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The mycorrhizal symbiosis alters the plant defence strategy in a model legume plant

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Abstract

Arbuscular mycorrhizal (AM) symbiosis modulates plant-herbivore interactions. Still, how it shapes the overall plant defence strategy and the mechanisms involved remain unclear. We investigated how AM symbiosis simultaneously modulates plant resistance and tolerance to a shoot herbivore, and explored the underlying mechanisms. Bioassays with *Medicago truncatula* plants were used to study the effect of the AM fungus *Rhizophagus irregularis* on plant resistance and tolerance to *Spodoptera exigua* herbivory. By performing molecular and chemical analyses, we assessed the impact of AM symbiosis on herbivore-triggered phosphate (Pi)- and jasmonate (JA)-related responses. Upon herbivory, AM symbiosis led to an increased leaf Pi content by boosting the mycorrhizal Pi-uptake pathway. This enhanced both plant tolerance and herbivore performance. AM symbiosis counteracted the herbivore-triggered JA burst, reducing plant resistance. To disentangle the role of the mycorrhizal Pi-uptake pathway in the plant's response to herbivory, we used the mutant line *ha1-2*, impaired in the H^+-ATPase gene *HA1*, which is essential for Pi-uptake via the mycorrhizal pathway. We found that mycorrhiza-triggered enhancement of herbivore performance was compromised in *ha1-2* plants. AM symbiosis thus affects the defence pattern of *M. truncatula* by altering resistance and tolerance simultaneously. We propose that the mycorrhizal Pi-uptake pathway is involved in the modulation of the plant defence strategy.

KEYWORDS

arbuscular mycorrhizal (AM) symbiosis, herbivory, jasmonates (JAs), phosphate (Pi), plant defence strategy, plant-microbe-insect interaction, plant resistance, plant tolerance

1 | INTRODUCTION

Plants are constantly challenged by a multitude of herbivore pests, which can impose significant costs to plant fitness. In response to herbivory, plants can employ diverse strategies to defend against herbivore attack, including resistance and tolerance (Rosenthal & Kotanen, 1994; Stowe et al., 2000; Strauss & Agrawal, 1999). Resistance reduces herbivore feeding or performance via plant physical or chemical traits, such as secondary compounds (Hanley et al., 2007; Mithöfer & Boland, 2012). Chemical defences against herbivore insects are controlled by complex signalling networks in which various hormones play major regulatory roles (Bari & Jones, 2009). The signalling cascade regulated by the plant hormone jasmonic acid (JA) and its derivatives, collectively referred to as jasmonates, is a central player in the regulation of plant responses to chewing herbivores (Kessler & Baldwin, 2002). Tolerance, on the other hand, minimises the fitness consequences of herbivore damage and it is primarily determined by plant physiological traits, such as innate growth rate and resource allocation patterns (Rosenthal & Kotanen, 1994; Strauss & Agrawal, 1999). Since resources available for allocation to defence are limited, it has been assumed that there is a trade-off between these two strategies (Leimu & Koricheva, 2006). Still, empirical evidence reveals that plants may exhibit a mixed pattern of defence strategies (Carmona & Fornoni, 2013; Núñez-Farfán et al., 2007). However, how environmental conditions drive the allocation to tolerance and resistance for a given plant genotype, and the molecular mechanisms orchestrating these processes, remain elusive so far. Recently, it has been demonstrated that plants under phosphate deficiency activate the JA pathway which enhances their defence against herbivory; and that this response is partially controlled by the *PHR1* (phosphate starvation response 1) transcription factor (Khan et al., 2016). These findings suggest that PHR1, together with other signalling components, may play a significant role in shifting from plant tolerance to resistance in environments where phosphate is limiting.

In addition to the plant intrinsic mechanisms for defence against herbivores, associations with beneficial soil microbes may result in enhanced plant performance upon herbivore attack (Shikano et al., 2017). Among beneficial microbes, arbuscular mycorrhizal (AM) fungi have received special attention because of their ubiquity and contribution to plant health (Begum et al., 2019; Miransari, 2010). AM fungi are able to establish AM symbiosis with *c.* 80% of all terrestrial plant species (Brundrett & Tedersoo, 2018). This symbiosis is the most ancient and widespread plant-microbe symbiosis and is considered a key step in plant evolution (Delaux & Schornack, 2021). AM symbiosis is characterised by the formation of differentiated, highly branched and tree-shaped structures called arbuscules in root cortical cells (Choi et al., 2018). The AM fungus provides the plant with an additional pathway of inorganic phosphate (Pi) uptake from the soil, the so-called mycorrhizal Pi-uptake pathway (Bucher, 2007). As a result, mycorrhizal roots have two pathways for Pi uptake: directly through the root (the direct pathway) and indirectly via transfer from mycorrhizal hyphae into root cortical cells (the mycorrhizal pathway) (Smith et al., 2011). On the one hand, the direct pathway involves Pi uptake directly from the soil solution via Pi transporters located in root epidermal cells and root hairs (Johri et al., 2015). On the other hand, the mycorrhizal Pi-uptake pathway involves the acquisition of Pi from the soil solution by the extra-radical mycelia and the subsequent translocation to the root via specific Pi transporters, which are located in the periarbuscular membrane of colonised root cells (Smith et al., 2011). From the Pi transporters identified in the *M. truncatula* genome (MtPT1-MtPT9; Cao et al., 2021), MtPT1-3 and MtPT5-6 are involved in the Pi acquisition via the direct Pi-uptake pathway (Cao et al., 2021; Chiou et al., 2001; Liu et al., 1998; Liu et al., 2008; Grunwald et al., 2009). MtPT4 was revealed as the only mycorrhiza-specific Pi transporter so far (Harrison et al., 2002) and is crucial for AM functioning in this plant species (Javot et al., 2007). The symbiotic Pi uptake by AM-specific Pi transporters further requires the activity of the AM-specific H^+-ATPase HA1 to generate the H^+ gradient across the periarbuscular membrane (Krajinski et al., 2014; Wang et al., 2014b). Indeed, the *ha1-2* mutant shows truncated arbuscules and is unable to acquire Pi via the mycorrhizal pathway (Krajinski et al., 2014). Both the direct and the mycorrhizal Pi-uptake pathways are regulated by the same Pi sensing-centred pathway (the PHR-centred network) (Shi et al., 2021). Physiological and molecular approaches have revealed that the contribution of the mycorrhizal pathway can represent up to nearly all plant Pi, and Pi uptake via the mycorrhizal pathway reduces the contribution of the direct (non-symbiotic) Pi uptake pathway (Smith et al., 2003, 2004). In return, the plant allocates photosynthesis-derived sugars and lipids to its fungal partner (Keymer et al., 2017; Shachar-Hill et al., 1995), establishing thus a tightly regulated exchange of nutrients between both partners.

Because plant resistance is assumed to be costly, and tolerance may be positively affected by the growth rate, the impact of AM symbiosis on plant growth, nutritional status and resource allocation may enhance plant defences against insect herbivores (Tao et al., 2016). Remarkably, several studies report that the enhancement of the plant nutritional status mediated by AM symbiosis can also improve food quality for herbivores, and thus positively affect insect herbivores (Frew et al., 2020; Hoffmann et al., 2009b; Real-Santillán et al., 2019). Therefore, the effect of AM symbiosis on plant defences against herbivores can range from beneficial to detrimental, depending on the ecological context (Minton et al., 2016; Bernaola et al., 2019). In addition, AM symbiosis may also increase plant resistance to insect herbivores by stimulating the plant innate immune system. Several studies demonstrate that AM symbiosis can boost plant defences in systemic tissues, rendering the entire plant more resistant to a variety of pathogens and pests (Formenti & Rasmann, 2019; Liu et al., 2007; Rivero et al., 2021; Schoenherr et al., 2019; Song et al., 2011). This phenomenon, called mycorrhiza-induced resistance (MIR), is generally associated with the priming of the plant immune system, resulting in faster and/or enhanced activation of the JA-regulated defences (Jung et al., 2012; Martinez-Medina et al., 2016; Pozo & Azcón-Aguilar, 2007). The defence priming associated with AM symbiosis provides the plant with a cost-effective mechanism of protection against pathogens and pests (Jacott et al., 2017; Sanmartín et al., 2020; Song et al., 2013, 2015).

By altering the plant´s growth, nutritional and immunity status, AM symbiosis may impact plant-herbivore interactions by affecting

both resistance and tolerance to herbivory. However, how exactly AM symbiosis simultaneously affects plant tolerance and resistance, and which are the underlying molecular mechanisms remain unclear. Here we hypothesise that AM symbiosis enhances plant defence against herbivory by altering the specific plant patterns of defence allocation to tolerance and resistance. Moreover, according to the impact of AM symbiosis on Pi uptake, and the recently discovered link between the Pi-related plant response and JA-mediated herbivore resistance (Khan et al., 2016), we further explored the potential involvement of the Pi-uptake response in the modulation of the plant defence strategy by AM symbiosis. Using *M. truncatula* as a model system, we found that mycorrhizal colonisation enhanced the nutritional status of the leaves, and partially counteracted the *Spodoptera exigua*-triggered burst of jasmonates, thus facilitating herbivore performance. At the same time, in mycorrhizal plants, herbivory led to a boost of the mycorrhizal Pi-uptake pathway, leading to over-compensatory plant growth. These results suggest that under herbivory pressure, mycorrhizal colonisation led to a shift of defence strategy in *M. truncatula*, towards a tolerance-dominant strategy. By using a *M. truncatula* line impaired in the mycorrhizal Pi-uptake pathway, our results revealed a relevant role of the herbivore-triggered boost of the mycorrhizal Pi-uptake pathway in the AM-mediated modulation of the plant defence strategy.

