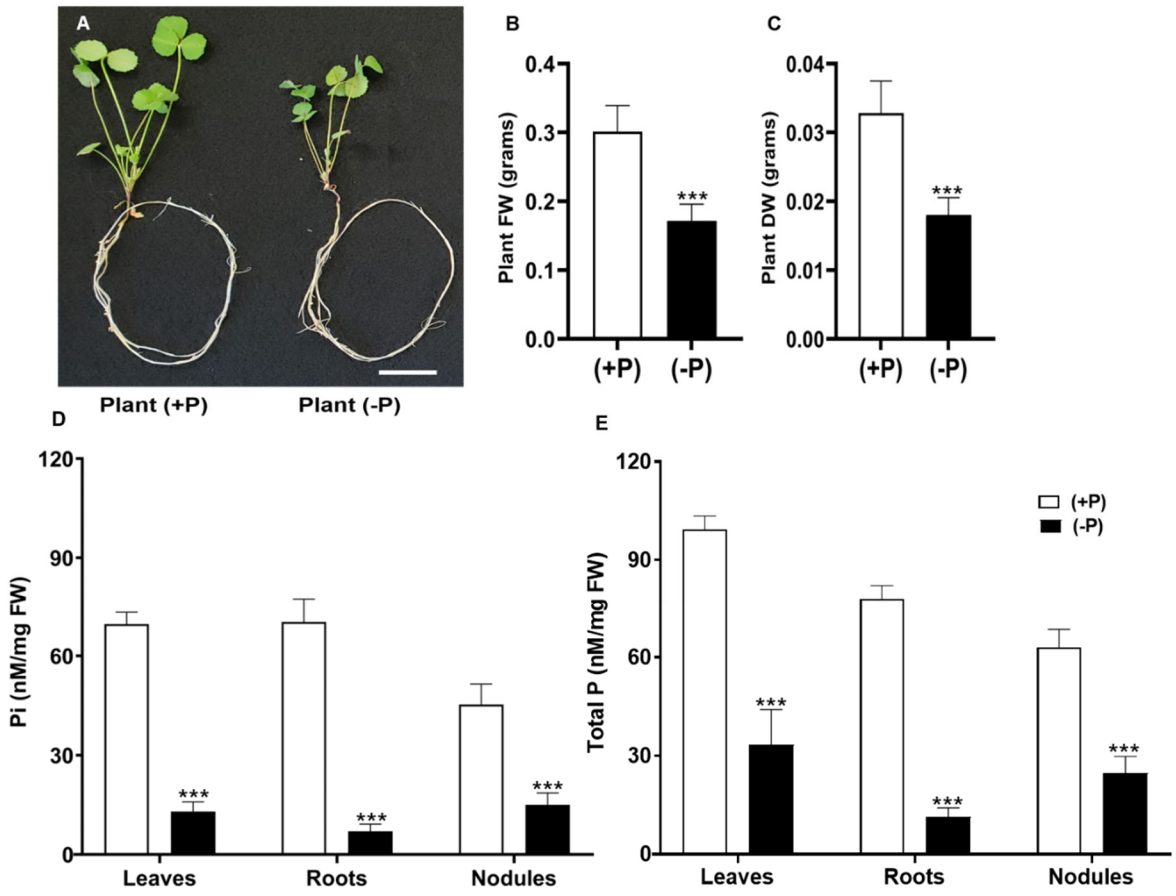
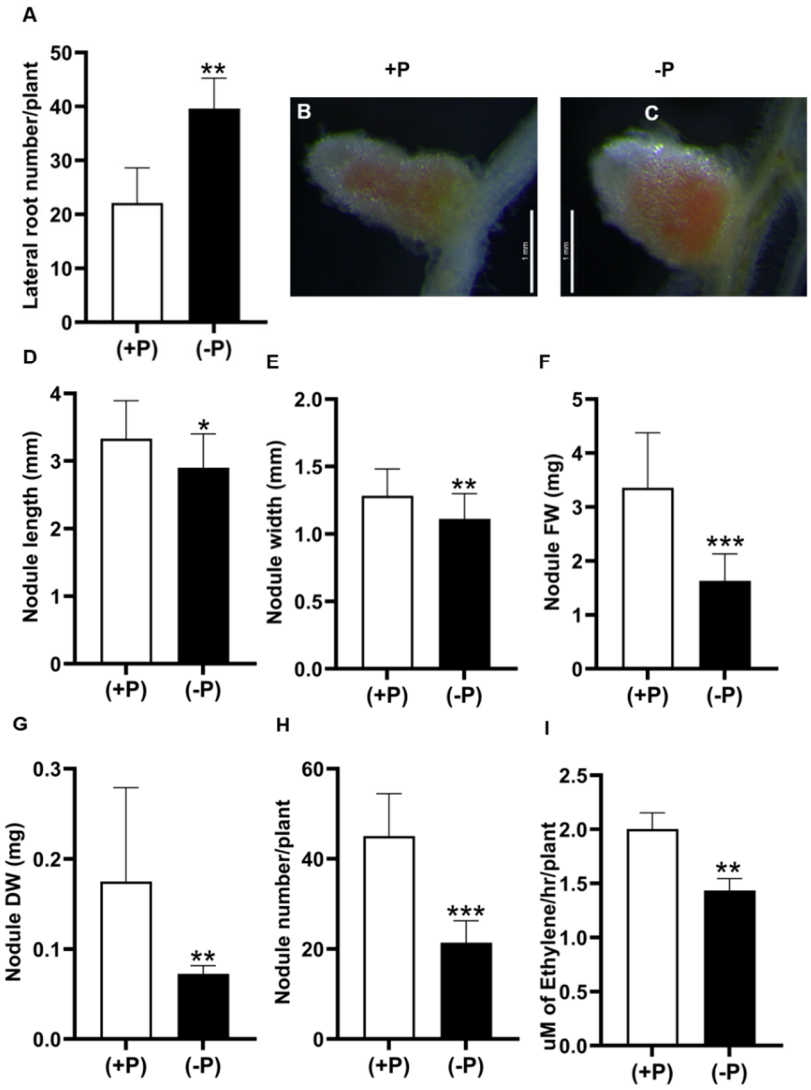
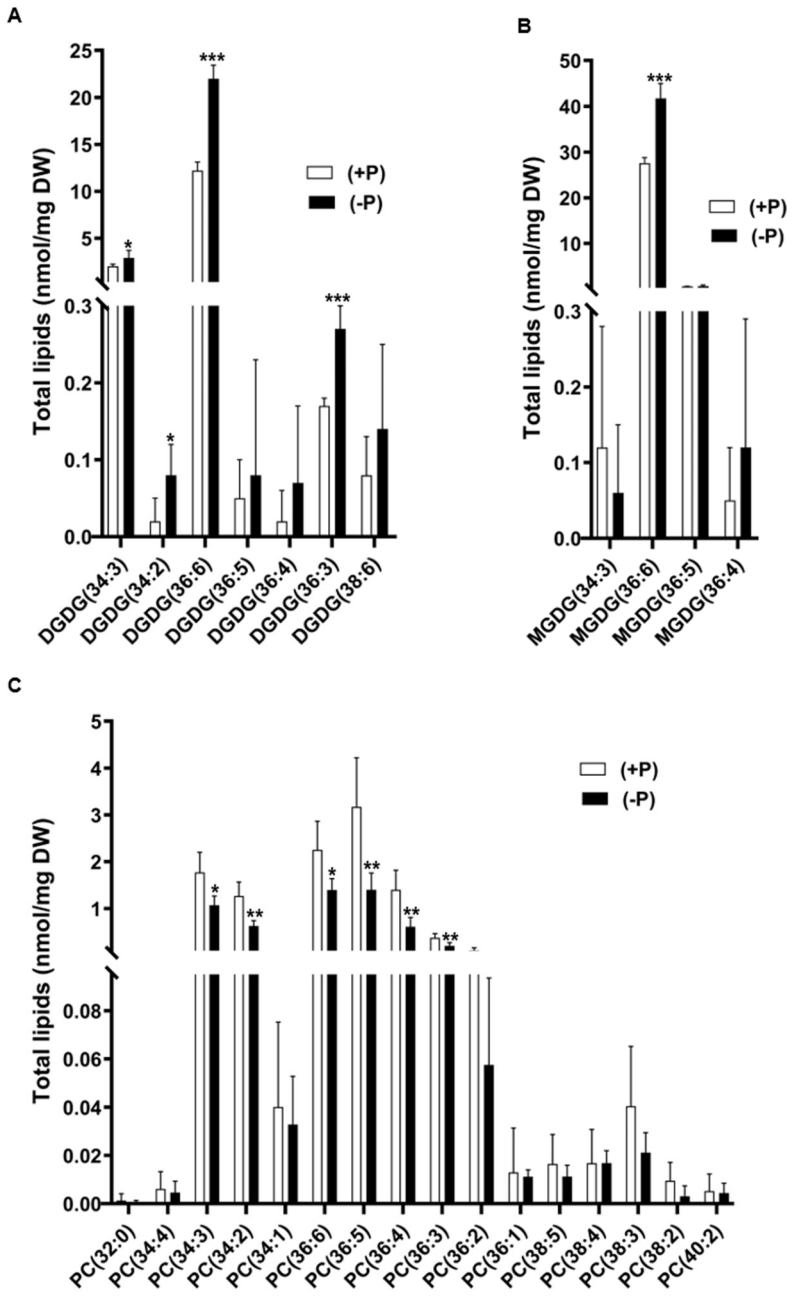


