



# OPEN Fungal endophytes influence soil organic carbon and nitrogen fractions promoting carbon sequestration and improving grain yield in soybean

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Fungal endophyte inoculants present a promising avenue for enhancing carbon sequestration in agricultural systems. These endophytes can significantly influence soil organic carbon (SOC) and nitrogen (N) fractions by modulating root exudation, soil aggregation, and organic matter decomposition. We investigated the effectiveness of commercial-stage fungal endophyte seed inoculants in an Australian soybean field trial to increase yield, total SOC, stable SOC fractions, and soil N. After one growing season, specific inoculants (*Thozetella* sp. and *Leptodontidium* sp.) and dosages increased soybean grain yield and stocks of soil organic matter (SOM) as aggregate occluded particulate organic matter (oPOM) C and N, and mineral-associated organic matter (MAOM) C and N in the topsoil layer (0–15 cm). Furthermore, positive correlations were established between grain yield and the stocks of oPOM (C and N) and MAOM (C and N) in the topsoil layer (0–15 cm). Importantly, increasing grain yield was significantly and positively associated with the proportion of oPOM-C and N stocks to total SOM stock, providing evidence of significant carbon sequestration in oPOM. However, the proportion of MAOM-C and N stock to total SOM stock decreased significantly with increasing grain yield, indicating higher proportion of MAOM is being turned over relative to other SOM fractions although the absolute amounts of MAOM-C and N remained stable. These findings suggest that fungal endophytes and dosages may have variable but potentially beneficial impacts on crop growth, yield and play a crucial role in altering SOM fractions. This alteration potentially leads to changed carbon sequestration strategies, emphasising the need for further research into fungal endophyte-mediated carbon sequestration mechanisms.

**Keywords** Fungal endophytes, Soil organic carbon (SOC), SOC sequestration, Density fractionation, Soybean

The application of fungal endophytes in influencing crop performance and soil organic carbon (SOC) and total nitrogen (TN) dynamics has attracted substantial attention in agricultural research in recent years<sup>1–5</sup>. Fungal endophytes establish symbiotic associations with host plants, residing within plant tissues without causing apparent harm<sup>1</sup>. Instead, they may confer benefits to their hosts by enhancing nutrient acquisition, improving tolerance to environmental stressors, and promoting plant growth and hormone production<sup>1,2,6</sup>. These fungi not only facilitate the uptake of essential macronutrients and micronutrients from soil and organic matter but also significantly reduce the necessity for phosphorus fertilisation through improved phosphorus acquisition<sup>7–9</sup>. Their role extends beyond nutrient dynamics, enhancing plant resistance to various biotic stresses<sup>3,10,11</sup>, and abiotic stresses<sup>12</sup> to their plant hosts, thus contributing to overall plant health and productivity.

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Fungal endophytes have also been reported to influence SOC and TN dynamics, impacting C sequestration and nutrient cycling in agroecosystems<sup>4,13</sup>. Their association with roots may modulate soil organic matter (SOM) and influence SOC and TN fractions by changing root exudation patterns, microbial activities, and soil aggregation<sup>14–16</sup>, thereby affecting SOC and N availability<sup>17,18</sup>. These interactions enhance the accumulation of SOC by promoting root biomass and stimulating microbial activity in the rhizosphere leading to changes in SOC and TN cycling processes<sup>19</sup>. While root structure and exudates generally contribute to soil aggregation, legumes are particularly notable due to their biological nitrogen fixation, high root turnover, and quality of root exudates, which uniquely improve C and N stoichiometry, promote microbial activity, SOC accumulation, and aggregate stability<sup>20,21</sup>.

These microbial processes may lead to the transformation of C into more stable forms<sup>22,23</sup>. For example, previous studies have shown inoculating wheat with endophytes enhanced SOC stability with particular impact on aggregate C fractions<sup>24</sup> and CO<sub>2</sub>-kinetics-derived “passive” SOC pools<sup>25</sup>. We therefore hypothesise that legume plants can influence SOC dynamics by promoting the formation of stable soil aggregates and enhancing the retention of organic matter within microaggregates, leading to increased concentrations of aggregate occluded particulate organic matter (oPOM) and mineral-associated organic matter (MAOM) fractions in the rhizosphere soil of legumes<sup>26–28</sup>. While the addition of endophytic fungal isolates to increase SOC concentration and stock has shown promise, the detailed mechanisms through which these fungi influence the formation of stable SOC fractions and the overall microbial composition in the soil remain less understood<sup>24,25,29</sup>. In particular, studying and predicting stability of SOM is complex, and requires separation into functional fractions to understand its dynamics and contributions to SOM. In addition to oPOM and MAOM fractions, SOM also include free particulate organic matter (fPOM) fraction, which is unprotected, light fraction primarily composed of plant residues (including root fragments). The two key fractions, oPOM and MAOM, both are fundamentally different in physical and chemical properties, mean residence times in soil, and responses to environment and land use changes<sup>30–34</sup>. The oPOM has some protection through occlusion in large aggregates, whereas MAOM is largely mineral associated which is sorbed to mineral surfaces, and has been protected by occlusion in aggregates, organo-mineral clusters, and micropores<sup>32</sup>. Therefore, persistence and stability of MAOM is much stronger and longer than oPOM<sup>19,35,36</sup>.