2 | MATERIALS AND METHODS

2.1 | Plant, fungal and insect material

We used *M. truncatula* cv. Jemalong line A17 (A17) in the bioassays, unless indicated otherwise. We further used the *Tnt1* insertion mutant homozygous for *ha1-2* (in the ecotype R108) and homozygous for the absence of *ha1-2* allele progeny of the same parent as wild type controls (WT *Tnt1*) (Krajinski et al., 2014). The AM fungus *Rhizophagus irregularis* isolate BEG141 (Biorize, Dijon, France) was used in all bioassays, unless indicated otherwise. For the production of the mycorrhizal inoculum, *R. irregularis* was maintained in open-pot cultures of *Allium porrum* in a growth chamber according to Devers et al. (2011). The inoculum was obtained on a sand-expanded clay mixture substrate, containing *R. irregularis* mycelia and spores, in an ex vitro system, in open-air conditions. *S. exigua* eggs were obtained from Entocare C.V. Biologische Gewasbescherming (Wageningen, The Netherlands). We maintained eggs, larvae and moths in a growth chamber under 24°C, 16-h light: 8-h dark and 45% relative humidity, and fed with an artificial diet according to Hoffman et al. (1966a). Moths were supplied with a 20% honey solution soaked in cotton balls (Bandoly et al., 2015).

2.2 | Plant growth and fungal inoculation

M. truncatula seeds were germinated as described previously (Branscheid et al., 2010). After germination, seedlings were transferred to 11 × 11 × 12 cm pots containing sterile sand-expanded clay mixture (1:1). Unless indicated otherwise, two seedlings were placed in one pot. Inoculation with *R. irregularis* was performed by mixing *R. irregularis* inoculum through the sand-expanded clay mixture at 5% (vol:vol) before transplanting. For non-mycorrhizal treatments, the same amount of substrate was used for obtaining the mycorrhizal inoculum, but non-inoculated with the mycorrhizal fungus was mixed through the sand-expanded clay mixture before transplanting. The plants were then placed in a completely randomised design in a glasshouse compartment, or in a climate chamber under conditions of 24± 3°C, 16-h light: 8-h dark, and 45% relative humidity. The plants were watered three times a week, 1 day with 50 ml distilled water and 2 days with 50 ml half-strength Hoagland nutrient solution (Hoagland & Arnon, 1950) containing 50 μM KH_2PO_4. Four weeks after transplanting, the plants were used for the experiments.

2.3 | Herbivore treatment

To assess the impact of mycorrhization and *S. exigua* on Pi and JA-related responses, 4 weeks after transplanting the plants, one 2nd instar *S. exigua* larva was placed on the youngest fully expanded leaf of each plant. The larva was contained on a single leaf for 2 days, using a round clip cage of 3 cm in diameter and 3.5 cm in height. Similarly, we mounted an empty clip cage on similar leaves of the plants not assigned to herbivore treatment. A total of six biological replicates (pots, containing two plants per pot) were assigned to each treatment. After 2 days of herbivory, the clip cages were removed and the shoots and roots of the plants were harvested. Shoot and root material were stored at −80°C for molecular and biochemical analyses. For the assessment of the impact of *R. irregularis* and *S. exigua* on plant biomass and total phosphorous (P) and carbon (C) content, 5 seedlings were transferred per pot as described above. Four weeks after transplanting, two (or three) 2nd instar *S. exigua* larvae were placed on the youngest fully expanded leaves of two (or three) plants in one cage for 2 days, using a round clip cage of 3 cm in diameter and 3.5 cm in height. Control plants received an empty clip cage on similar leaves. A total of six biological replicates (pots, containing five plants per pot) were assigned to each treatment. After 2 days of herbivory, the plants were harvested.

2.4 | Determination of larval growth consumed leaf area and shoot dry weight

After 1, 2, 3 and 6 days of feeding, the larvae were removed, and their body mass was recorded. We limited the time span of the bioassay to 6 days after the herbivore challenge based on a preliminary experiment which showed that 9 days after herbivory, all the leaf material in the clip cages was consumed by the herbivores. At 1, 2, 3 and 6 days after the herbivore challenge, plants were harvested and the damaged leaf was photographed for the estimation of the removed area. The leaf area consumed was calculated using

the ImageJ software (https://imagej.nih.gov/). For the determination of plant dry mass, the shoots of plants were oven-dried at 70°C for 3 days. After recording the shoot dry mass, the shoot material was homogenised and passed through a sieve (particle size ≤63 µm). The homogenised material was used for the analysis of shoot P and C.

2.5 | Wounding of plants

Four weeks after transplanting, trifoliate leaves were wounded with a serrated 18/8 forceps by squeezing the surface of the youngest fully expanded leaf, vertically along the main vein (Landgraf et al., 2012). The plants were mechanically wounded once per day for 2 consecutive days. A total of six biological replicates (pots, containing two plants per pot) were assigned to each treatment. Two days after the first wounding, the plants were harvested. Shoot material was stored at −80°C for molecular and biochemical analysis.

2.6 | Arbuscular mycorrhiza quantification

Root colonisation by *R. irregularis* was estimated by root staining with trypan blue, according to Phillips & Hayman (1970). Quantification of the different fungal structures was performed according to Trouvelot et al. (1986), using a bright field microscope (Axioskop; ZEISS). Molecular quantification of *R. irregularis* within the roots was performed by qRT-PCR, analysing the *R. irregularis* constitutively expressed gene *RiTEF* (*Translation Elongation Factor*; Seddas et al., 2009), relative to the reference gene *MtH3L* (MTR_4g097170), which encodes the histone 3 of *M. truncatula* (Wang et al., 2014b).

2.7 | Real-time quantitative RT-PCR

Total RNA was isolated from about 100 mg fresh sample (root or leaf) by using the InviTrap® spin plant RNA mini kit (STRATEC Molecular), according to manufacturer instructions. The quality and quantity of RNA were checked using a Nanodrop 2000 (Thermo Fisher Scientific) and by gel electrophoresis (1% agarose). Traces of DNA were removed by treating 2 µg of the extracted RNA with 2 U/µl of DNase I (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) following the manufacturer's instructions. Complementary DNA synthesis, real-time quantitative PCR reactions and relative quantification of specific messenger RNA levels were performed as described in Supporting Information Methods 1, and by using the gene-specific primers described in Table S1.

2.8 | Determination of phytohormones

Phytohormones (OPDA, JA, JA-Ile, ABA and SA) were extracted and quantified from 100 mg of leaf material following the protocol described by (Mbaluto et al., 2020). The extraction solution contained a deuterated form of each phytohormone (40 ng each) as the internal standards (i.e., $^{2}H_6$-JA, $^{2}H_6$-JA-Ile, $^{2}H_6$-ABA, and $^{2}H_6$-SA). Data acquisition and processing were performed using the 'MS data Review' software (Bruker MS Workstation, version 8.2; Bruker). Phytohormone levels were calculated based on the peak area of the corresponding internal standard and the fresh mass of plant material (ng/mg FW).

2.9 | Determination of Pi, phosphorus, carbon and nitrogen content

Soluble Pi was determined in leaves according to the method of Itaya and Ui (1966) with some modifications. A mixture of 50 µl supernatant and 300 µl colour reagent (1 volume 4.2% $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ in 5 M HCl, 3 volumes 0.2% malachite green dye in water) was incubated with 325 µl ddH_2O, 1.5 ml 1 M HCl in a 5 ml-tube at room temperature for 1 h. The concentration of soluble Pi was calculated using a standard curve made by 500 µM K_2HPO_4 solution.

For the determination of shoot phosphorous (P) content, 0.1 g of oven-dried powder was placed on PFA vessels and was supplemented with 2.5 ml of HNO_3 and 0.25 ml of H_2O_2. A Mars 6 microwave closed system (CEM GmbH) was used for acid digestion. Analysis of diluted acid extracts was carried out using an inductively-coupled plasma optical emission spectrometer (Thermo Scientific™ iCAP™ 7400 ICP-OES Duo).

For the determination of shoot carbon (C) and nitrogen (N) content, around 10 mg of oven-dried powder was processed in an elemental analyzer (Vario EL cube, Elementar Analysensysteme GmbH) by dry combustion a in a furnace at a temperature exceeding 950°C and was analysed by a thermal conductivity detector.

2.10 | Statistical analysis

All data sets were analyzed using the software R (R studio v1.3.1093, R version 4.0.3). Firstly, normality and homogeneity of variance were verified using Shapiro–Wilk and Levene's tests, respectively. For the herbivore performance experiment, we used a generalised linear model (GLM) consisting of mycorrhizal inoculation as a model explanatory factor. The data of gene expression, hormone levels, shoot fresh and dry weight, caterpillar weight and eaten leaf area, shoot phosphorus and carbon and leaf Pi contents were analyzed using GLM and additional Bonferroni post hoc test for comparisons. To meet the requirements of normality and homogeneity of variances, the relative expression of *miR399b*, *miR399c* and *MtPI* were square-root transformed, and relative expression of *MtAOC1* was $\log_{10}+1$ transformed before they were analyzed using GLM, as explained above. The results of one-way and two-way analysis of variances are shown in Table S2.

3 | RESULTS

3.1 | Mycorrhizal colonisation enhances *S. exigua* performance and stimulates an over-compensatory shoot growth

We first explored the impact of mycorrhizal colonisation on the resistance and tolerance of *M. truncatula* to herbivory. With this aim, we assessed the impact of mycorrhizal colonisation on larval growth and consumed leaf area as proxies for plant resistance. The larval mortality rate after 6 days of herbivory was close to 0% in all treatments. We found that caterpillars feeding on leaves of mycorrhizal plants gained a higher weight compared to caterpillars feeding on leaves of non-mycorrhizal plants (Day 6: $F_{1,21} = 31.98$; $p < 0.001$; Figure 1a). Along the same lines, caterpillars feeding on leaves of mycorrhizal plants removed more leaf area compared to those feeding on non-mycorrhizal plants (Day 6: $F_{1,15} = 29.31$; $p < 0.001$; Figure 1b,c). This reveals that mycorrhizal colonisation facilitated the performance of *S. exigua*. To assess the impact of mycorrhizal colonisation on plant tolerance to herbivory, we measured shoot growth as a proxy for plant tolerance to herbivory. We found that in non-mycorrhizal plants, shoot herbivory did not result in shoot biomass reduction (Figure 2a), indicating some degree of plant tolerance to *S. exigua* herbivory. Interestingly, herbivore-challenged mycorrhizal plants showed higher shoot dry biomass, compared to not herbivore-challenged mycorrhizal plants (Figure 2a), even though when mycorrhizal plants lost a larger leaf area due to herbivory (Figure 1b). This suggests that mycorrhizal colonisation stimulated an over-compensatory plant growth (vegetative growth) upon herbivory. We also found that mycorrhizal colonisation enhanced shoot total P content, compared to non-mycorrhizal plants (Figure 2b). Interestingly, in mycorrhizal plants, herbivory led to a significant increase in shoot total P content compared to not herbivore-challenged plants (Figure 2b). Along the same lines, we found that mycorrhizal colonisation increased shoot C and N contents (Figure S2).