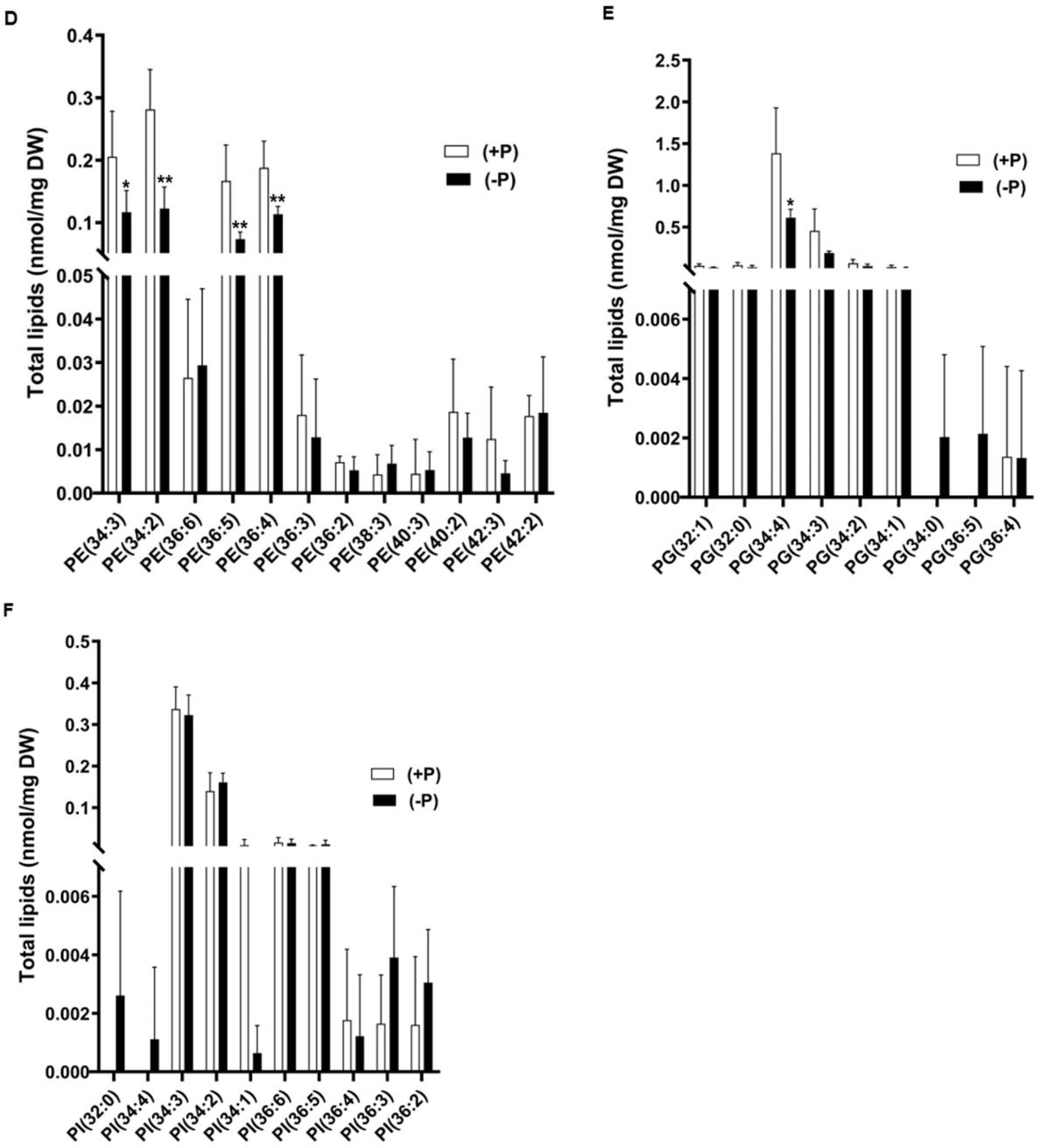
Supplemental Figure S1. Characteristics of *Medicago truncatula* in phosphorus (P) depleted conditions. A. Appearance of wild-type R108 under sufficient phosphorus (+P) and absence of phosphorus (–P) after 28 days post inoculation (DPI) with *Sinorhizobium meliloti* Rm 41. Scale bar 2cm; **B.** Average Fresh weight (FW); **C.** Average Dry weight (DW). N = 15 for B and C. **D.** Inorganic Phosphate (Pi); **E.** Total P in leaves, roots and nodules of plants grown under +P and –P conditions, N=6. The values are mean ± SD. Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; ***, P ≤ 0.001.