Despite the growing recognition of the importance of fungal endophytes in agroecosystems, there remains a need for more empirical data to understand the influence of endophytic fungi and their dosages on host plants, and soil biota, including crop productivity and soil health<sup>5,37</sup>. At optimum inoculant levels, plants reward beneficial fungi with increased carbohydrate supply, leading to enhanced nutrient exchange and plant performance<sup>38,39</sup>. However, excessive fungal inoculation may result in carbon resource competition, where high microbial loads compete for limited root exudates, potentially reducing nutrient uptake efficiency and plant benefits<sup>40,41</sup>. Additionally, plant immune receptors may become desensitized to excessive fungal signalling, leading to reduced symbiotic efficiency<sup>39</sup>. This effect has been observed in rhizosphere interactions, where excess fungal exudates suppress plant receptor sensitivity, altering mycorrhizal recruitment and reducing benefits at higher inoculation dosages<sup>39,42</sup>.

This study aimed to evaluate the performance of two pre-screened and carefully selected fungal endophytes in soybean production, focusing on their role in plant growth, SOM and SOM fractions sequestration. The selection of inoculants and dosage (low, medium and high) levels was informed by extensive prior research and screening processes to ensure their effectiveness in promoting soybean growth and improving soil health. The fungal strains used, AU-2359 (*Thozetella* sp.) and AU-4873 (*Leptodontidium* sp.), are classified as dark septate endophytes (DSE) with facultative endophytism, meaning they associate with plant roots for part of their lifecycle but can also persist as free-living saprophytes in the soil. The field site was located in a semi-arid region, posing challenges related to soil carbon stabilisation<sup>43</sup>, and making it an ideal environment for evaluating the potential of these fungi in enhancing SOC dynamics within a soybean cropping system.

## Results

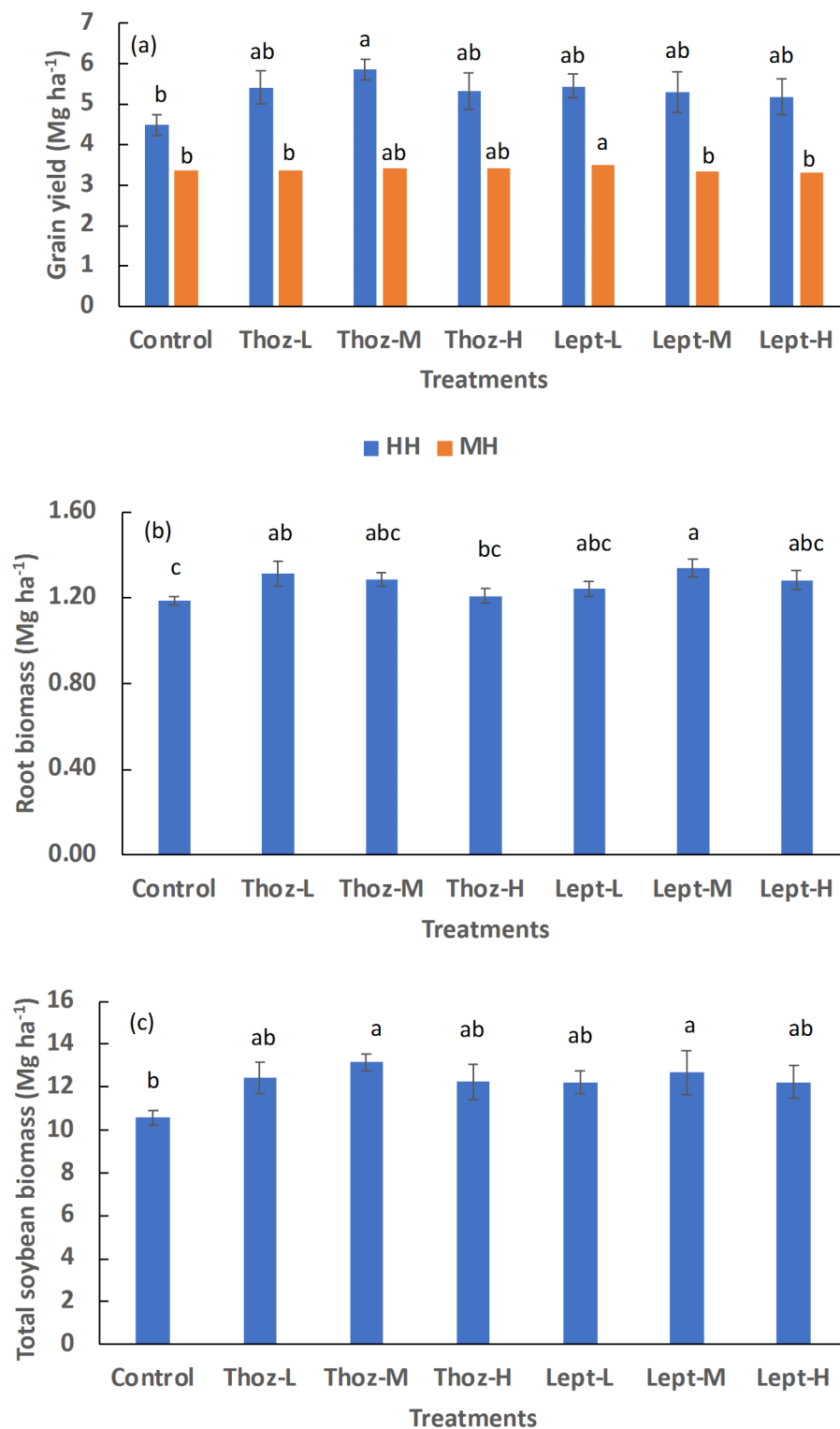
### Soybean grain yield, root biomass and total soybean biomass