To uncouple the nutritional effects, due to enhanced Pi contents in mycorrhizal plants, from the over-compensatory growth observed in mycorrhizal plants upon herbivory, we performed an experiment with non-mycorrhizal plants under different Pi application levels: low Pi (0.05 mM), standard Pi (0.5 mM) and high Pi (1 mM). We analyzed the effect of the different Pi application levels on herbivore performance and plant tolerance to herbivory (Figure S3). We found that a higher plant Pi supply enhanced *S. exigua* weight and herbivore-removed leaf area, indicating that a higher Pi plant supply facilitated the herbivore performance. Remarkably, shoot weight was not affected by herbivory, regardless of the Pi application level, suggesting that the over-compensatory plant growth displayed by the mycorrhizal plants (Figure 2a) is not just a mere effect of AM symbiosis-mediated Pi enhancement. Taken together, our results show that in *M. truncatula* plants, mycorrhizal colonisation by *R. irregularis* enhanced the performance of *S. exigua* larvae, but at the same time it stimulated an over-compensatory shoot growth.

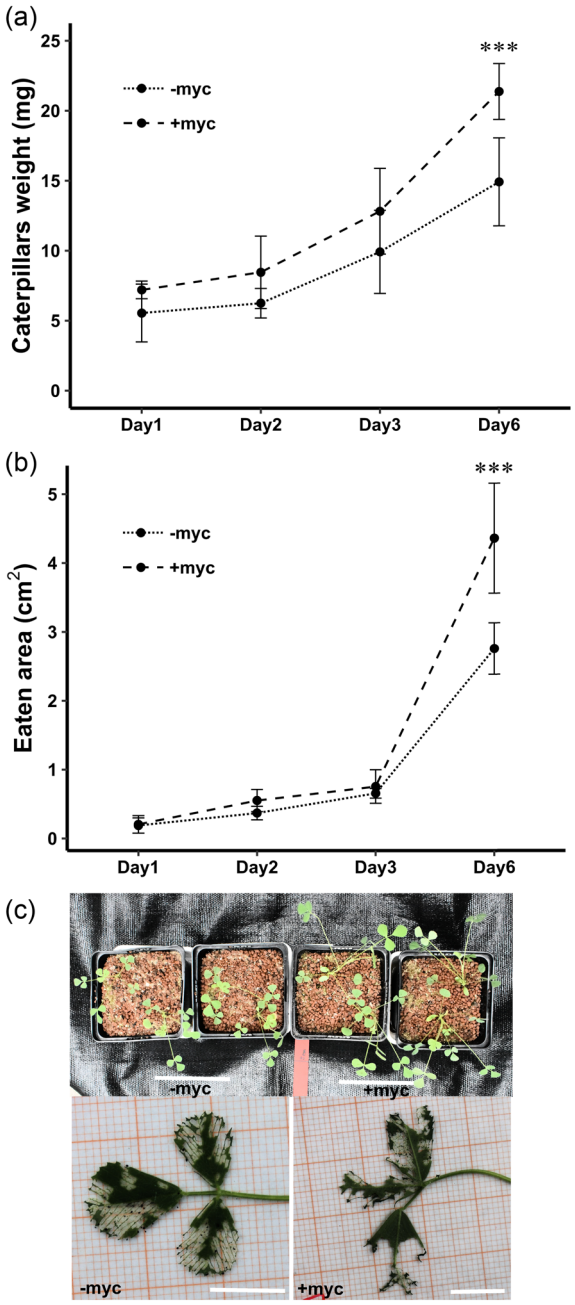

FIGURE 1 Impact of arbuscular mycorrhizal (AM) colonisation on the performance of *Spodoptera exigua* larvae on *Medicago truncatula* A17 leaves. (a) Weight gain of *S. exigua* larvae feeding on leaves of *M. truncatula* plants. (b) Leaf area removed by *S. exigua* larvae feeding on leaves of *M. truncatula* plants. (c) Representative photographs of leaves of *M. truncatula* plants 5 days after *S. exigua* feeding. Plants were not inoculated (−myc) or inoculated (+myc) with the AM fungus *R. irregularis*, and 4 weeks later, each plant was challenged with one 2nd instar *S. exigua* larva. Data are mean ± *SD* (in a, $n \geq 10$; in b $n \geq 8$). The white bar in (c) denotes 1 cm. In (a) and (b), asterisks indicate significant differences between values from non-inoculated and inoculated plants (Student's *t* test, ***$p < 0.001$).

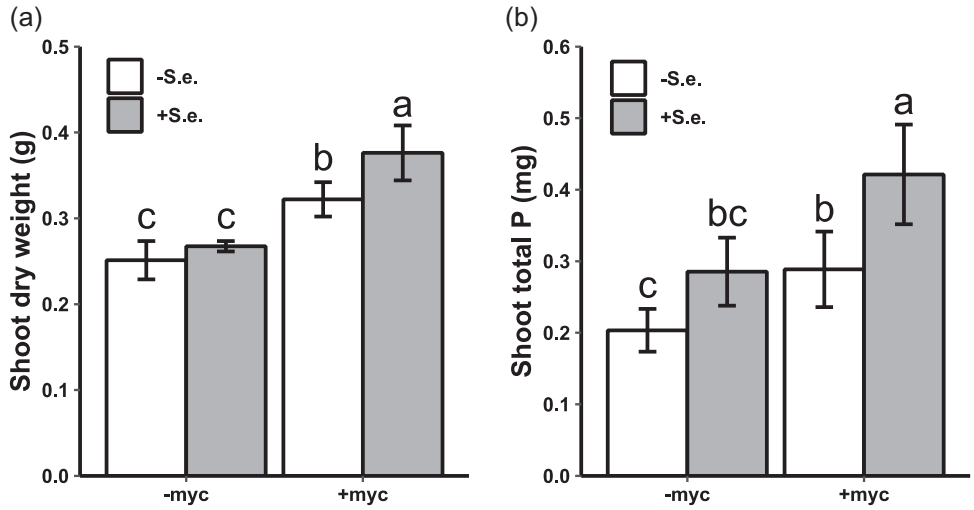

FIGURE 2 Impact of shoot herbivory and AM colonisation on shoot biomass and total P content of *Medicago truncatula* A17 plants. (a) Shoot dry weight, (b) shoot total P in *M. truncatula* plants. Plants were not inoculated (−myc) or inoculated (+myc) with the AM fungus *R. irregularis*, and 5 weeks later, five plants in one pot were not challenged (−S.e.) or leaf challenged (+S.e.) with five 2nd instar *S. exigua* larvae. Shoot dry weight, total P content were assessed 2 days after herbivore challenge. Data are mean ± *SD* ($n \geq 4$). Each biological replicate consists of pooled shoot material from five plants growing in one pot. Different letters indicate significant differences between treatments according to generalised linear model (GLM) and post hoc tests adjusted by Bonferroni method at $p < 0.05$. ns, not significant

3.2 | Mycorrhizal colonisation enhances the Pi uptake triggered by shoot herbivory specifically through the mycorrhizal pathway

We found that in response to herbivory, the mycorrhizal colonisation led to over-compensatory shoot growth and a higher increase of total P in the leaves (Figure 2a,b). We thus aimed to further investigate the impact of the mycorrhizal colonisation and herbivore challenge on the plant Pi uptake. We assessed the expression level of a set of plant genes related to Pi transport and Pi starvation response, in roots or leaves of *M. truncatula* plants.

In the case of non-mycorrhizal plants, we found that in comparison to herbivore-unchallenged plants, shoot herbivory stimulated the expression of Pi transporters encoding genes *MtPT1* (Chiou et al., 2001; Liu et al., 1998), *MtPT2* (Liu et al., 1998), *MtPT3* (Liu et al., 2008) and *MtPT6* (Cao et al., 2021) in the root, all being related to the direct Pi-uptake pathway (Figure 3a). By contrast, herbivory did not affect the expression of the root Pi transporter *MtPT5* (Liu et al., 2008; data not shown). We further observed, in non-mycorrhizal plants, a slight increase (although not statistically significant) in leaf Pi concentration triggered by herbivory, after 2 days of herbivore feeding, compared to herbivore-unchallenged plants (Figure 3b). Along the same lines, in non-mycorrhizal plants, shoot herbivory led to a downregulation of the root Pi-starvation-related genes *MtPLD* (encoding Phospholipase D; Branscheid et al., 2010), *MtSPX1*, *MtSPX2* and *MtSPX3* (orthologous genes of the SPX domain proteins in rice and *Arabidopsis*; Puga et al., 2014; Wang et al., 2014a) (Figure 4a), compared to herbivore-unchallenged plants. Similarly, shoot herbivory led to a downregulation of the leaf Pi-starvation-related precursors of microRNAs *miR399b* and *miR399c* (Branscheid et al., 2010; Li & Yu, 2018) (Figure 4b). These results suggest that shoot herbivory triggers the Pi transport at the transcriptional level, and thus reduces the Pi stress in non-mycorrhizal *M. truncatula* plants.