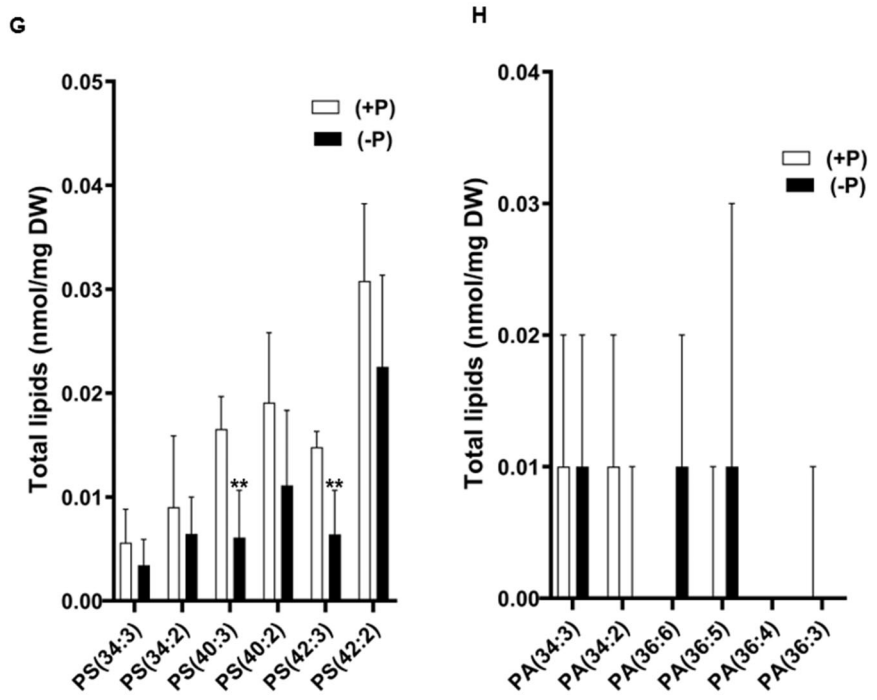
Supplemental Figure S2. Characteristics of nodulated root system of *Medicago truncatula* R108 in phosphorus (P) depleted conditions. A. Lateral root number; **B** and **C.** Images of nodules from plants grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions respectively, scale bar - 1mm; **D.** Nodule length; **E.** Nodule width; **F.** Nodule fresh weight (FW); **G.** Nodule dry weight (DW); **H.** Nodule number. N = 15 for figures A, D, E, F, G and H. **I.** Acetylene reduction activity assayed using whole plant 28 dpi with *Sinorhizobium meliloti* Rm41; N=5. The values are mean ± SD. Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



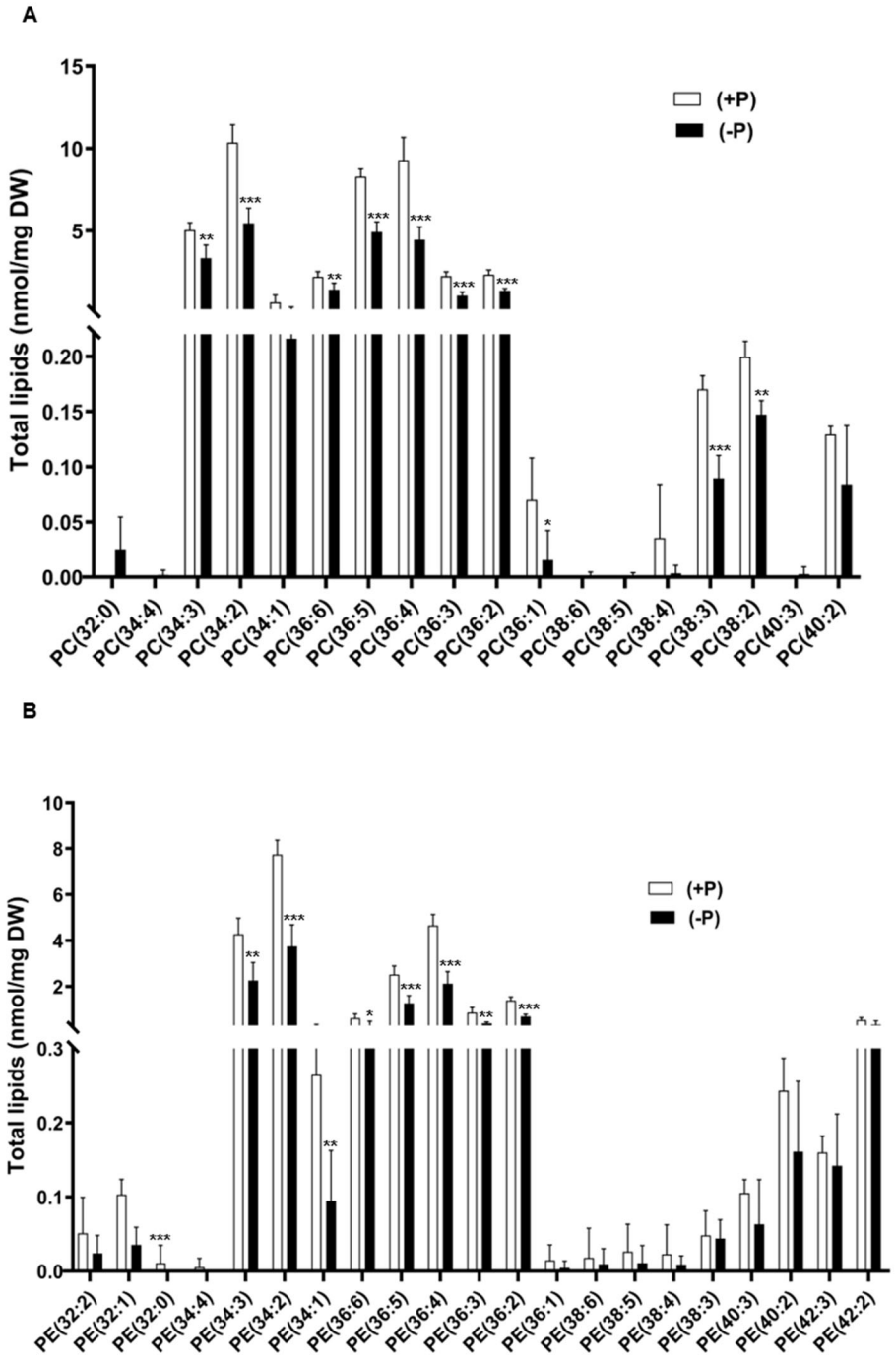
Supplemental Figure S3. Total polar glycerolipids from leaves of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium meliloti* Rm41. **A.** Digalactosyldiacylglycerol (DGDG); **B.** Monogalactosyldiacylglycerol (MGDG); **C.** Phosphatidylcholine (PC). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with standards. The values are mean ± SD (N=5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