Hand-harvested (HH) grain yield was significantly higher ( $P < 0.05$ ) in the *Thozetella* sp. medium dosage (Thoz-M), while machine-harvested (MH) grain yield was higher in the *Leptodontidium* sp. low dosage (Lept-L) compared to the control treatment (Fig. 1a). On average, inoculated treatments yielded 18% more grain yield (5.3 Mg ha<sup>-1</sup>) than the control (4.5 Mg ha<sup>-1</sup>) in hand-harvested samples. Root biomass yield (0–20 cm depth) in the control (1.19 Mg ha<sup>-1</sup>) was significantly lower than that in the Thoz-L (1.31 Mg ha<sup>-1</sup>) and *Leptodontidium* sp. medium dosage (Lept-M) treatments (1.34 Mg ha<sup>-1</sup>) (Fig. 1b). Similarly, Thoz-M and Lept-M showed significantly greater total plant biomass compared to the control (Fig. 1c).

A closer examination of machine-harvested grain yield (Supplementary Figure, S1) showed distinct inoculant-specific responses to increasing dosages. *Thozetella* showed a positive trend, with grain yield progressively increasing with higher dosages. In contrast, *Leptodontidium* showed inverse pattern, where higher inoculant dosages led to a decline in grain yield. A select group of treatments (Thoz-M, Thoz-H, and Lept-L) achieved relatively higher grain yields than the control in machine-harvested samples (Supplementary Figure, S1). While there was a positive correlation between the machine-harvested and hand-harvested grain yields (Supplementary Figure S2), hand-harvested mean grain yield data produced higher values (between 4.5 and 6.0 Mg ha<sup>-1</sup>) than the machine-harvested (between 3.3 and 3.5 Mg ha<sup>-1</sup>; Fig. 1a; Supplementary Figure S1).

### SOC and TN stocks after the crop harvest

The soil treated with fungal inoculant, *Thozetella* sp. at medium to high doses (Thoz-M and Thoz-H) tended to have higher SOC stocks, and the fungal inoculant, *Leptodontidium* sp. at low dose (Lept-L) had significantly higher SOC stocks at 0–15 cm depth than the control, although at 15–30 cm depth, SOC stocks were similar for



**Fig. 1.** Effect of fungal inoculant treatments on (a) soybean grain yield in hand-harvested (HH) and machine-harvested (MH) samples, (b) soybean root biomass, and (c) total soybean plant biomass. Where Thoz-L, M and H are low, medium, and high dosages of inoculant *Thozetella* sp., and Lept-L, M and H are low, medium, and high dosages of inoculant *Leptodontidium* sp. Bars represent standard error of the means (SE, n = 8). Mean values with letters not in common differ significantly at  $P < 0.05$ .

the treatments on equivalent soil mass basis since bulk density was not significantly affected by the treatments (Fig. 2a).

Also, consistent with the trends observed in machine-harvested grain yield (Supplementary Figure S1), SOC and TN stocks showed distinct responses to increasing inoculant dosages between *Thozetella* sp. (Thoz) and *Leptodontidium* sp. (Lept). *Thozetella* showed a positive dose-dependent response, with SOC and TN stocks progressively increasing at higher dosages. In contrast, *Leptodontidium* showed an inverse pattern, where increasing inoculant dosages led to a decline in SOC and TN stocks (Fig. 2a,b).

Cumulative SOC stock at 0–30 cm depth in Lept-L treatment was 6 Mg ha<sup>-1</sup> higher than the control ( $P < 0.05$ ), and in Thoz-M and Thoz-H treatments, SOC stocks were 4 Mg ha<sup>-1</sup> higher than the control but not significantly different ( $P > 0.05$ ) (Fig. 2a).

The stocks of TN (Fig. 2b) followed similar trend to that of SOC, since these values were closely correlated ( $r = 0.95$ ;  $P < 0.001$ ). Again, TN stocks in Thoz-M, Thoz-H, and Lept-L treatments were higher ( $P < 0.05$ ) than the control and contained 0.4–0.5 Mg ha<sup>-1</sup> of TN more than that in the control treatment. At 15–30 cm depth, TN stocks were similar in all treatments.

C:N ratio of SOM varied from 13.2 to 14.0 (mean value, 13.8) at 0–15 cm depth, but higher (14.8) at 15–30 cm depth although similar in all treatments (Fig. 2c).