In comparison to non-mycorrhizal plants, we found that, in the absence of herbivory, mycorrhizal colonisation triggered the root expression of the Pi transporter encoding gene *MtPT4* (Harrison et al., 2002; Javot et al., 2007), which is specific to the mycorrhizal Pi-uptake pathway (Figure 3c). Interestingly, without herbivory, mycorrhizal colonisation led to a downregulation of *MtPT6* and *MtPT3* in roots, although the latter effect was not statistically significant (Figure 3a), and did not affect the expression of *MtPT1* and *MtPT2* in roots, compared to non-mycorrhizal plants (Figure 3a). In herbivore-unchallenged plants, we found a higher concentration of Pi in leaves of mycorrhizal plants, compared to non-mycorrhizal plants (Figure 3b). Accordingly, mycorrhizal colonisation, in the absence of herbivory, led to a downregulation of the Pi-starvation-related genes *MtPLD*, *MtSPX1* and *MtSPX3* in the roots and *miR399b* and *miR399c* in leaves, compared to non-mycorrhizal plants (Figure 4a,b). A similar expression of *MtSPX2* was found in roots of non-mycorrhizal and mycorrhizal plants. These results show that, in the absence of herbivory, mycorrhizal colonisation enhances *M. truncatula* Pi uptake specifically via the mycorrhizal pathway. Remarkably, in mycorrhizal plants, shoot herbivory did not significantly affect the expression of *MtPT1*, *MtPT2*, *MtPT3*, and *MtPT6*, compared to mycorrhizal plants without herbivores (Figure 3a). By contrast, shoot herbivory led to an upregulation of *MtPT4* (Figure 3c), and to a higher Pi concentration (Figure 3b), compared to mycorrhizal plants without herbivores. Accordingly, we found a higher expression level of the *R. irregularis*

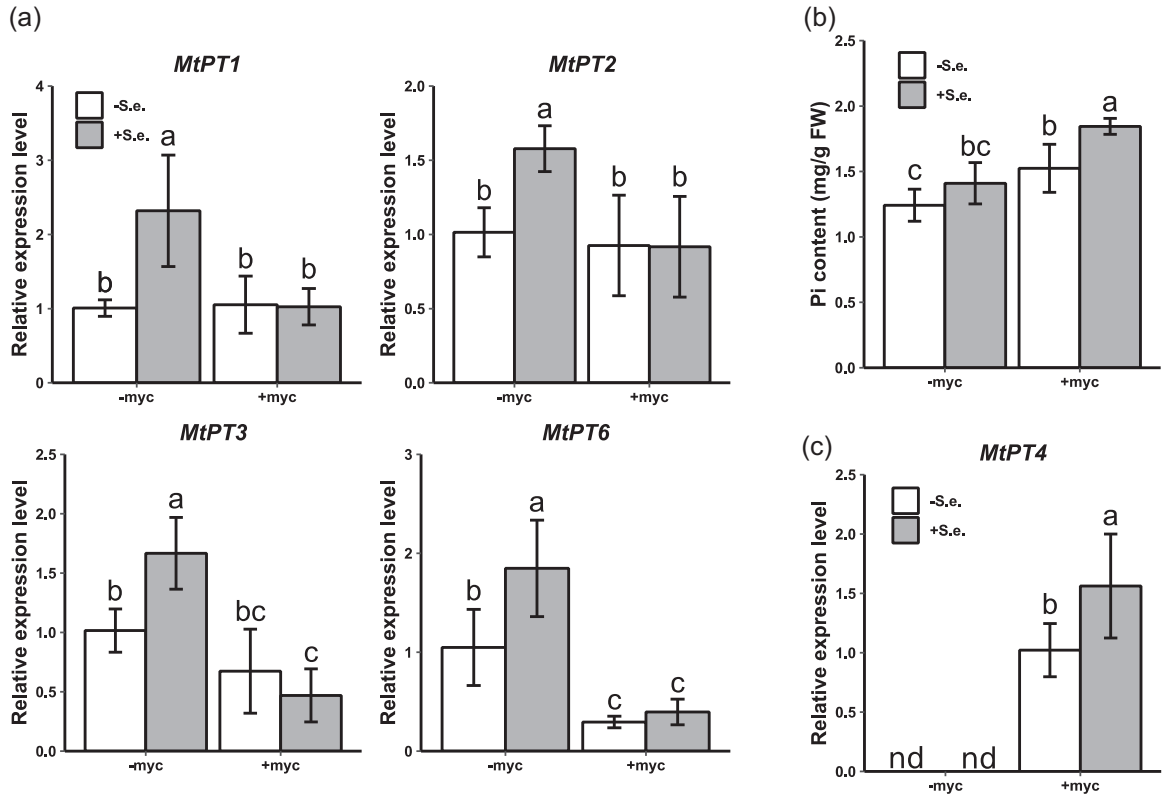

FIGURE 3 Impact of shoot herbivory and AM colonisation on the expression of Pi transporter genes and leaf Pi content in *Medicago truncatula* A17 plants. (a) Relative expression of the Pi transporter genes *MtPT1*, *MtPT2*, *MtPT3* and *MtPT6* in roots. (b) Pi content in leaves. (c) Relative expression of the mycorrhizal specific transporter gene *MtPT4* in roots. Plants were not inoculated (−myc) or inoculated (+myc) with the AM fungus *R. irregularis*, and 4 weeks later, each plant was not challenged (−S.e.) or leaf challenged (+S.e.) with one 2nd instar *S. exigua* larva. Relative transcript accumulation and Pi content were assessed 2 days after herbivore challenge. Data are mean ± *SD* ($n \geq 3$). Each biological replicate consists of pooled root (a, c) or leaf (b) material from two plants growing in one pot. In (a) and (c) the results are normalised to the *MtH3L* gene expression in the same samples. In (a) the results are expressed relative to those found in plants not inoculated with *R. irregularis* and not challenged with *S. exigua*. In (c) the results are expressed relative to those found in plants inoculated with *R. irregularis* and not challenged with *S. exigua*. Different letters indicate significant differences between treatments according to GLM and post hoc tests adjusted by Bonferroni method at $p < 0.05$. nd, not detected

constitutively expressed gene *RiTEF* (*Translation Elongation Factor*; Seddas et al., 2009) in roots of herbivore-challenged plants, compared to plants without herbivores (Figure S1). However, this increase did not correlate with the histological quantification of arbuscule abundance (Table S3). In the mycorrhizal plants, shoot herbivory did not significantly affect the expression of the root Pi-starvation-related genes *MtPLD*, *MtSPX1*, *MtSPX2*, *MtSPX3*, *miR399b* and *miR399c* compared to non-herbivore-challenged plants (Figure 4a,b). Altogether, these results show that in *M. truncatula* plants, mycorrhizal colonisation by *R. irregularis* enhances the Pi uptake activated by shoot herbivory, specifically through the mycorrhizal pathway.

3.3 | Mycorrhizal colonisation partially counteracts the JA burst triggered by shoot herbivory

According to the pivotal role of JAs in plant resistance to herbivory, we next aimed to investigate whether mycorrhizal colonisation might affect the burst of JAs triggered by herbivore feeding. For this purpose, we measured the content of the different JAs—jasmonic acid (JA); its precursor *cis*−12-oxo-phytodienoic acid (OPDA); and its conjugated form JA-isoleucine (JA-Ile), in plant leaves. We further addressed the expression of the JA biosynthesis genes *MtAOS* (encoding ALLENE OXIDE SYNTHASE; Adolfsson et al., 2017), *MtAOC1* (encoding ALLENE OXIDE CYCLASE; Isayenkov et al., 2005) and *MtOPR* (encoding OPDA REDUCTASE; Sun et al., 2018) in leaves. We found that in non-mycorrhizal plants, shoot herbivory led to an increase in the level of OPDA, JA and JA-Ile in leaves, compared to herbivore-unchallenged plants (Figure 5a). Accordingly, in non-mycorrhizal plants shoot herbivory triggered an upregulation of *MtAOC1* and *MtOPR* (Figure 5b). Shoot herbivory also led to a slight increase (although not statistically significant) of *MtAOS* expression level (Figure 5b). By contrast, shoot herbivory did not significantly affect ABA and SA contents (Figure S4). In leaves of plants without herbivores, we found similar levels of OPDA, JA and JA-Ile, and similar expression