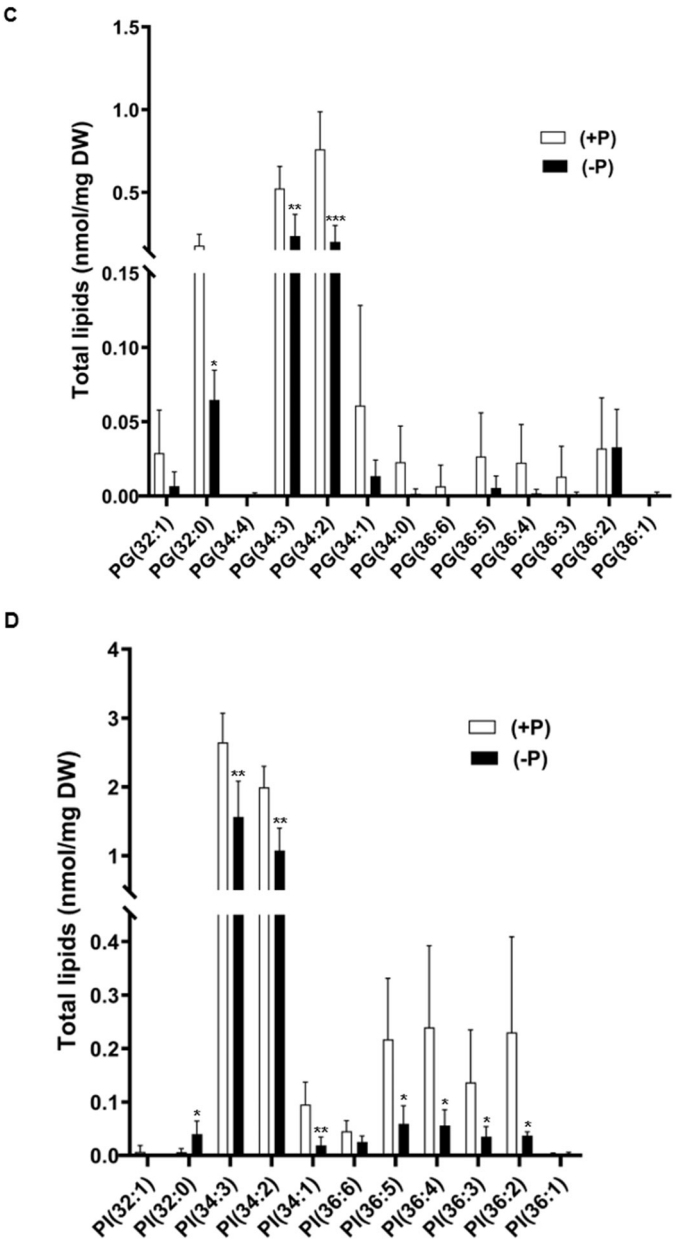
Supplemental Figure S3. Total polar glycerolipids from leaves of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium meliloti* Rm41. **D.** Phosphatidylethanolamine (PE); **E.** Phosphatidylglycerol (PG); **F.** Phosphatidylinositol (PI). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.



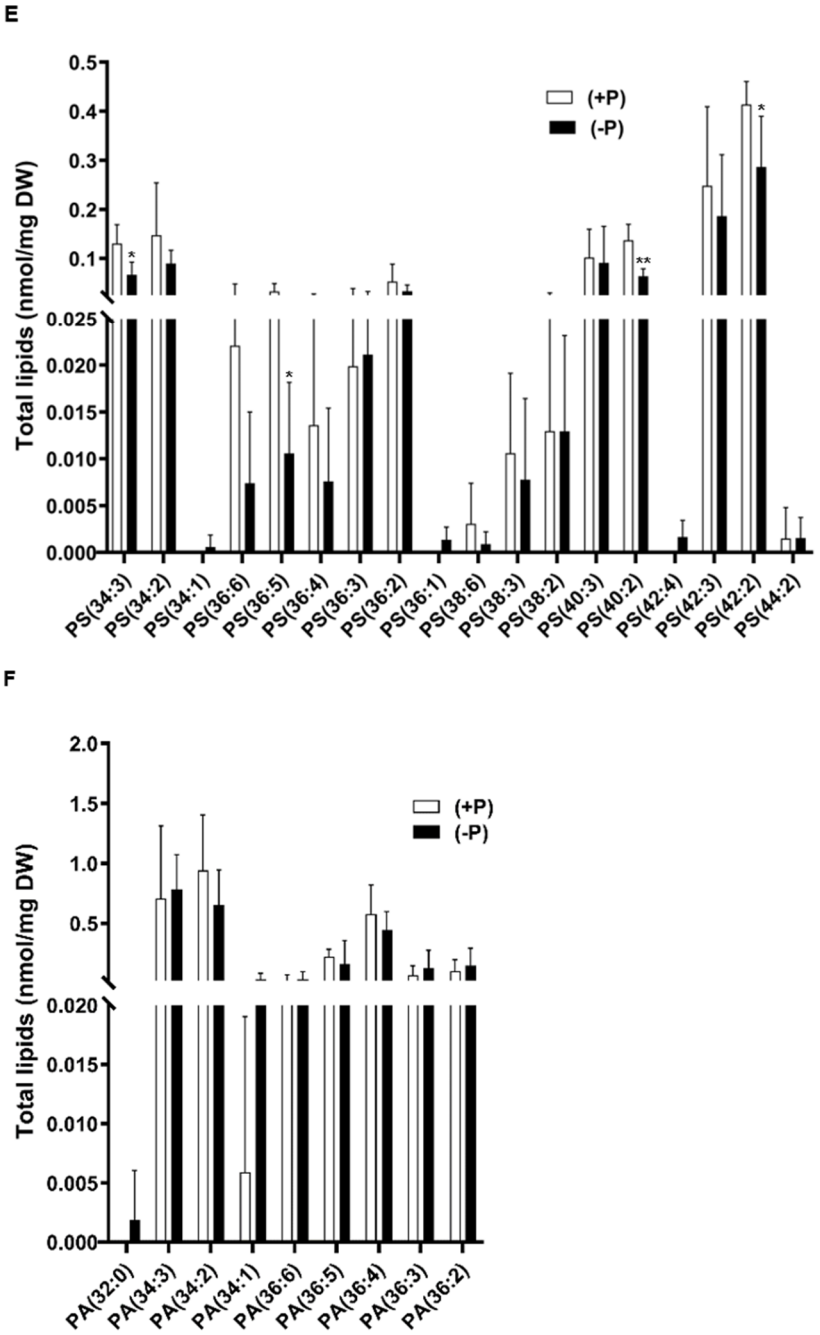
Supplemental Figure S3. Total polar glycerolipids from leaves of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium meliloti* Rm41. **G.** Phosphatidylserine (PS); **H.** Phosphatidic acid (PA). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