### SOM fractions

Density and size separation of SOM into fPOM, oPOM and MAOM were significantly affected by the treatments. Proportions of soil fractions, recovery of SOM-C and SOM-N, and concentrations of C and N in various SOM fractions (fPOM, oPOM and MAOM) are presented in Supplementary Table S1. Stocks and proportions of C and N for SOC fractions are respectively presented in Fig. 3 and Table 1. Specific inoculum treatments and dosages resulted in greater stocks of SOC fractions compared with the control (Fig. 3a). For example, stock of fPOM fraction was significantly higher for Thoz-L treatment, whereas for oPOM, Thoz-H, Lept-L and Lept-M treatments, and for MAOM, Lept-L treatment were significantly higher, compared to the control (Fig. 3a). Similarly, Thoz-L, Thoz-M and Lept-L treatments resulted in greater fPOM stocks of TN compared with the control, whereas Thoz-H, and Lept-L treatments had greater oPOM, and Thoz-M, Thoz-H and Lept-L had greater MAOM fractions of TN compared with the control (Fig. 3b).

Note, fPOM stocks of C and N showed a decreasing trend with increasing dosages for both *Thozetella* sp. and *Leptodontidium* sp. In contrast, oPOM and MAOM stocks of C and N followed similar patterns to the total SOC and TN stocks observed earlier. Specifically, *Thozetella* showed a positive response, with increasing dosages enhancing oPOM and MAOM stocks of C and N, whereas *Leptodontidium* showed a negative response, where higher dosages led to declines in these fractions (Fig. 3a,b).

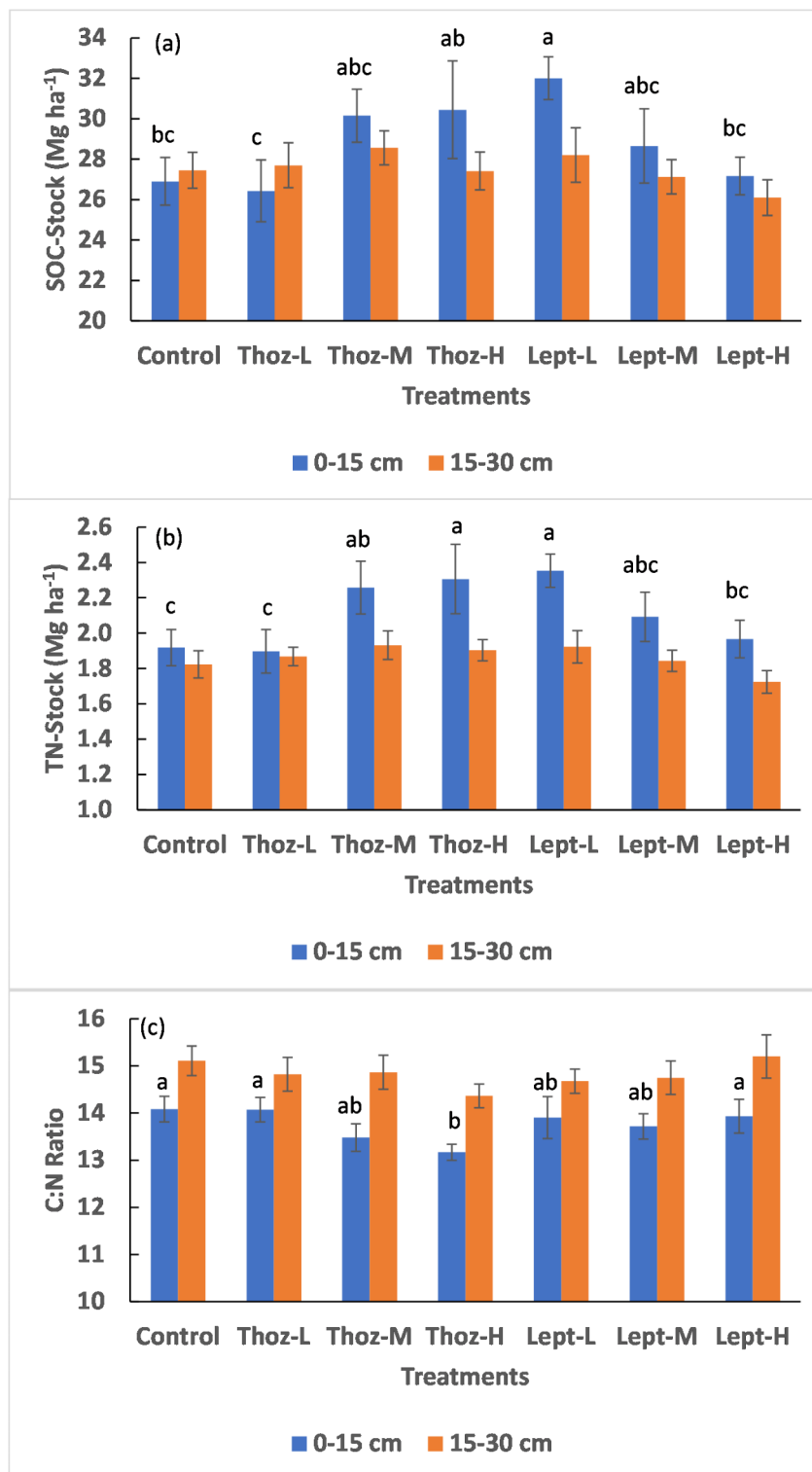
Eighty-eight percent (88.1%) of SOC was present as MAOM-C; both fPOM-C and oPOM-C were present in similar proportions, 5.4% and 6.4%, respectively (Table 1). However, the endophyte treatments significantly affected the distribution of each fraction. For fPOM, the Thoz-L treatment had the highest proportion (8.2%), followed by Thoz-M (6.2%), with no significant difference between them, and fPOM proportion in the control (4.8%) was significantly lower than Thoz-L. The oPOM proportions were significantly higher in Thoz-H and Lept-M than the control. For MAOM-C, the control treatment had the highest proportion (89.7%) and differed significantly from the Thoz-L treatment (86.2%). However, there were no significant differences between the control and other inoculant treatments (ranging between 87.1% and 89.1%) for MAOM-C fraction of SOC. Comparison between 0–15 cm and 15–30 cm depths showed that the soil at the 0–15 cm depth had significantly higher proportion of oPOM than that at 15–30 cm depth (6.4% versus 3.1%); distribution of SOC between the two depths for fPOM-C and MAOM-C was not significantly different (Table 1).