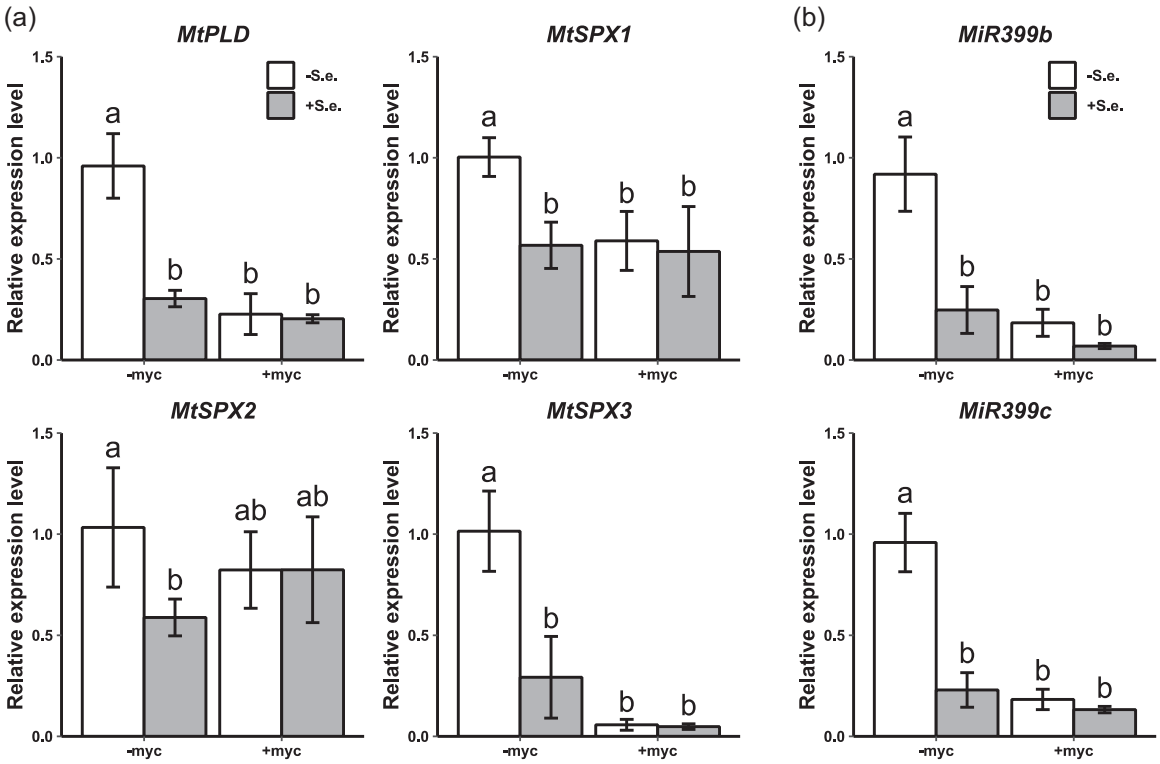

FIGURE 4 Impact of shoot herbivory and AM colonisation on the expression of *Medicago truncatula* A17 Pi starvation marker genes. (a) Relative expression of Pi starvation marker genes *MtPLD*, *MtSPX1*, *MtSPX2* and *MtSPX3* in roots. (b) Relative expression of precursors of Pi starvation marker microRNAs *MiR399b* and *MiR399c* in leaves. Plants were not inoculated (−myc) or inoculated (+myc) with the AM fungus *R. irregularis*, and 4 weeks later, each plant was not challenged (−S.e.) or leaf challenged (+S.e.) with one 2nd instar *S. exigua* larva. Relative transcript accumulation was assessed 2 days after herbivore challenge. Data are mean ± *SD* ($n \geq 3$). Each biological replicate consists of pooled root (a) or leaf (b) material from two plants growing in one pot. The results are normalised to the *MtH3L* gene expression in the same samples, and expressed relative to those found in plants not inoculated with *R. irregularis* and not challenged with *S. exigua*. Different letters indicate significant differences between treatments according to GLM and post hoc tests adjusted by Bonferroni method at $p < 0.05$

levels of *MtAOS* and *MtAOC1* in non-mycorrhizal and mycorrhizal plants (Figure 5a,b). Remarkably, in plants without herbivores, mycorrhizal colonisation led to a decrease in the expression level of *MtOPR* compared to non-mycorrhizal plants (Figure 5b). This indicates that a well-established AM symbiosis is not associated with the direct activation of the JA biosynthesis pathway in *M. truncatula* leaves. Strikingly, after herbivore feeding, we found a lower accumulation of OPDA, JA and JA-Ile, and lower expression levels of *MtAOC1* and *MtOPR* in leaves of mycorrhizal plants, compared to non-mycorrhizal plants (Figure 5a,b). Indeed, similar levels of JA and JA-Ile, and *MtOPR* expression were found in mycorrhizal plants, regardless of the herbivore challenge (Figure 5a,b). Along the same lines, shoot herbivory in mycorrhizal plants led to a generally weaker herbivore-triggered activation of the *M. truncatula proteinase inhibitor* gene *MtPI* in leaves, compared to the enhancement of *MtPI* expression triggered by the herbivore in leaves of non-mycorrhizal plants (Figure S5). These results reveal that mycorrhizal colonisation by *R. irregularis* counteracts, at least partially, the JAs burst triggered by herbivore feeding in *M. truncatula* shoots.

3.4 | Mycorrhizal colonisation increases Pi uptake upon wounding and counteracts wound-triggered JA burst

We next investigated the potential impact of AM symbiosis on Pi uptake and the JA burst after wounding. With this aim, we performed a separate bioassay in which non-mycorrhizal and mycorrhizal plants were mechanically wounded. We found that in non-mycorrhizal plants, mechanical wounding neither affected leaf Pi concentration nor shoot fresh weight, compared to non-wounded plants (Figure 6a,b), but it did lead to a higher accumulation of JA and JA-Ile (Figure 6c,d). In non-wounded plants, mycorrhizal colonisation increased leaf Pi concentration, as observed before (Figure 3b), and also increased shoot fresh weight, compared to non-mycorrhizal plants (Figure 6a,b). In addition, a reduced accumulation of JA was observed in leaves of mycorrhizal plants compared to non-mycorrhizal plants (Figure 6c), whereas mycorrhizal colonisation did not affect leaf JA-Ile accumulation (Figure 6d). Remarkably, in mycorrhizal plants, the mechanical wounding treatment triggered an enhanced accumulation of leaf Pi and shoot fresh biomass,

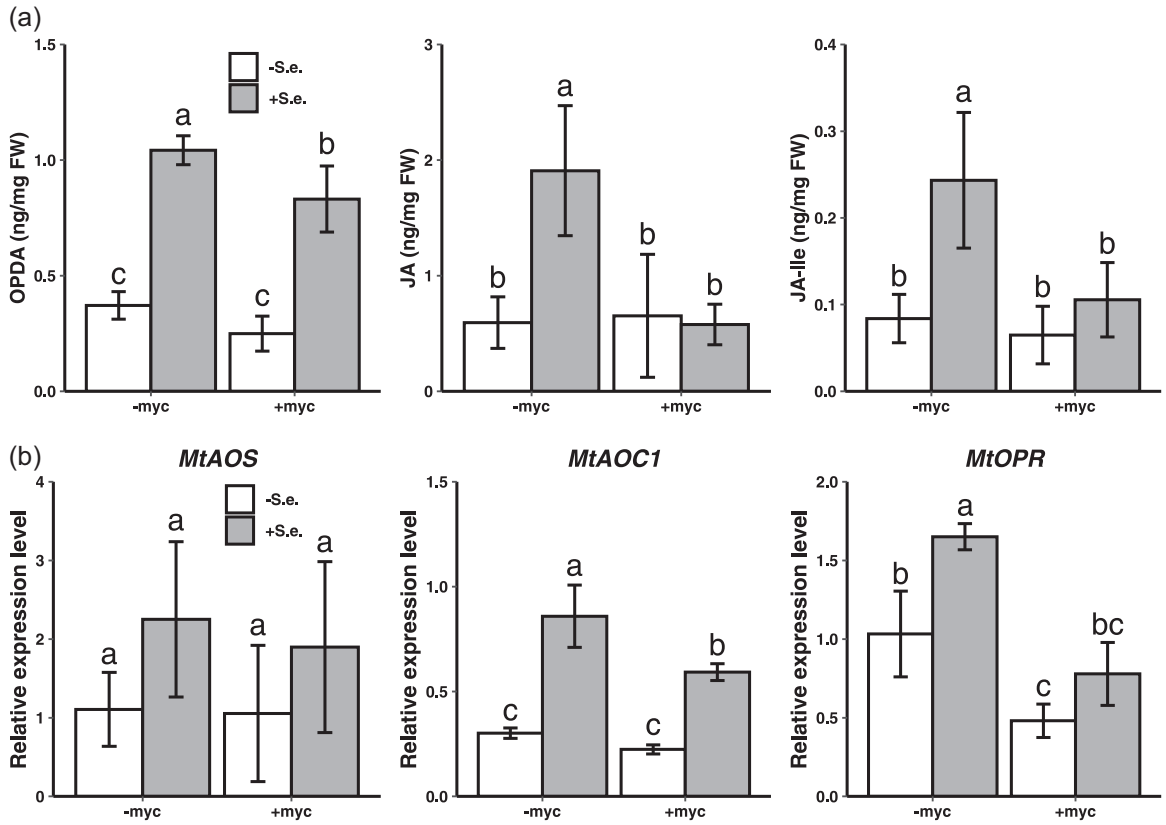

FIGURE 5 Impact of shoot herbivory and AM colonisation on the jasmonate biosynthesis pathway in *Medicago truncatula* A17 leaves. (a) The level of OPDA, JA and JA-Ile in leaves. (b) Relative expression of *MtAOS*, *MtAOC1* and *MtOPR* in leaves. Plants were not inoculated (−myc) or inoculated (+myc) with the AM fungus *R. irregularis*, and 4 weeks later, each plant was not challenged (−S.e.) or leaf challenged (+S.e.) with one 2nd instar *S. exigua* larva. Levels of jasmonates and relative transcript accumulation were assessed 2 days after herbivore challenge. Data are mean ± *SD* ($n \geq 3$). Each biological replicate consists of pooled leaf material from two plants growing in one pot. In (b) the results are normalised to the *MtH3L* gene expression in the same samples, and expressed relative to those found in plants not inoculated with *R. irregularis* and not challenged with *S. exigua*. Different letters indicate significant differences between treatments according to GLM and post hoc tests adjusted by Bonferroni method at $p < 0.05$

compared to non-wounded plants (Figure 6a,b). In addition, a lower accumulation of leaf JA and JA-Ile was triggered by the mechanical wounding treatment in mycorrhizal plants, compared to that observed in non-mycorrhizal wounded plants (Figure 6c,d). Our results suggest that, in analogy to plants under herbivore challenge, mycorrhizal colonisation by *R. irregularis* leads to an increase in leaf Pi uptake after wounding, and also partially counteracts the wound-triggered jasmonate burst in *M. truncatula* leaves.

3.5 | Mycorrhizal-mediated enhancement of herbivore performance is abolished in the *M. truncatula ha1-2* mutant line

Our results show that under herbivore pressure, mycorrhizal plants enhanced the mycorrhizal Pi-uptake pathway and that this response was concomitant with the enhanced performance of the herbivore. To investigate the relevance of the Pi-uptake pathway on the observed herbivore phenotype, we established a bioassay using the *M. truncatula ha1-2* mutant. This mutant (ecotype R108) harbours a *Tnt1*-insertion in the gene encoding the H^+-ATPase HA1 (R108) and is unable to take up Pi via the mycorrhizal pathway (Krajinski et al., 2014; Wang et al., 2014b). Plant's homozygote for the absence of the *Tnt1* insertion within the MtHA1-encoding gene and derived in the progeny of *Mtha-1* heterozygous plants served as the respective wild type control. We first found that, indeed, under our experimental conditions, *MtPT4* transcripts in mycorrhizal plants were significantly less abundant in the *ha1-2* mutant than in the WT (Figure 7a), and that the *M. truncatula ha1-2* mutant failed to show a positive growth response to mycorrhizal colonisation (Figure 7b).

