synthesis of phenolic compounds. However, simultaneous analysis of flavonoid accumulation and phenylpropanoid gene expression would be required to confirm this.

A comparison of endogenous root phenolics in plants challenged with wild-type and *NodC*⁻ mutants of *M. loti* clearly showed that the plant response 1 week after infection was broadly similar but varied in detail according to the infecting strain. Nine out of the 14 affected peaks tended to accumulate or decrease in response to both wild-type or *NodC*⁻ mutant strains. This suggests that most changes in root secondary metabolite profiles detected at this stage are independent of Nod factor signalling. Nod factor is required for rhizobial entry, infection thread initiation and nodule formation (Riely et al., 2004; Oldroyd and Downie, 2008). However, other rhizobial factors including β -glycans, exopolysaccharides (EPS) and lipopolysaccharides (LPS) are also known to play important roles at the earliest stages of the interaction by mediating root attachment, participating in infection thread formation and avoiding plant defence reactions in indeterminate nodules (Jones et al., 2007). In determinate nodulating hosts, the role of these rhizobial surface molecules is still unclear, although the ability to form efficient nodules of *M. loti* mutants deficient in LPS and/or cyclic

glycans but not EPS, was reduced (Hotter and Scott, 1991; Lepeck et al., 2002; Turska-Szewczuk et al., 2009). Thus, the common changes in the level of the phenolic compounds after inoculation with both *M. loti* strains detected here may be induced by the plant recognition of these other rhizobial factors. Interestingly, this Nod factor-independent recognition led to accumulation of precursors such as flavanones and chalcones, while relative levels of end-products such as flavonol glycosides decreased. Flavanones and chalcones are well-known *nod* gene inducers of *Rhizobium leguminosarum* and *Sinorhizobium meliloti* (Maxwell et al., 1989; Zaat et al., 1989); however, they were inefficient at stimulating synthesis of Nod factor in *M. loti* for which only tetronic acid has been shown to be active to date (López-lara et al., 1995; Gagnon and Ibrahim, 1998; Steele et al., 1999). Thus, the biological significance of the accumulation of precursors after recognition of *M. loti* by *L. japonicus* is still not clear. However, in accordance with the plant defence suppresser function of rhizobial LPS and EPS (Tellström et al., 2007; Jones et al., 2008), it is unlikely that their accumulation is associated with plant defence since the characteristic *L. japonicus* phytoalexins, i.e., the isoflavans vestitol and sativan (Robbins et al., 1995) were not detected.

The remaining 5 peaks were found to be modulated only in response to one of the strains. Interestingly, most of them (4/5) were only, or were more strongly, accumulated in response to the Nod factor-producing strain, NZP 2037 (Table 2), which is also the strain inducing the most drastic changes in root hair morphology (Table 1). Nod factor activates a complex signalling pathway in host roots and induces coordinated events in both root hair and root cortical cells, ultimately leading to nodule formation (Riely et al., 2004; Oldroyd and Downie, 2008). Nod factor was also shown to induce changes in root phenolic content. In the root hair, Nod factor was shown to activate flavonoid gene expression (McKhann et al., 1997)

1 and the subsequent production and exudation of *nod* gene inducer flavonoids (Recourt et al.,
2 1991). Simultaneously, localised induction of flavonoid gene expression and subsequent
3 accumulation of flavonoids were detected in response to Nod factor in cortical cells preceding
4 auxin transport inhibition and nodule primordia formation (Djordjevic et al., 1997; Mathesius
5 et al., 1998a, b). Here, the levels of four peaks: two flavanones, one coumestan and an
6 undetermined compound were increased in response to wild-type strain of *M. loti* 7 days after
7 inoculation (Table 2). This stage of the interaction corresponded to the maximum *nod* gene
8 inducing activity of *Vicia sativa* root exudates (Recourt et al., 1991). Thus, some of the
9 changes observed in *L. japonicus* root profile may be related to this increase in *nod* gene
10 inducing activity. The role of coumestan in the *L. japonicus*-*M. loti* interaction is not known.
11 However, isoflavonoids such as coumestans and isoflavones have been described as the
12 potent *nod* gene inducers from determinate nodulating species such as soybean (Kape et al.,
13 1992). While isoflavones were shown not to induce *M. loti nod* gene expression (López-Lara
14 et al., 1995), coumestans have not been tested. It may thus be possible that the coumestan
15 detected here acts as *nod* gene inducers in this rhizobial species also. Alternatively, in
16 addition to their role as a *nod* gene inducer from indeterminate nodulating species, flavanones
17 were also shown to be potent auxin transport inhibitors (Maxwell et al., 1989; Zaat et al.,
18 1989; Mathesius et al., 1998b). Hence, accumulation of these compounds may have been
19 induced in *L. japonicus* roots to mediate Nod factor signalling in cortical cells allowing auxin
20 recruitment. Determination of the exact roles of these accumulated compounds requires
21 further study.

22
23 Finally, one of the peaks, P2, was significantly increased in roots inoculated with the
24 *NodC*⁻ mutant strain. Examination of this peak indicated the presence of one flavonol
25 glycoside and a coumarin, relating it to defence processes. This suggests that *NodC*⁻ strain,

1 lacking Nod factor synthesis ability, was not able to completely suppress the host defence
2 processes previously reported during nodulation and symbiosis by compatible rhizobia (Shaw
3 and Long, 2003).