The N proportions of TN present in fPOM, oPOM and MAOM at 0–15 cm depth were 1.7, 8.2 and 89.9%, respectively (Table 1). That is, similar to C, most of the soil N was present in MAOM. However, the fungal inoculant treatments significantly affected the distribution of TN proportions among different SOM fractions. The soil from the Thoz-L treatment contained the highest proportion of fPOM-N, which was significantly different from the control, Thoz-H, Lept-M and Lept-H treatments but similar to Thoz-M and Lept-L treatments. The highest proportion of oPOM-N was in the soil from the Thoz-H treatment, which was 28% higher than the control. However, the proportion of MAOM-N was the highest in the control treatment although it was significantly higher than that in the Thoz-H treatment only. At 15–30 cm depth, the proportion of fPOM-N was only 0.6%, and oPOM-N was 6.2%, significantly lower than proportions in 0–15 cm layer, while MAOM-N was 3% higher ( $P < 0.05$ ) than that in the 0–15 cm layer and constituted almost 93% of TN (Table 1).

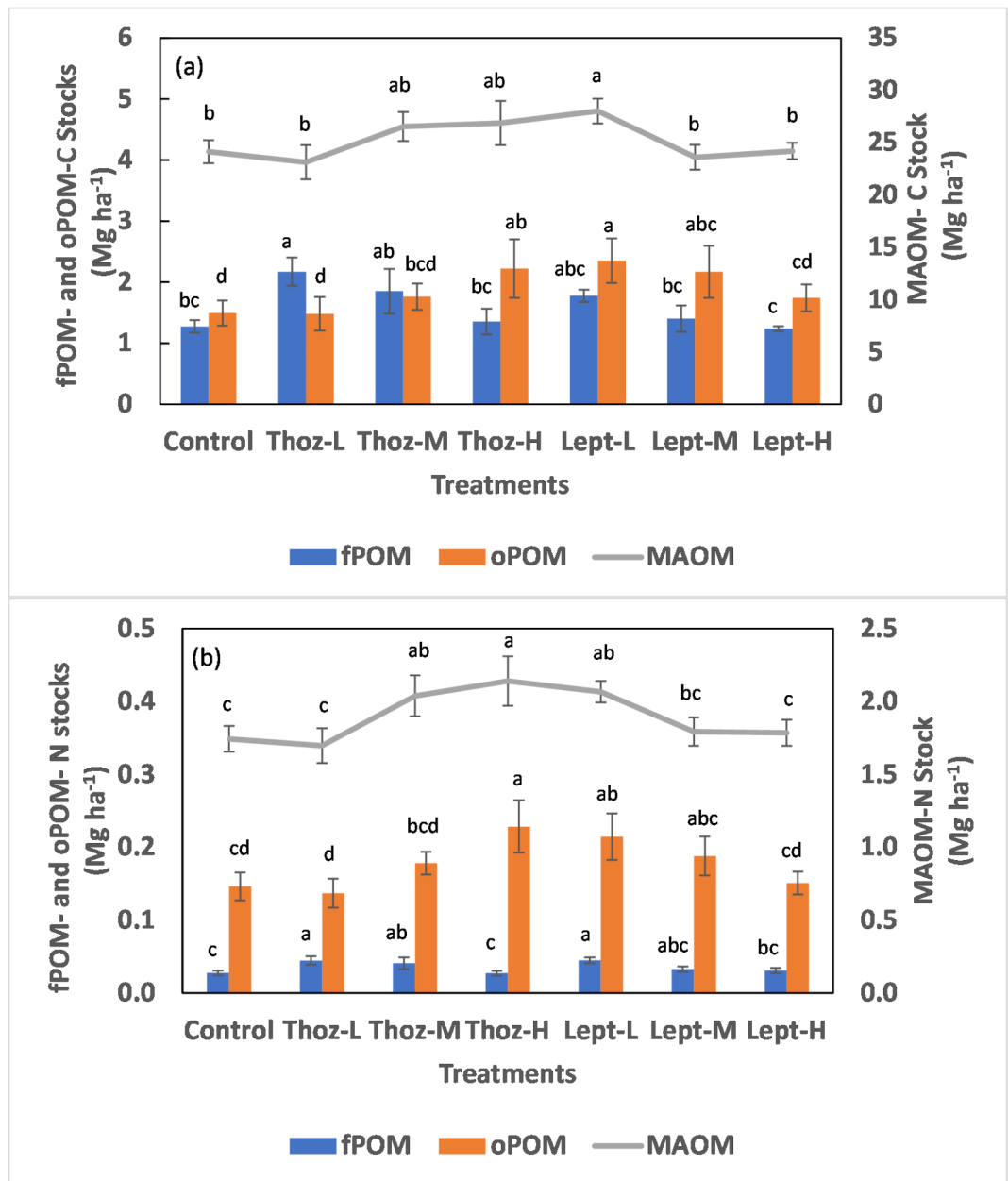
The mean C:N ratios of fPOM, oPOM and MAOM at 0–15 cm depth were 39.8, 9.2 and 11.9, respectively, and showed only small variations among treatments including the control (Supplementary Figure, S3). At 15–30 cm depth, C:N ratio of the fPOM fraction was much higher (80–100) than that in the 0–15 cm layer.