We further found that in the WT plants, mycorrhizal colonisation led to an increase in the weight of *S. exigua* larvae compared to non-mycorrhizal plants (Figure 8a), similarly to that observed in the A17 line (Figure 1a). Along the same lines, mycorrhizal colonisation led to an increase in the leaf area removed by the herbivore, compared to non-mycorrhizal plants (Figure 8b). Interestingly, in the *ha1-2* mutant, mycorrhizal colonisation failed in increasing the weight of *S. exigua* (Figure 8a). Indeed, no differences in the herbivore weight were observed in larvae feeding on mycorrhizal and non-mycorrhizal *ha1-2* plants (Figure 8a). Moreover, mycorrhizal colonisation did not affect the leaf area

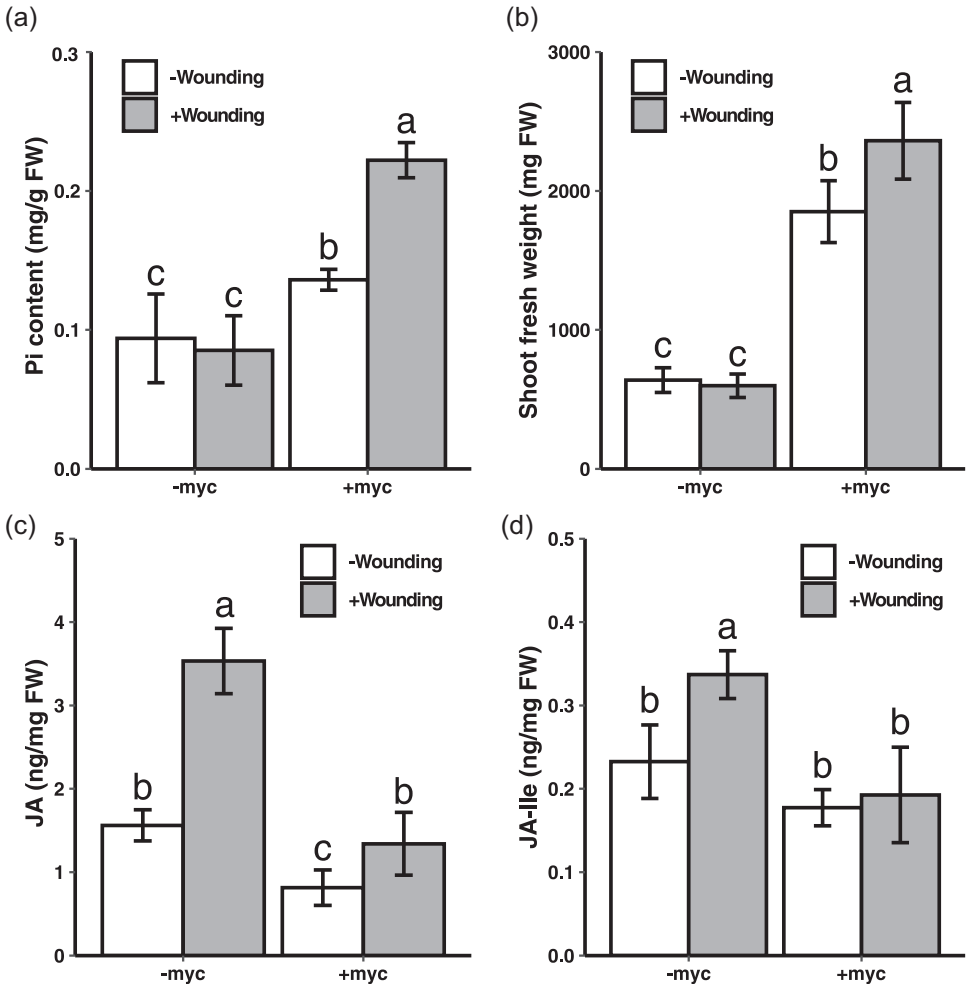

FIGURE 6 Impact of wounding and AM colonisation on Pi, biomass and accumulation of jasmonates in *Medicago truncatula* A17 leaves. (a) Leaf Pi content, (b) shoot biomass, (c) leaf JA and (d) leaf JA-Ile levels. Plants were not inoculated (−myc) or inoculated (+myc) with the AM fungus *R. irregularis*, and 4 weeks later, each plant was not wounded (−wounding) or mechanically wounded (+wounding) once per day during two consecutive days. Pi content, plant growth, JA and JA-Ile levels were assessed 2 days after 1st wounding. Data are mean ± *SD* ($n \geq 3$). Each biological replicate consists of pooled leaf (a, c, d) or shoot (b) material from two plants growing in one pot. Different letters indicate significant differences between treatments according to GLM and post hoc tests adjusted by Bonferroni method at $p < 0.05$

removed by herbivores in the *ha1-2* line (Figure 8b). These findings show that the mycorrhizal-mediated enhancement of *S. exigua* performance is abolished in the *ha1-2* mutant.

We finally assessed the impact of mycorrhizal colonisation on herbivore-triggered leaf Pi accumulation and JA burst in the *ha1-2* line. Under herbivory pressure, in non-mycorrhizal plants, there were no significant differences in Pi concentration among the WT and the *ha1-2* line (Figure 8c). In WT plants, mycorrhizal colonisation led to a higher level of Pi concentration compared to that in non-mycorrhizal plants (Figure 8c). Strikingly, in the *ha1-2* line, mycorrhizal colonisation of herbivore-challenged plants also led to a higher Pi concentration compared to non-mycorrhizal plants. Remarkably, in herbivore-challenged plants, the mycorrhizal wild type plants showed a significantly higher Pi level than mycorrhizal *ha1-2* plants. These results suggest that root colonisation by *R. irregularis* and shoot herbivores still induced Pi uptake of *ha1-2* mutants, independent of the mycorrhizal Pi-uptake pathway. Strikingly, in WT, we observed a higher JA-Ile accumulation in herbivore-challenged mycorrhizal plants, compared to that observed in non-mycorrhizal plants (Figure 8d). Still, an even higher increase of leaf JA-Ile accumulation mediated by mycorrhizal colonisation was observed in the *ha1-2* line (Figure 8d). In summary, these data suggest that mycorrhizal colonisation of *ha1-2* mutants did not positively affect *S. exigua* performance. This is likely to be based on the loss of a functional mycorrhizal Pi-uptake pathway in this mutant.

4 | DISCUSSION

AM symbiosis can affect plant resistance and tolerance to insect herbivores, thus altering the overall plant defence strategy (Bernaola & Stout, 2021; Frew et al., 2020; Vannette & Hunter, 2009). Noticeably, most of the studies on the effects of AM symbiosis on plant defences against insect herbivores focus on either tolerance or resistance separately (but see Tao et al., 2015). By contrast, tolerance

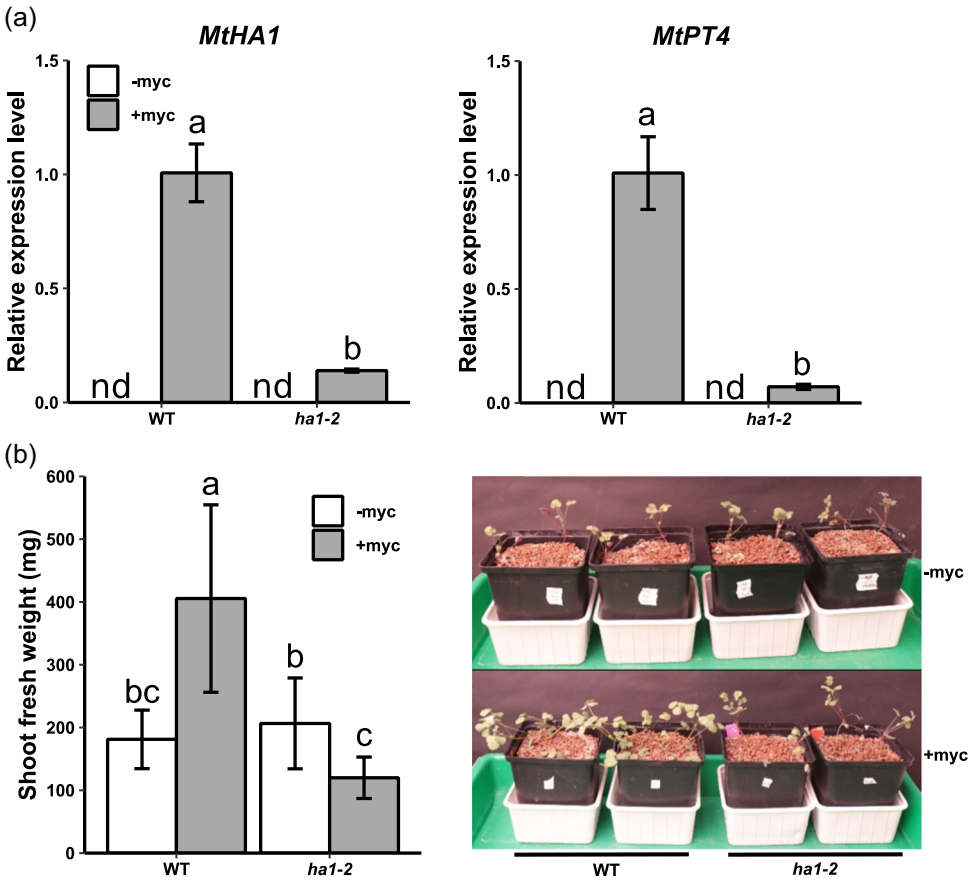

FIGURE 7 Impact of mycorrhizal colonisation on the phenotype of *Medicago truncatula ha1-2* (R108) plants. (a) Relative expression of *MtHA1* and *MtPT4* in roots of wild type and *ha1-2* lines. (b) Shoot fresh weight and representative photographs of wild type and *ha1-2* plants. Plants were not inoculated (−myc) or inoculated (+myc) with the AM fungus *R. irregularis*. Data are mean ± *SD* (in a, $n \geq 3$; in b $n \geq 11$). For gene expression level, each biological replicate consists of pooled root material from two plants growing in one pot. For shoot fresh weight, each biological replicate represents one plant growing in one pot. The results in (a) are normalised to the *MtH3L* gene expression in the same samples, and expressed relative to wild type plants inoculated with *R. irregularis*. Different letters indicate significant differences between treatments according to GLM and post hoc tests adjusted by Bonferroni method at $p < 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

and resistance collectively determine the effects of herbivory on plant fitness, and examining one defence strategy in isolation may lead to erroneous predictions on how AM symbiosis affects the plant defence overall (Tao et al., 2015). Moreover, the molecular mechanisms underlying the impact of AM symbiosis on the overall defence strategy of the plant against herbivory remain obscure. Here, we show that the AM fungus *R. irregularis* affects the resistance and tolerance of *M. truncatula* plants to the insect herbivore *S. exigua* simultaneously. Interestingly, our study shows that AM symbiosis led to a shift of the plant defence strategy towards a tolerance-dominant strategy.