4
5 The present study revealed many quantitative changes in the soluble phenolic profile
6 of *L. japonicus* roots in response to its symbiont *M. loti*. While Nod factor signalling
7 specifically led to increases in 4 phenolic compounds, most of the changes observed might be
8 induced by *M. loti* surface polysaccharides such as EPS and LPS, since they are detected in
9 response to both wild-type and *NodC*⁻ mutant *M. loti* strains. Work is in progress to purify and
10 characterise the three components specifically induced in roots inoculated with the wild-type
11 strain to determine their specific involvement in the *L. japonicus*-*Mesorhizobium* interaction.

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19 20 LITERATURE CITED

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18 PATENTS

- 19 Process for extracting polar phytochemicals (2002) EP 03706756.8
- 20 Method for monitoring the quality of a herbal medicine (2002) EP03706757.6

TABLES

Table 1. Evaluation of root hair deformation of *L. japonicus* roots at 1 week post-inoculation with 2 *M. loti* strains as compared to non-inoculated controls. Assessment was performed by counting the number of typical symbiotic deformations on root hairs observed from 100 random fields of view under light microscope at X20 magnification.

Treatment	Root hair deformation (%)			
	Branching	Swelling	Curling	Total
Non-inoculated Control	3.3	1.2	1.2	5.7
+ <i>NodC</i> ⁻ mutant strain PN 4047	3.0	3.0	3.9	9.9
+ wild-type strain NZP 2037	12.4	7.1	30.3	49.8

Table 2: Characteristics of the phenolic peaks or group of co-eluting peaks significantly affected 1 week after inoculation with *M. loti* NZP 2037 (wild-type) and PN 4047 (*NodC*⁻) mutant strains.

Peaks	Retention Time	Phenolic class ^a	Relative Proportion (%) ^{b,c}	Quantitative Importance ^d	Changes (%)	
					NZP 2037	PN 4047
P2	13.6	M - U	1.6 (0.3)	I	---	56.1
P4/7	16.5	M - P/FI	4.0 (0.6)	I	-23.3	-27.9
P12/14	19.0	FI	3.9 (0.4)	I	-14.8	-23.7
P15/17	19.6	FI	0.7 (0.09)	m	-30.5	-30.8
P32	25.4	Co	0.2 (0.02)	m	58.9	17.6
P34	27.0	U	0.1 (0.03)	m	-37.9	-65.3
P43	8.4	M - P	0.9 (0.1)	m	40.4	27.5
P51	15.2	P	8.4 (1.1)	M	-11.6	-24.4
P54/56	32.0	P	5.4 (0.5)	M	23.8	28.0
P59	34.1	M - U	0.5 (0.03)	m	117.9	---
P60	37.2	Fa	2.9 (0.2)	I	27.4	---
P62	39.5	Fa	0.3 (0.03)	m	21.2	---
P79	40.4	M - U	0.1 (0.02)	m	65.0	24.2
P81	43.5	Ch	0.05 (0.005)	m	14.4	14.8

^a Classification performed according to UV spectra. M: mixture of compounds, U: undetermined class, P: phenolic precursors, FI: flavonols, Fa: flavanones, Co: coumestans, Ch: chalcones.

^b Relative importance of the phenolic peak in relation to total phenolics in non-inoculated roots.

^c Values in brackets correspond to standard error for n = 9.

^d Quantitative discrimination of compounds as M: major components (>5% total phenolics), I: intermediate components (between 1 and 5% of total phenolics) and m: minor components (< 1% of total phenolics).

^e Dashes represent differences not statistically significant (p > 0.05).

FIGURE LEGEND

Figure 1. Rhizobial influence on the polar secondary metabolite profile of *L. japonicus* root extracts. Comparison of HPLC-PDA chromatograms obtained from the soluble phenolic fraction obtained after 70% methanol extraction of approximately 100 mg freeze-dried *L. japonicus* roots kept non-inoculated or 1 week after inoculation with *M. loti* wild-type N2P 2037 or non-nodulating, *NodC*⁻ mutant, PN 4047. Chromatograms were obtained after separation of the crude methanol extract on a C₁₈ reverse-phase column using a gradient of 0 to 100% methanol in 50 min and monitored by the PDA detector at 280, 340 and 380 nm. Quantitative differences between treatments were detected at all three monitored wavelengths. To simplify the figure, only one representative chromatogram obtained at 340 nm for each treatment is represented.

Figure 2. Rhizobial influence on the phenolic class content of *L. japonicus* roots 1 week after inoculation. Changes were determined by comparing root phenolic content from *L. japonicus* maintained non-inoculated (■) or inoculated with either *M. loti* strain N2P 2037 (□), a wild-type nodulating strain, or *M. loti* strain PN 4047 (□), a non-nodulating *NodC*⁻ mutant. Each detected peak from the HPLC-PDA chromatograms was assigned to a particular class according to its UV spectra characteristics. Quantitative estimation of each identified phenolic class was then assessed by summing the area of each individual peak assigned to each class. The different detected classes are P: phenolic precursors, Fl: flavonols, Fa: flavanones, O: others, Co: coumestans, Ch: chalcones and Pt: pterocarpenes. 'Others' represents peaks from either undetermined classes or co-eluting compounds showing unreadable UV spectra. Horizontal bars represent standard error for n = 9.

1
2 **Figure 3.** UV spectra characteristics of interesting phenolic peaks from *L. japonicus* roots, 1
3 week after inoculation with *M. loti* strains. Numbering was assigned arbitrarily for each
4 monitored peak from HPLC-PDA chromatograms. Black numbers represent unique peaks of
5 mixed or pure compounds whereas underlined, double underlined or italicised numbers
6 indicated groups of co-eluting peaks quantified together. Each underlined, double underlined
7 or italicised peak belong to the same co-eluting group. UV spectra were collected from 240 to
8 400 nm by the PDA detector during HPLC separation.

9

Figure 1

Figure 2

Figure 3