### Relationships between soil organic matter and its fractions and soybean yield

The stocks of both SOC and TN were significantly correlated with soybean grain yields (machine or hand-harvested) (Table 2). However, the stocks of oPOM-C and oPOM-N were more closely correlated than the total SOC and TN with soybean grain yield ( $P < 0.001$ ). Similar correlations were observed when oPOM-C as a proportion of SOC and oPOM-N as a proportion of TN were considered ( $r = 0.67$ ,  $P < 0.001$  for oPOM-C;  $r = 0.61$ ;  $P < 0.001$  for oPOM-N) with machine harvested grain yield. Although the stocks of MAOM-C and MAOM-N were positively correlated with soybean yield, when MAOM-C as a proportion of SOC and MAOM-N as a proportion of TN were considered, the correlations with grain yield were negative ( $r = -0.37$ ,  $P < 0.01$  for MAOM-C;  $r = -0.61$ ;  $P < 0.001$  for MAOM-N). This suggests that the stocks of oPOM increased by a greater percent than MAOM. Stocks of oPOM (C and N) increased around fivefold, whereas stocks of MAOM (C and N) increased around twofold with increasing grain yields. SOC:TN ratio, and fPOM-C:fPOM-N ratios were



**Fig. 2.** Effect of fungal inoculants on (a) SOC-stock, (b) TN-stock, and (c) C:N ratio at 0–15 and 15–30 cm depths after the crop harvest. Where Thoz-L, M and H are low, medium, and high dosages of inoculant *Thozetella* sp., and Lept-L, M and H are low, medium, and high dosages of inoculant *Leptodontidium* sp. Bars represent standard error of the means (SE, n = 8). Mean values for the top layer (0–15 cm) with letters not in common differ significantly at  $P < 0.05$ . No significant differences in SOC, TN, and C:N for 15–30 cm layer.



**Fig. 3.** Effect of fungal inoculant treatments on (a) SOC stocks and (b) N stocks for fPOM, oPOM and MAOM at 0–15 cm depth. Where Thoz-L, M and H are low, medium, and high dosages of inoculant *Thozetella* sp., and Lept-L, M and H are low, medium, and high dosages of inoculant *Leptodontidium* sp. Bars represent standard error of the means (SE, n = 8). Mean values with letters not in common differ significantly at  $P < 0.05$ .

negatively correlated with the grain yield but the oPOM-C:oPOM-N ratio was positively correlated with the grain yield (Table 2).

The trends in the correlations between oPOM, MAOM and the root biomass were similar to that with the grain yield, although these reached the level of significance only with oPOM-C, both with its stock ( $r = 0.31$ ,  $P < 0.05$ ) and its proportion of SOC ( $r = 0.35$ ,  $P < 0.01$ ) (Table 2).