We first found that caterpillars performed better and removed more leaf biomass in AM plants compared to non-mycorrhizal plants, suggesting that AM symbiosis increases plant susceptibility to the herbivore. Similarly, several studies have reported positive effects of AM symbiosis on herbivore performance (Bernaola & Stout, 2021; Maurya et al., 2018; Real-Santillán et al., 2019). Despite this seemingly enhanced plant susceptibility mediated by AM symbiosis, we found that herbivory damage led to a significant increase in the shoot biomass of the mycorrhizal plants, indicating the plant´s ability to overcompensate for herbivore damage. It is noticeable that in non-mycorrhizal plants, herbivory did not affect shoot biomass, which might indicate that *M. truncatula* plants have a degree of tolerance for herbivory. Still, our results propose that AM symbiosis enhances the compensatory growth (vegetative growth) capacity of *M. truncatula* plants following herbivory, thus increasing plant tolerance to herbivore damage. Along the same lines, several studies demonstrate that AM symbiosis can enhance plant tolerance to herbivory (Bernaola & Stout, 2021; Frew et al., 2020). Here we found that AM symbiosis, on the one hand, facilitated the herbivore performance, and on the other hand, stimulated an over-compensatory growth capacity upon herbivore challenge. This promotes a mutually beneficial relationship between the plant and the herbivore, where both the plant and herbivore benefit each other (Agrawal, 2000; Garcia & Eubanks, 2019). It is remarkable that herbivory increased the root colonisation by the AM fungus, as observed at the molecular level. Herbivory effects on AM colonisation have been reported mainly as negative based on the premises of the carbon-limitation

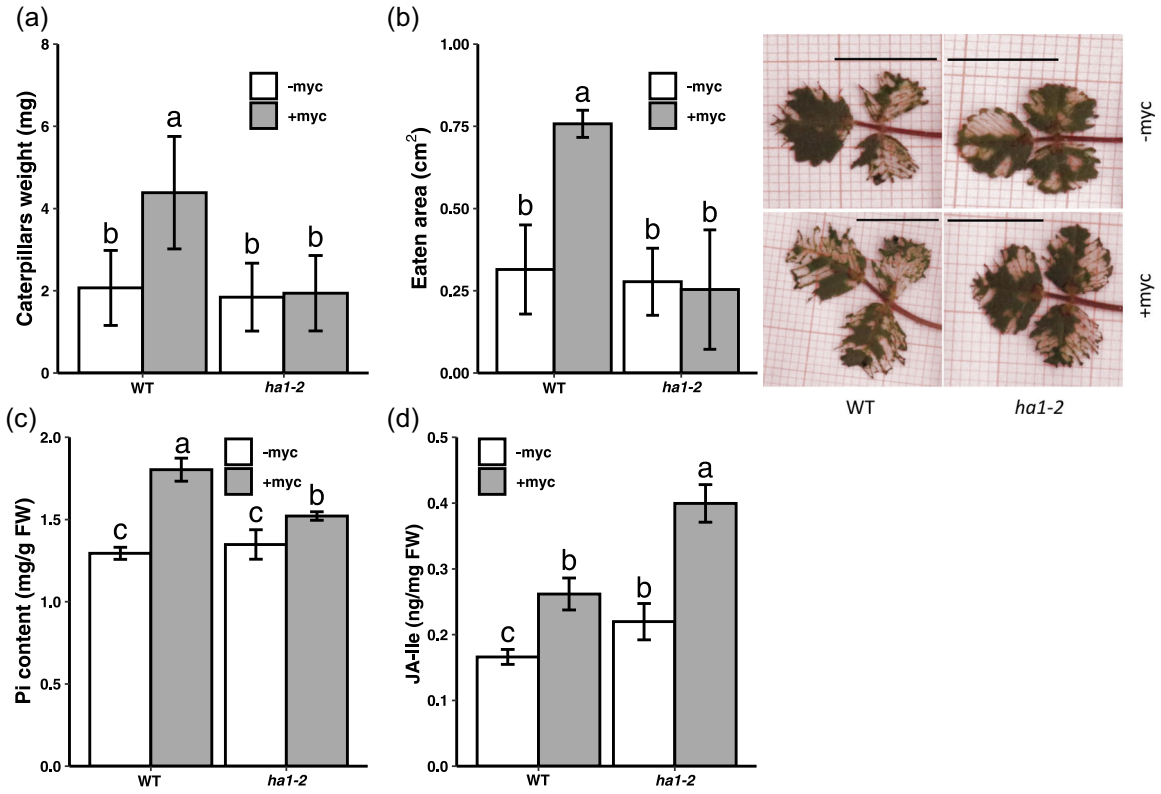

FIGURE 8 Impact of mycorrhizal colonisation on herbivore performance and Pi uptake and JA-Ile content in *Medicago truncatula ha1-2* (R108) plants. (a) Weight gain of *S. exigua* larvae feeding for 2 days on leaves of wild type and *ha1-2* plants. (b) Leaf area removed by *S. exigua* larvae feeding for 2 days on leaves of wild type and *ha1-2* plants (left panel), and representative photographs of leaves of wild type and *ha1-2* 2 days after *S. exigua* feeding (right panel). (c) Pi and (d) JA-Ile levels in leaves of wild type and *ha1-2* plants 2 days after *S. exigua* feeding. Plants were not inoculated (−myc) or inoculated (+myc) with the AM fungus *R. irregularis*, and 4 weeks later, each plant was challenged with one 2nd instar *S. exigua* larva. Data are mean ± *SD* (in a, $n \geq 10$; in b, $n \geq 8$; in c and d, $n \geq 3$). In c and d, each biological replicate consists of pooled leaf material from two plants growing in one pot. Different letters indicate significant differences between treatments according to GLM and post hoc tests adjusted by Bonferroni method at $p < 0.05$. JA, jasmonate; Pi, phosphate. [Color figure can be viewed at wileyonlinelibrary.com]

hypothesis, which predicts a reduced mycorrhizal growth following the removal of aboveground biomass (Ghering & Whitham, 2002; Wardle et al., 2004). However, there is some evidence that this may not always be the case, depending on the extent of the herbivory, the type of mycorrhiza, or the plant species (Barto & Rillig, 2010). In *M. truncatula*, it has been shown that repeatedly performed mechanical wounding of leaves increases the colonisation of roots with a mycorrhizal fungus (Landgraf et al., 2012). The over-compensatory growth capacity upon herbivory observed here in the mycorrhizal plants, may lead to a greater amount of photosynthetic tissue, which could better support the mycorrhizal fungi. The ability of AM symbiosis to provide plant tolerance to herbivory is associated with the enhanced capacity of the mycorrhizal plants to take up P from the soil (Real-Santillán et al., 2019; Tao et al., 2015). Indeed, the P content is shown to strongly affect plant tolerance to herbivory (Quijano-Medina et al., 2019; Strauss & Agrawal, 1999; Tao et al., 2015). We found that in non-mycorrhizal plants, shoot herbivory systemically triggered the expression level of the root Pi transporters *MtPT1*, *MtPT2*, *MtPT3* and *MtPT6* involved in the direct Pi-uptake pathway, and led to a slight increase (although not significant) in leaf Pi content. Shoot herbivory further led to a repression of the Pi-related stress response in *M. truncatula* shoots and roots. This indicates that the activation of the Pi-uptake machinery is part of the systemic response triggered in *M. truncatula* roots by shoot herbivory, most likely as a tolerance mechanism to herbivore damage. In the absence of shoot herbivory, mycorrhizal colonisation triggered the expression of the AM-specific Pi transporter *MtPT4*, leading to an increased Pi content and shoot biomass, and a repression of the Pi-related stress response. Remarkably, shoot herbivory further enhanced the expression of *MtPT4* in the mycorrhizal roots. Moreover, in contrast to what was observed in non-mycorrhizal plants, shoot herbivory did not affect the expression of the high-affinity Pi transporters involved in the direct Pi-uptake pathway (*MtPT1*, *MtPT2*, *MtPT3* and *MtPT6*) in the mycorrhizal plants. This indicates that mycorrhizal colonisation shifts the herbivore-triggered Pi-uptake strategy in the plant, from the direct Pi-uptake pathway to the mycorrhizal Pi-uptake pathway. Interestingly, the enhancement of the mycorrhizal Pi-uptake pathway by herbivore feeding was concomitant with a boosted accumulation of Pi content. This suggests that the mycorrhizal Pi-uptake pathway is more efficient in taking up Pi upon herbivore feeding, compared to the direct pathway. Accordingly, mycorrhizal plants showed a higher tolerance

capacity to herbivore damage compared to non-mycorrhizal plants, even displaying over-compensatory shoot (vegetative) growth. It has been reported that the plant capacity of compensation for herbivory depends on the severity of herbivore damage (Quijano-Medina et al., 2019; Ramula et al., 2019). In our study, we found that the herbivore damage level was higher in the mycorrhizal plants. Therefore, to assess whether the differences in the compensatory responses observed between the mycorrhizal and non-mycorrhizal plants were driven by differences in the herbivore damage level among the treatments, we performed a bioassay in which we mechanically wounded the plants to homogenise the damage level. Our study demonstrated that, independent of the differences in the damage intensity between mycorrhizal and non-mycorrhizal plants, mycorrhizal colonisation led to a higher level of leaf Pi content after leaf damage, and an enhanced tolerance compared to non-mycorrhizal plants. Overall, our results suggest that mycorrhizal colonisation enhances the tolerance of *M. truncatula* plants to herbivore damage, and point to a prominent role of the activation of Pi uptake, specifically via the mycorrhizal pathway, as a mechanism in the enhanced tolerance.