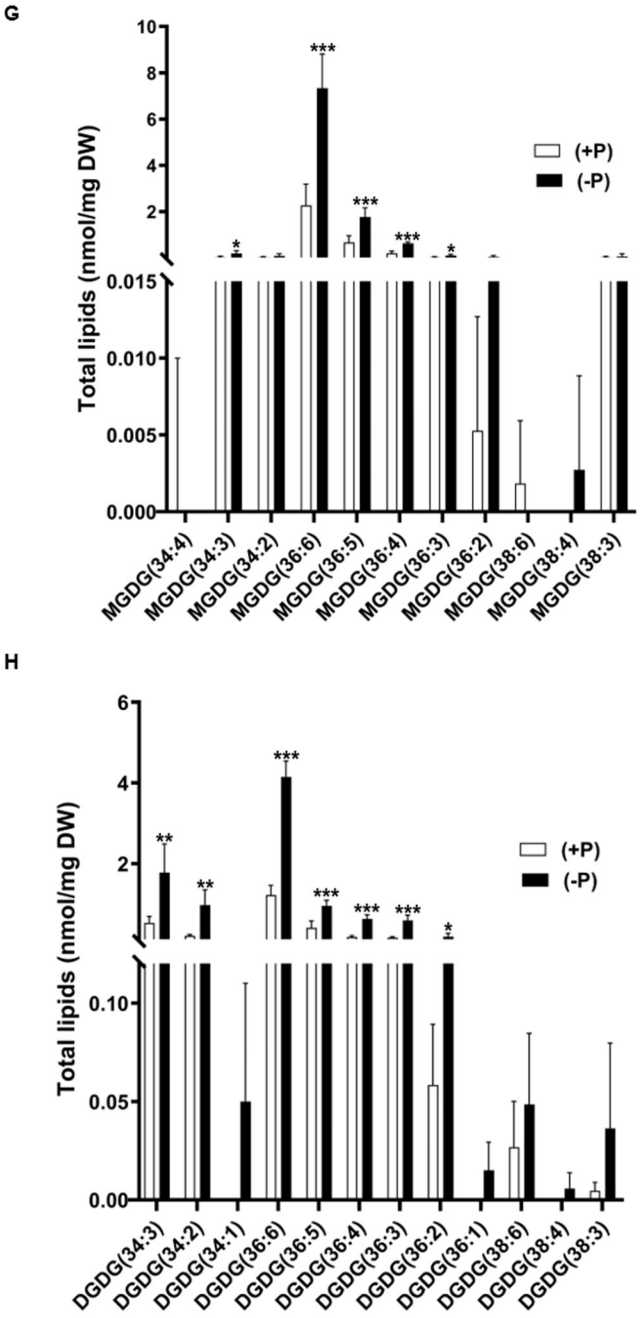
Supplemental Figure S4. Total polar glycerolipids from roots of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium meliloti* Rm41. **A.** Phosphatidylcholine (PC); **B.** Phosphatidylethanolamine (PE). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.



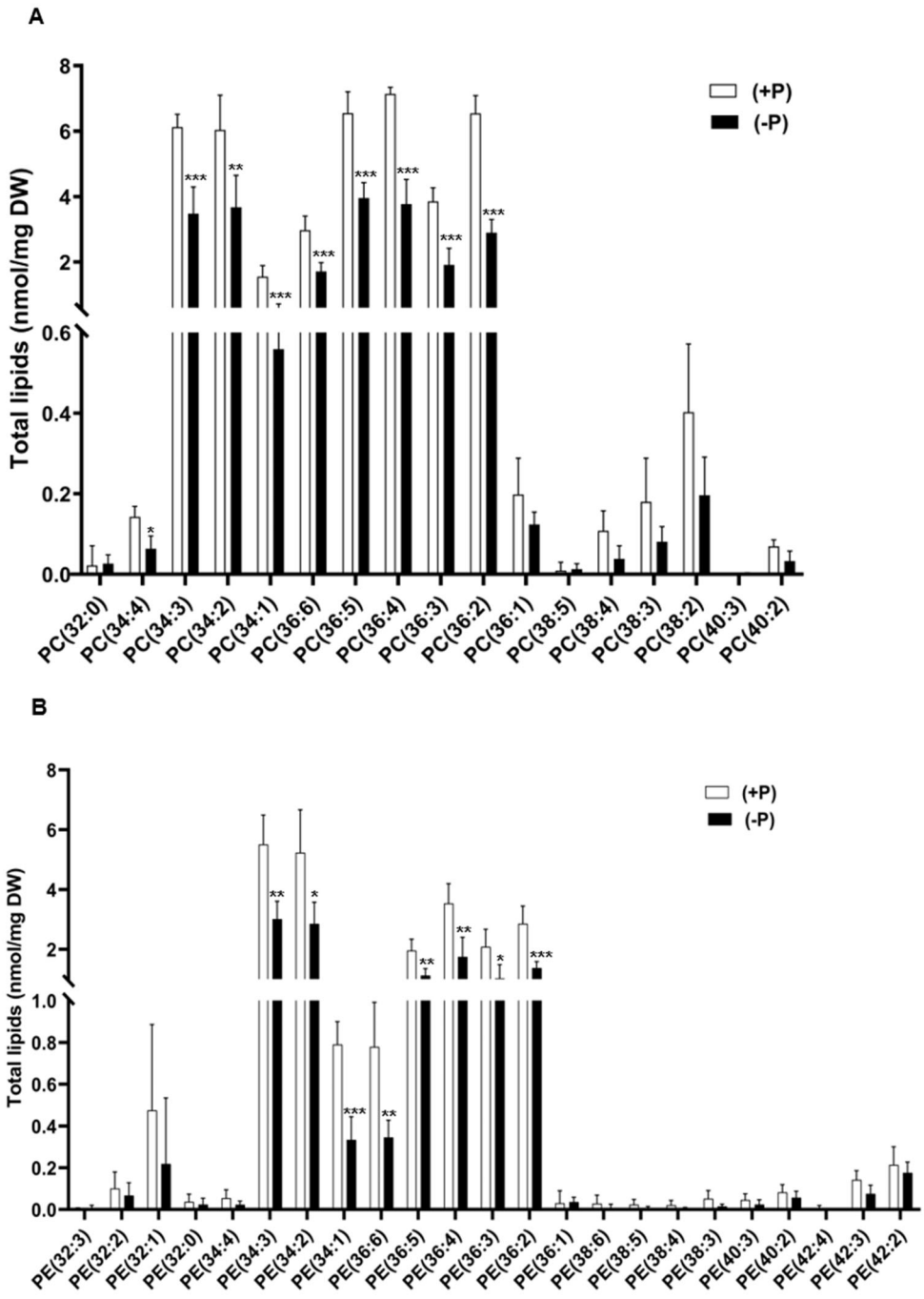
Supplemental Figure S4. Total polar glycerolipids from roots of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium meliloti* Rm41. **C.** Phosphatidylglycerol (PG); **D.** Phosphatidylinositol (PI). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.