To visualise the association of stocks of oPOM (C and N) and MAOM (C and N) with grain yield, linear regressions between machine harvested grain yields and stocks of oPOM (C and N) and MAOM (C and N) were constructed (Fig. 4). Positive linear regressions were observed for stocks of oPOM (C and N) and MAOM (C and N). Increasing stocks of oPOM (C and N) and MAOM (C and N) significantly increased grain yield. However, stocks of oPOM were more closely related to grain yield ( $R^2 = 0.51$ , for C,  $R^2 = 0.58$  for N stocks;  $P < 0.001$  for both C and N stocks, Fig. 4a,b) compared to stocks of MAOM ( $R^2 = 0.14$   $P < 0.05$  for C stock;  $R^2 = 0.23$ ,  $P < 0.01$  for N stock, Fig. 4c,d).

| Treatment       | Proportion of total SOC (%) |              |              | Proportion of TN (%) |              |              |
|-----------------|-----------------------------|--------------|--------------|----------------------|--------------|--------------|
|                 | fPOM-C                      | oPOM-C       | MAOM-C       | fPOM-N               | oPOM-N       | MAOM-N       |
| Control         | 4.8±0.40 b                  | 5.5±0.64 bc  | 89.7±0.77 a  | 1.4±0.14 bc          | 7.5±0.67 d   | 91.0±0.78 a  |
| Thoz-L          | 8.2±0.87 a                  | 5.3±0.55 c   | 86.2±0.70 b  | 2.4±0.18 a           | 7.3±0.51 d   | 89.1±0.81 ab |
| Thoz-M          | 6.2±1.33ab                  | 5.8±0.61 bc  | 88.0±1.41 ab | 1.9±0.39 ab          | 8.0±0.53 bcd | 90.1±0.81 ab |
| Thoz-H          | 4.6±0.75 b                  | 7.4±1.00 a   | 88.1±1.03 ab | 1.3±0.19 c           | 9.6±0.90 a   | 89.0±0.93 b  |
| Lept-L          | 5.4±0.46 b                  | 6.9±1.05 ab  | 87.7±1.05 ab | 1.9±0.16 abc         | 8.9±0.93 abc | 89.3±1.03 ab |
| Lept-M          | 5.2±0.77 b                  | 7.7±1.15 a   | 87.1±1.12 ab | 1.6±0.19 bc          | 9.1±0.77 ab  | 89.2±0.76 ab |
| Lept-H          | 4.6±0.26 b                  | 6.3±0.62 abc | 89.1±0.58 ab | 1.5±0.11 bc          | 7.6±0.46 cd  | 90.9±0.54 a  |
| Mean (0–15 cm)  | 5.4±0.33 A                  | 6.4±0.33 A   | 88.1±0.40 A  | 1.7±0.09 A           | 8.2±0.29 A   | 89.9±0.33 A  |
| Mean (15–30 cm) | 5.0±0.36 A                  | 3.1±0.12 B   | 89.5±1.52 A  | 0.6±0.05 B           | 6.2±0.20 B   | 92.7±0.35 B  |

**Table 1.** Proportions of soil organic carbon (SOC) and total soil nitrogen (TN) in free particulate organic matter (fPOM), aggregate occluded particulate organic matter (oPOM) and mineral-associated organic matter (MAOM) fractions at 0–15 cm depth, and comparison with the mean values at 15–30 cm depth. Where Thoz-L, M and H are low, medium, and high dosages of inoculant *Thozetella* sp., and Lept-L, M and H are low, medium, and high dosages of inoculant *Leptodontidium* sp. + / – represents standard error of the means (SE, n = 8). Different lower-case letters represent significant difference at  $P < 0.05$  between treatment means, upper case letters for the depth means.

| SOM and fractions   | MH grain yield | HH grain yield | Root biomass | Total soybean biomass |
|---------------------|----------------|----------------|--------------|-----------------------|
| SOC stock           | 0.43**         | 0.41**         | 0.13         | 0.42**                |
| TN stock            | 0.54***        | 0.49***        | 0.21         | 0.50***               |
| fPOM-C stock        | –0.10          | –0.08          | –0.15        | –0.09                 |
| oPOM-C stock        | 0.72***        | 0.56***        | 0.31*        | 0.56***               |
| MAOM-C stock        | 0.34**         | 0.34**         | 0.10         | 0.36**                |
| fPOM-N stock        | 0.29*          | 0.28*          | 0.22         | 0.31*                 |
| oPOM-N stock        | 0.74***        | 0.53***        | 0.25         | 0.52***               |
| MAOM-N stock        | 0.46***        | 0.45***        | 0.18         | 0.46***               |
| fPOM-C (% SOC)      | –0.21          | –0.20          | –0.20        | –0.22                 |
| oPOM-C (% SOC)      | 0.67***        | 0.53***        | 0.35**       | 0.53***               |
| MAOM-C (% SOC)      | –0.37**        | –0.26*         | –0.11        | –0.25*                |
| fPOM-N (% TN)       | 0.06           | 0.06           | 0.09         | 0.08                  |
| oPOM-N (% TN)       | 0.61***        | 0.43**         | 0.24         | 0.41**                |
| MAOM-N (% TN)       | –0.61***       | –0.40**        | –0.25        | –0.40**               |
| SOC:TN ratio        | –0.53***       | –0.48***       | –0.34*       | –0.47**               |
| fPOM-C:fPOM-N ratio | –0.33*         | –0.32*         | –0.36*       | –0.36*                |
| oPOM-C:oPOM-N ratio | 0.32*          | 0.29**         | 0.23         | 0.40**                |
| MAOM-C:MAOM-N ratio | –0.16          | –0.18          | –0.30*       | –0.18                 |

**Table 2.** Pearson correlation coefficients between SOM and its fractions and soybean machine-harvested (MH) and hand-harvested (HH) grain yield, root biomass yield and total soybean plant yield (n = 56).