Besides enhancing plant tolerance, we found that mycorrhizal colonisation facilitated herbivore performance. Previous studies demonstrate that the mycorrhizal-mediated enhanced susceptibility can be driven by the increase of the nutritional content in the leaf of the mycorrhizal plants (Bennett et al., 2006; Hoffmann et al., 2009b). Accordingly, we found a higher level of Pi, N and C in the leaf of the mycorrhizal plants, which are important indicators of plant nutrient quality for herbivores (Hoffmann et al., 2009b). Interestingly, besides the impact of the mycorrhizal colonisation on plant nutrition, our data prompt that mycorrhizal colonisation interfered with the JA burst mounted by the plant upon herbivore attack. In the non-mycorrhizal plants, shoot herbivory triggered the activation of the JA biosynthesis pathway, which is the master regulator of chemical defences against herbivores (Kessler & Baldwin, 2002). By contrast, this response was partially blocked in the mycorrhizal plants, indicating that the mycorrhizal colonisation impairs, at least partially, the activation of the herbivory-triggered JA pathway. As the activation of the JA-response by herbivory can be influenced by the extent of damage, and also the specific timing of the damage (Quijano-Medina et al., 2019; Tiffin, 2002), we assessed the impact of the mycorrhizal colonisation in mechanically wounded plants. In the mechanically wounded plants, the timing and the extent of damage were similar among the mycorrhizal and non-mycorrhizal plants. Similar to what was observed upon herbivory, the mycorrhizal colonisation reduced the wound-triggered JAs burst. Our data indicate that the enhanced herbivore performance mediated by mycorrhizal colonisation is driven not only by the improvement of the nutritional status, but most likely also by interfering with the immune response mounted by the plant upon herbivory. An increasing amount of evidence is showing that mycorrhizal colonisation can strongly affect the plant immune responses (Jung et al., 2012). Remarkably, the majority of these studies show that mycorrhizal colonisation can induce systemic resistance against herbivores, mainly by priming JA-regulated chemical defences (Gruden et al., 2020; Rivero et al., 2021; Song et al., 2013). Although we do not have a specific explanation for these apparent discrepancies, several studies point out that the impact of the AM fungi on plant chemical defences is highly genotype- and context-dependent (Bernaola & Stout, 2019; Fernández et al., 2014; Minton et al., 2016). It is remarkable that most of the studies reporting mycorrhizal-mediated priming of the JA-defences use tomato as a model plant, where the growth and nutritional status of the plant is overall, not improved by the mycorrhizal symbiosis. These results suggest that the plant genotype might exert a strong influence on the specific defence strategy that is shaped by the mycorrhizal symbiosis. Taken together, our results suggest that in *M. truncatula* plants mycorrhizal colonisation led to a shift in the defence strategy against herbivory, from a more resistance dominant strategy to a more tolerance dominant strategy.

The molecular mechanisms underlying the capability of the AM fungi for modulating the plant defence strategy remain poorly understood. We aimed to explore the possible role(s) of the mycorrhizal Pi-uptake pathway in the mycorrhizal-mediated modulation of the plant defence strategy. We found that in the *ha1-2* plants, which are impaired in Pi uptake via the mycorrhizal pathway (Krajinski et al., 2014; Wang et al., 2014b), the mycorrhizal-enhanced susceptibility was blocked. This indicates the involvement of the mycorrhizal Pi-uptake pathway in the enhanced susceptibility mediated by AM symbiosis. Interestingly, although to a lesser extent compared to the wild type, the colonisation by *R. irregularis* (non-functional mycorrhizal colonisation) still led to increased Pi levels in *ha1-2* herbivore-challenged plants. This suggests that in herbivore-challenged plants, further mechanisms unrelated to the mycorrhizal Pi-uptake pathway, are likely to contribute to the increased Pi levels. Recently, a link has been reported between the Pi-related plant response and JA-mediated resistance to insect herbivory (Chevalier et al., 2019; Khan et al., 2016; Li & Yu, 2018). Along the same lines, we found that in herbivore-challenged *ha1-2* plants, where the enhancement of Pi content mediated by the mycorrhizal colonisation was greatly compromised, mycorrhizal colonisation led to a higher level of JA-Ile, compared to that in WT plants. Interestingly, this enhanced level of JA-Ile in herbivore-challenged *ha1-2* mycorrhizal plants was not associated with a lower herbivore performance. These results might suggest that the impairment of herbivore-triggered increase in Pi content in the *ha1-2* plants is more determinant for the suppression of the mycorrhiza-mediated enhancement of herbivore performance in the *ha1-2*, than the boosted accumulation of JA-Ile in these plants. It is remarkable that in the herbivore-challenged WT plants, the mycorrhizal colonisation also led to a higher level of JA-Ile. Currently, we do not have a clear explanation for this specific phenotype, but it could be related to unknown side effects associated with the *Tnt1* retrotransposon insertion in the WT (Grandbastien et al., 1997; Melayah et al., 2001; Mhiri et al., 1997). Still, we found that in *ha1-2* plants, the impact of root colonisation on the JA-Ile level was amplified, suggesting a role for the mycorrhizal Pi-uptake pathway in the plant resistance phenotype mediated by the mycorrhizal symbiosis.

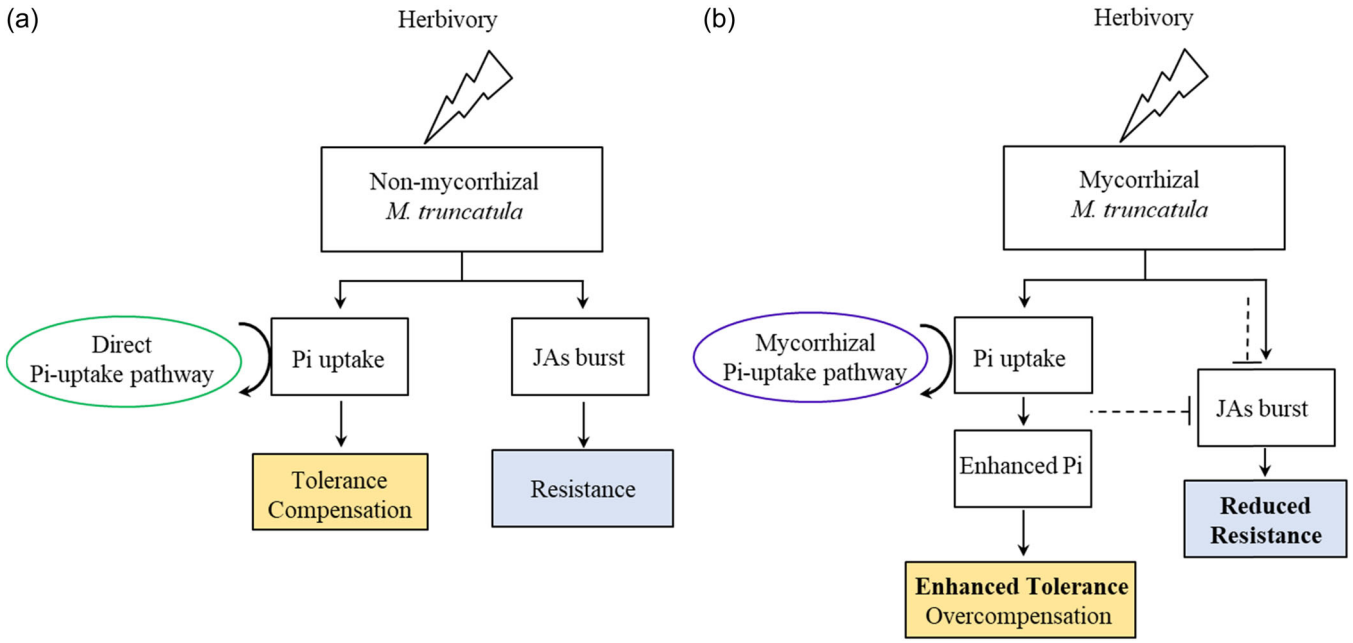

FIGURE 9 Proposed model for the modulation of the plant defence strategy mediated by the mycorrhizal symbiosis in *Medicago truncatula* plants. In non-mycorrhizal plants (a), shoot herbivory triggers systemically the activation of the direct (non-symbiotic) Pi-uptake pathway, and a burst of jasmonates (JAs) in the leaves. This leads to a specific tolerance/resistance plant phenotype. In the presence of the mycorrhizal symbiosis (b), shoot herbivory triggers specifically the activation of the mycorrhizal Pi-uptake pathway, which leads to an enhanced level of Pi in the leaves and stimulates an over-compensatory plant growth, enhancing plant tolerance to herbivory. The mycorrhizal symbiosis further partially counteracts the burst of jasmonates triggered by herbivory, leading to a reduction of the plant resistance to the herbivore. The specific mechanisms involved are still unknown (dotted lines). We proposed that the already described link between Pi- and JAs-signalling might, at least partially, mediate this phenotype. The reduced resistance promoted by the mycorrhizal symbiosis also contributes to herbivory facilitation. Pi, phosphate. [Color figure can be viewed at wileyonlinelibrary.com]

All in all, our results indicate that the presence of *R. irregularis* can strongly affect the defence pattern of *M. truncatula* plants at different levels (Figure 9). The mycorrhizal colonisation enhances the nutritional status of the plant and interferes with the plant resistance phenotype facilitating the herbivore performance. On the other hand, the mycorrhizal colonisation boosts the Pi uptake triggered by the herbivore, specifically via the mycorrhizal pathway, leading to a higher tolerance to damage. Our results further suggest a role of the mycorrhizal Pi-uptake pathway as a mechanism involved in the modulation of the plant defence strategy by AM symbiosis.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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