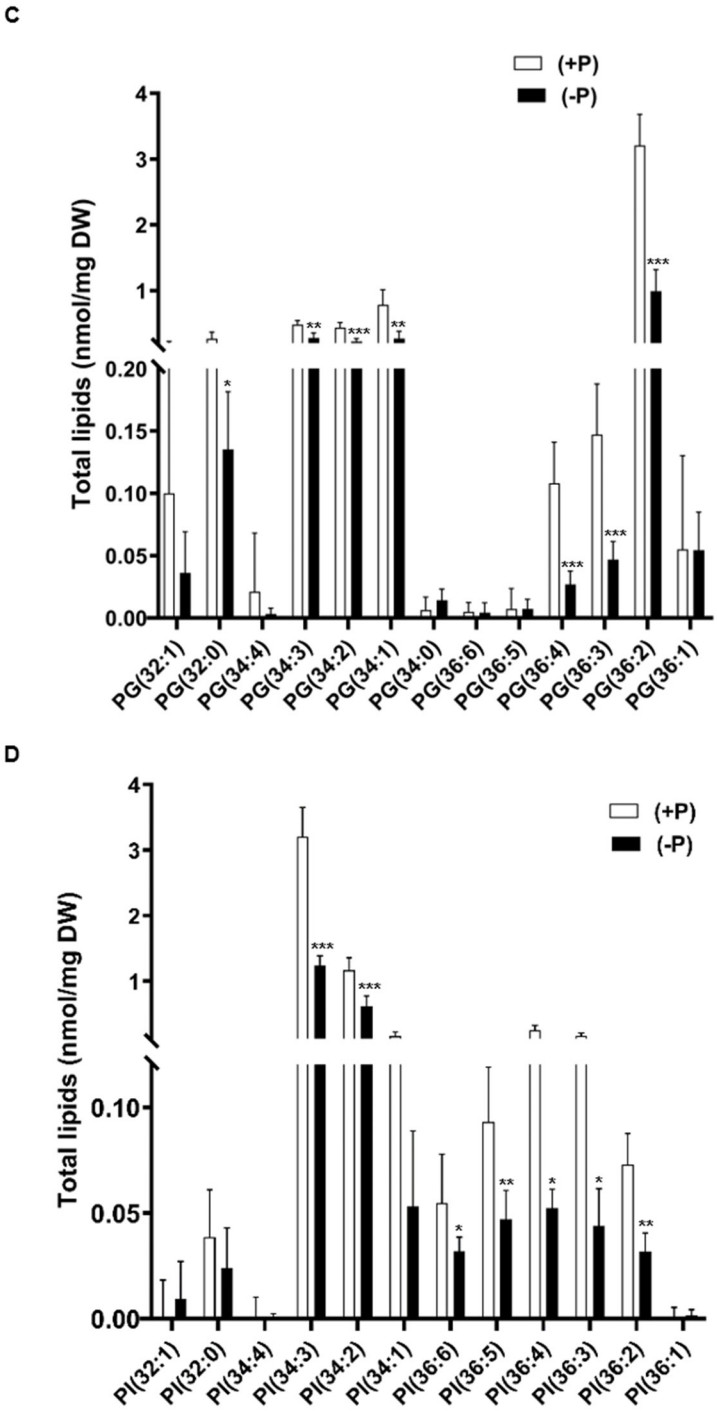
Supplemental Figure S4. Total polar glycerolipids from roots of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium meliloti* Rm41. **E.** Phosphatidylserine (PS); **F.** Phosphatidic acid (PA). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.



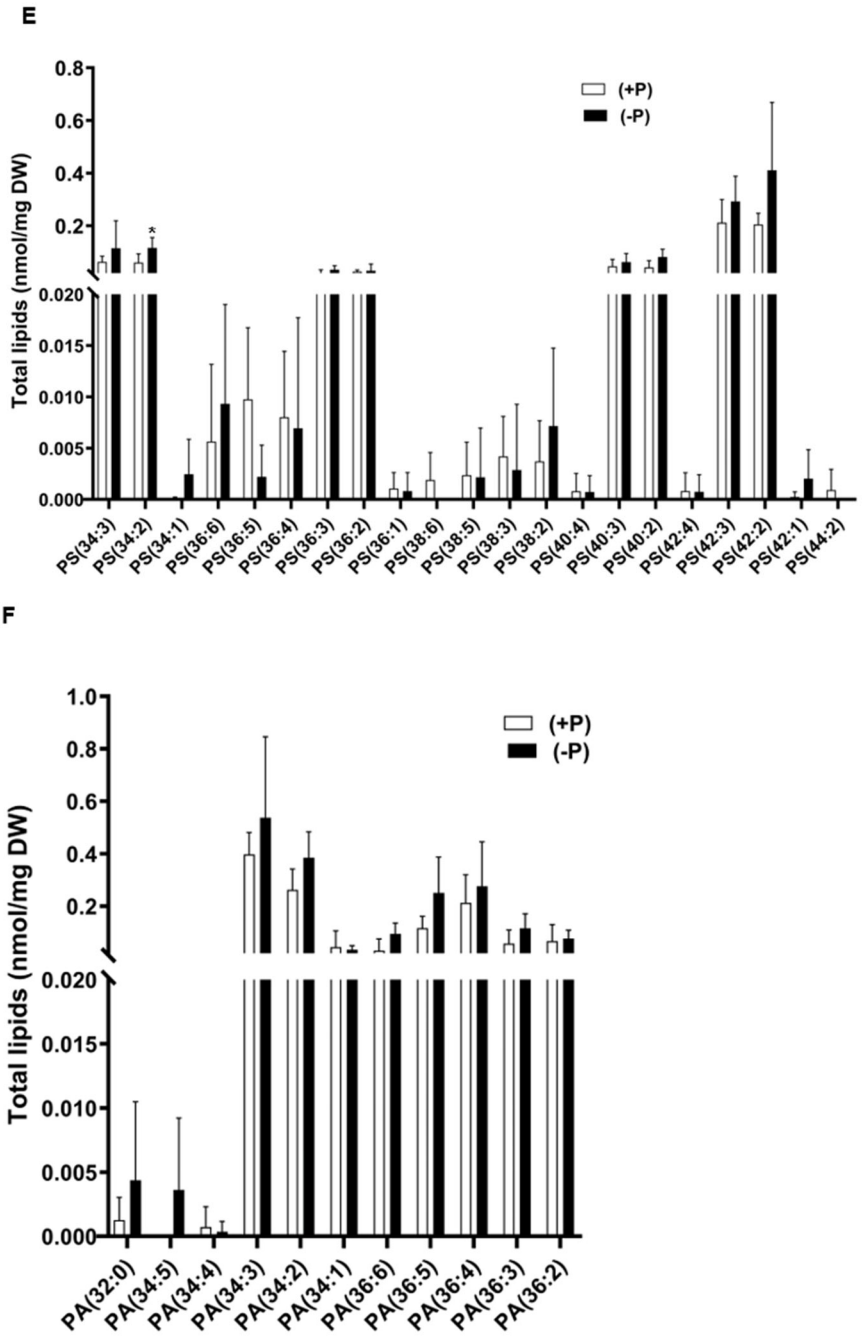
Supplemental Figure S4. Total polar glycerolipids from roots of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium meliloti* Rm41. **G.** Monogalactosyldiacylglycerol (MGDG); **H.** Digalactosyldiacylglycerol (DGDG). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.



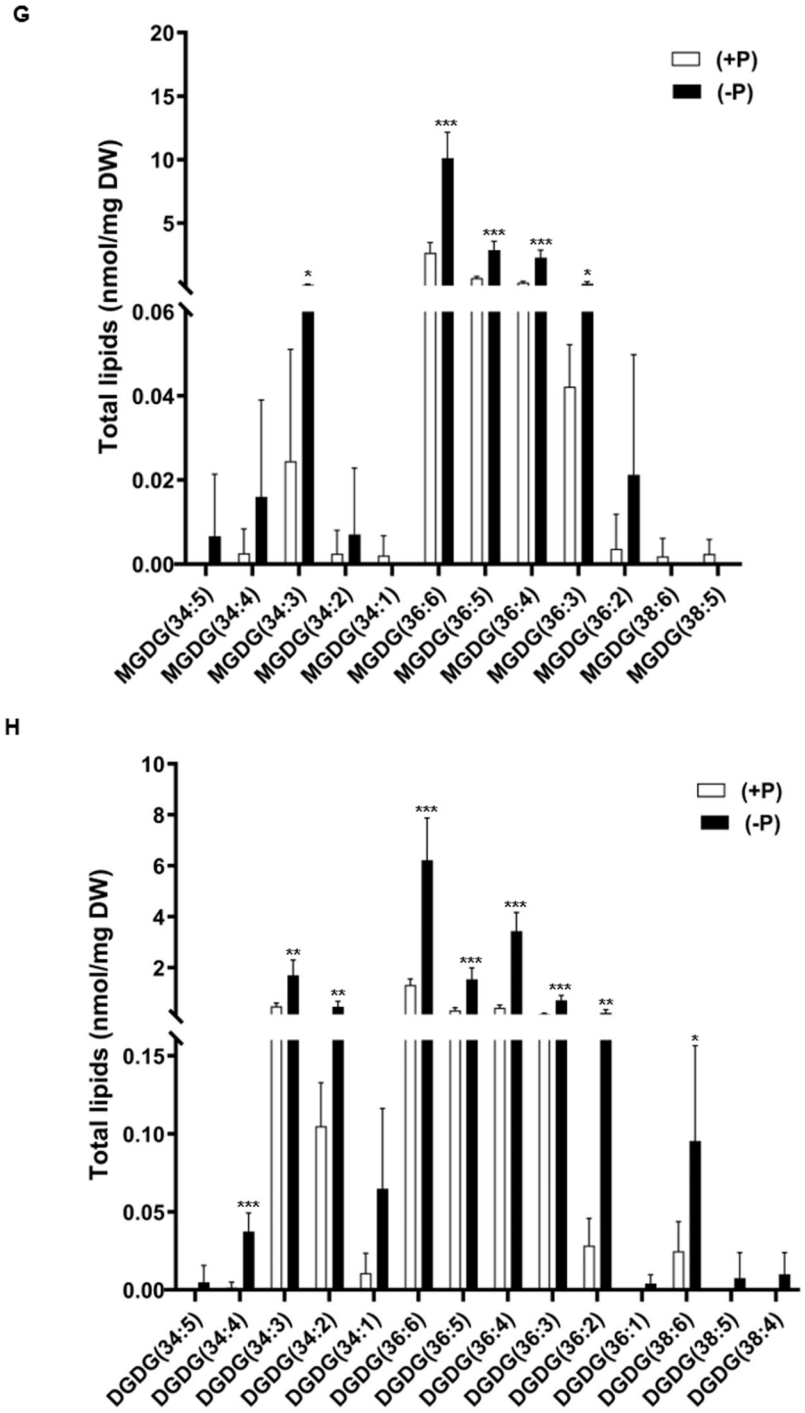
Supplemental Figure S5. Total polar glycerolipids from nodules of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium. meliloti* Rm41. **A.** Phosphatidylcholine (PC); **B.** Phosphatidylethanolamine (PE). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.



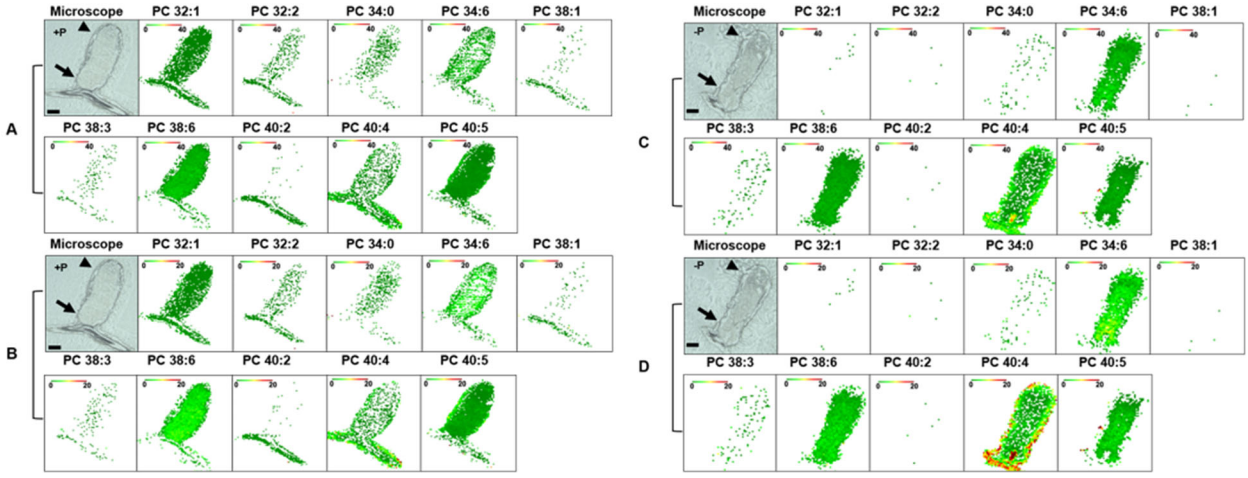
Supplemental Figure S5. Total polar glycerolipids from nodules of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium. meliloti* Rm41. **C.** Phosphatidylglycerol (PG); **D.** Phosphatidylinositol (PI). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.



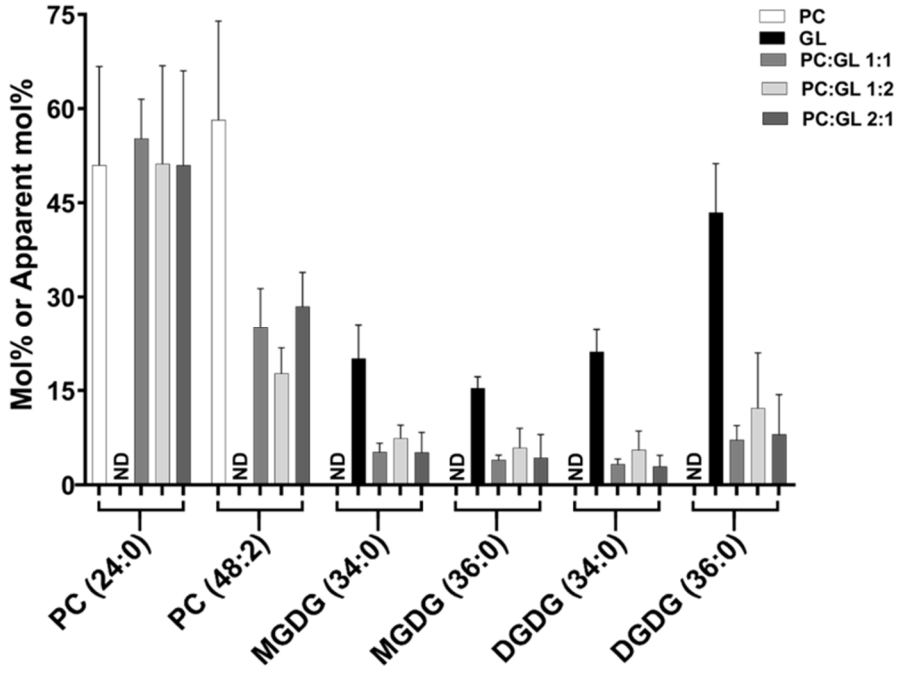
Supplemental Figure S5. Total polar glycerolipids from nodules of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium. meliloti* Rm41. **E.** Phosphatidylserine (PS); **F.** Phosphatidic acid (PA). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



Supplemental Figure S5. Total polar glycerolipids from nodules of *Medicago truncatula* R108 plants. Plants were grown under sufficient phosphorus (+P) and absence of phosphorus (–P) conditions for 28 days after inoculation with *Sinorhizobium. meliloti* Rm41. **G.** Monogalactosyldiacylglycerol (MGDG); **H.** Digalactosyldiacylglycerol (DGDG). Values are expressed as total lipids (nmol/mg dry weight (DW) of tissue) after normalizing with the amount of respective standards. The values are mean ± SD (N =5). Asterisks indicate a significant difference between the control plants (+P) and test plants (-P), as determined using one-way ANOVA analysis; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.



Supplemental Figure S6. MALDI-MS imaging of phosphatidylcholine (PC) metabolites in *Medicago truncatula* nodules. Bright field images of nodules cross section (25µm thick) before coating with DHB matrix grown under sufficient phosphorus (+P); **A**, **B**, and absence of phosphorus (–P); **C**, **D** are shown on the left. The distributions of selected PC molecular species are shown with fixed mol % to show absolute distribution profiles; A, C and adjusted mol % to show relative distribution profiles; B, D. Bars = 500µm. Proximal zone is indicated by arrowhead and distal zone by arrow. MALDI scale shows mol% with green and red representing low and high levels, respectively. Representative images of N = 5 each for +P and –P.



Supplemental Figure S7. Suppression of galactolipids by phosphatidylcholine (PC) in matrix-assisted laser desorption/ionization (MALDI). Phosphatidylcholine (PC) standards (PC 24:0 and PC 48:2) and galactolipid (GL) standards containing monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG); (MGDG 34:0, MGDG 36:0, DGDG 34:0 and DGDG 36:0) were spotted on a slide. Standards were spotted individually and in different ratios as follows: PC, GL, PC/GL 1/1, PL/GL, 1:2 and PL/GL 2/1. The mol% and apparent mol% in PL/GL mixtures were determined by MALDI-MS. N=4. ND=not detected. The values are mean ± SD.

Supplemental Table S1. Phosphate (Pi) levels in media during *Medicago truncatula* growth.

Days post-inoculation	Total phosphate levels		Days post-inoculation	Total phosphate levels	
	Replete, µM	Deprived, µM		Replete, µM	Deprived, µM
1	53.2 ± 4.4	0.0 ± 0.3	**15**	45.2 ± 1.7	0.0 ± 0.6
2	51.2 ± 1.3	0.6 ± 0.1	**16**	45.5 ± 0.2	0.1 ± 0.3
3	51.9 ± 3.6	0.1 ± 0.3	**17**	40.5 ± 1.7	0.2 ± 0.6
4	51.5 ± 3.7	0.0 ± 0.3	**18**	42.4 ± 2.5	0.0 ± 0.3
5	55.1 ± 7.6	0.4 ± 1.2	**19**	44.5 ± 4.6	0.2 ± 0.3
6	48.9 ± 3.7	0.0 ± 0.3	**20**	45.1 ± 1.5	0.0 ± 0.0
7	45.7 ± 1.7	0.2 ± 0.6	**21**	44.2 ± 3.7	0.1 ± 0.6
8	47.4 ± 2.0	0.1 ± 1.3	**22**	55.5 ± 8.8	0.2 ± 1.1
9	50.5 ± 5.1	0.0 ± 0.1	**23**	49.7 ± 3.1	0.2 ± 0.6
10	44.4 ± 2.2	0.2 ± 0.3	**24**	49.7 ± 2.6	0.4 ± 0.7
11	47.4 ± 3.0	0.4 ± 0.7	**25**	43.4 ± 5.1	0.1 ± 1.1
12	46.2 ± 0.0	0.6 ± 0.1	**26**	42.5 ± 1.6	1.7 ± 2.1
13	47.1 ± 3.0	0.4 ± 0.7	**27**	46.7 ± 1.8	0.3 ± 0.3
14	41.9 ± 1.1	0.1 ± 0.7	**28**	44.2 ± 2.2	0.6 ± 0.6

Phosphate (Pi) levels were assayed daily in aeroponic chambers in Pi replete and Pi deprived growth media. Data are mean values +/- SD. N=3.

Supplemental Dataset S1. Suppression study of phosphatidylcholine (PC) on galactolipid detection in matrix-assisted laser desorption/ionization (MALDI).

Results

To address the phenomenon of ion suppression of galactolipids by phosphatidylcholine (PC), we used similar parameters to those employed for imaging the nodule cross sections by MALDI-MS imaging. We observed lower intensities of galactolipids (MGDG 34:0, 36:0 and DGDG 34:0, 36:0) as reported by MALDI-MS when galactolipid standards were spotted in different ratios with PC compared to galactolipid standards alone (Fig S7). When the galactolipid mixture was individually spotted on a MALDI slide, MGDG 34:0 and 36:0 comprised 20 and 15 mol%, while DGDG 34:0 and 36:0 was 20 and 45 mol%, respectively. When this galactolipid standard mix was spotted with PC standard in 1:1 ratio, MGDG 34:0 and 36:0 decreased to an apparent 5 and 4 mol% and DGDG 34:0 and 36:0 to an apparent 3 and 7 mol% respectively (Fig S7). The recovery of MGDG and DGDG 34:0 and 36:0 signal in mol% was negligible when galactolipid and PC standard mixes were spotted in a 2:1 ratio. Further suppression of galactolipids by PC was observed when spotted in a 1:2 ratio. In that case DGDG 34:0 had the lowest apparent abundance of 3 mol% (Fig S7). In contrast, the PC 24:0 signal showed no ion suppression by galactolipids, while PC 48:2 showed moderate suppression by galactolipids, but not nearly as severe as the suppressive effect of PC on galactolipid (Fig S7).

Discussion

In an effort to evaluate the suppression phenomenon of PC on galactolipids (MGDG and DGDG), we carried out a study using similar parameters that were used for imaging nodule cross sections in MALDI-MS imaging. As shown in Fig S7, we observed lower intensities of galactolipids when spotted in different

ratios with phospholipids compared to when galactolipids were spotted alone. The reduction in galactolipid signal was at least 75% when spotted along with PC standards compared to being spotted without PCs. This study also evaluated the suppression of PC species by galactolipids in MALD-MS imaging. We found PC 24:0 was not suppressed while PC 48:2 showed moderate suppression by galactolipids, but the amount of suppression was independent of the ratio of PC to galactolipids in the spot sampled by MALDI-MS. We interpret this to mean that if galactolipids are induced in nodules in all parts of the nodule but in varying amounts depending on developmental stage, they will not interfere with PC imaging results. PC has previously been shown to cause ion suppression in MALDI-MS imaging of cotton embryos triacylglycerols (Horn et al., 2012) other phospholipids (PS, PE, PI) from a mixture (Petkovic et al., 2001), tripalmitin from beef and egg yolk (Emerson et al., 2010) and various other studies (Fuchs et al., 2009; Fuchs et al., 2010; Woodfield et al., 2017). The reduction of apparent intensities of other lipids including galactolipids in MALDI-MS imaging by PC thereby makes it difficult to make qualitative or quantitative conclusions. Technological improvements in metabolite and lipid imaging (Stopka et al., 2018; Colin and Jaillais, 2020) will likely improve our understanding of glycerolipid distribution in nodules beyond.

Materials and methods

Galactolipids (GL) and phospholipids (PL) standards that were obtained from KLRC were spotted individually and together in different ratios (1:1 PL/GL, 1:2 PL/GL and 2:1 PL/GL) on slides. Matrix (DHB) was sublimed on slides containing the spotted standards and subjected to MALDI-MS imaging as described in the main manuscript methods section.

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