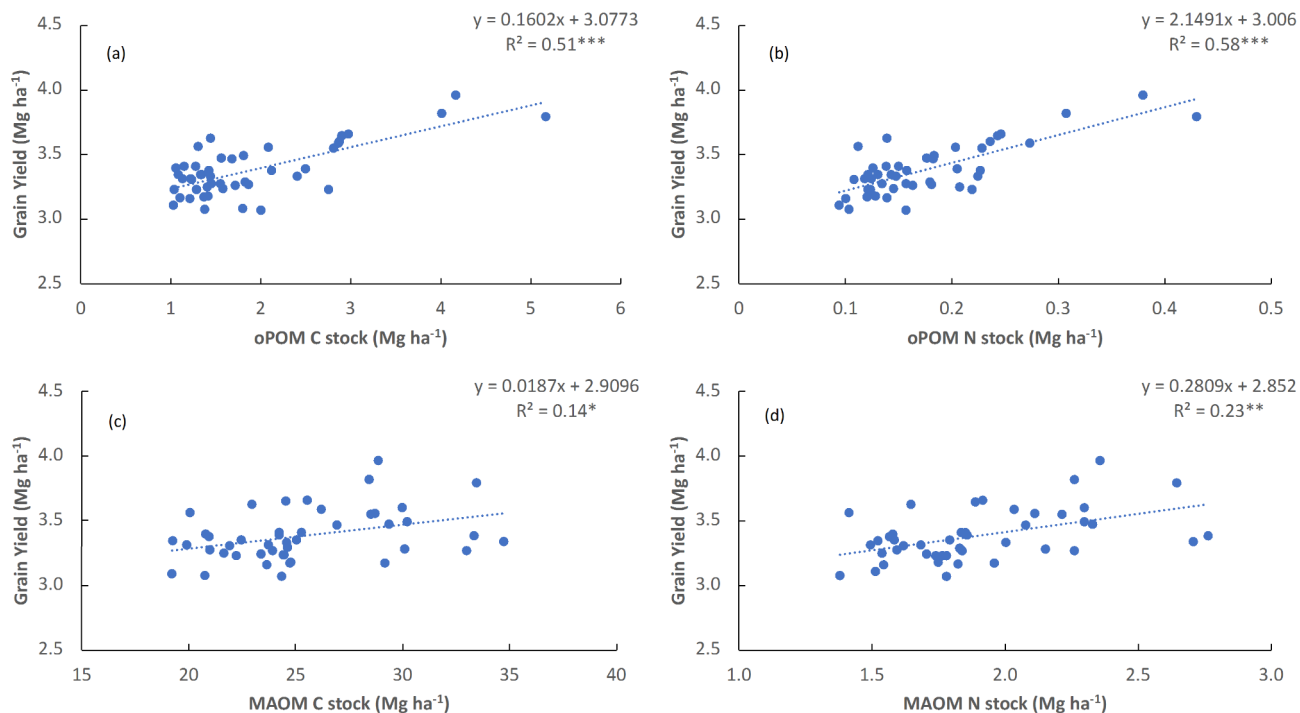
### Microbial C and N biomass

Microbial biomass C (MBC) was relatively higher in fungal endophyte-treated soils compared to the control (Fig. 5a). In particular, Thoz-H and Lept-L treatments showed 75% and 87% greater ( $P < 0.05$ ) MBC than the control. In contrast, microbial N biomass N (MBN) showed no significant differences among treatments (Fig. 5b), indicating that fungal inoculation had a more pronounced effect on MBC dynamics than on MBN accumulation. It is important to note here that the pattern of MBC responses to increasing dosages closely aligned with the trends observed for total SOC-C, TN, and oPOM and MAOM-C and N stocks in *Thozetella* treatments. Whereas, for *Leptodontidium*, the declining trend in MBC with increasing dosages was less pronounced.

### Effect of selected inoculants and dosages on Arbuscular mycorrhizal colonisation

We quantified arbuscular mycorrhizal fungi (AMF) colonisation by assessing the presence of arbuscules in root segments of soybean plants across selected treatments (Control, Thoz-M, Thoz-H, Lept-L and Lept-M) (Fig. 6). Treatments were chosen based on post flowering differences in plant growth.

Our results showed a significant effect of inoculant and their dosages on AMF colonisation. AMF colonisation was significantly higher in the Thoz-M and Lept-M treatments compared to control (Fig. 6). This highlights the potential synergistic interactions between fungal endophytes and AMF in enhancing root colonisation.



**Fig. 4.** Linear relationships between SOM fractions (soil organic C, SOC; total nitrogen, TN), MAOM and oPOM at 0–15 cm depth and soybean grain yields. **(a)** SOC stock of oPOM-C, **(b)** TN stock of oPOM-N, **(c)** SOC stock of MAOM-C, and **(d)** TN stock of MAOM-N. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

Microphotographs showed the presence of vesicles and hyphal coils, with the qualitative confirmation of dark septate hyphae (Fig. 7).

## Discussion

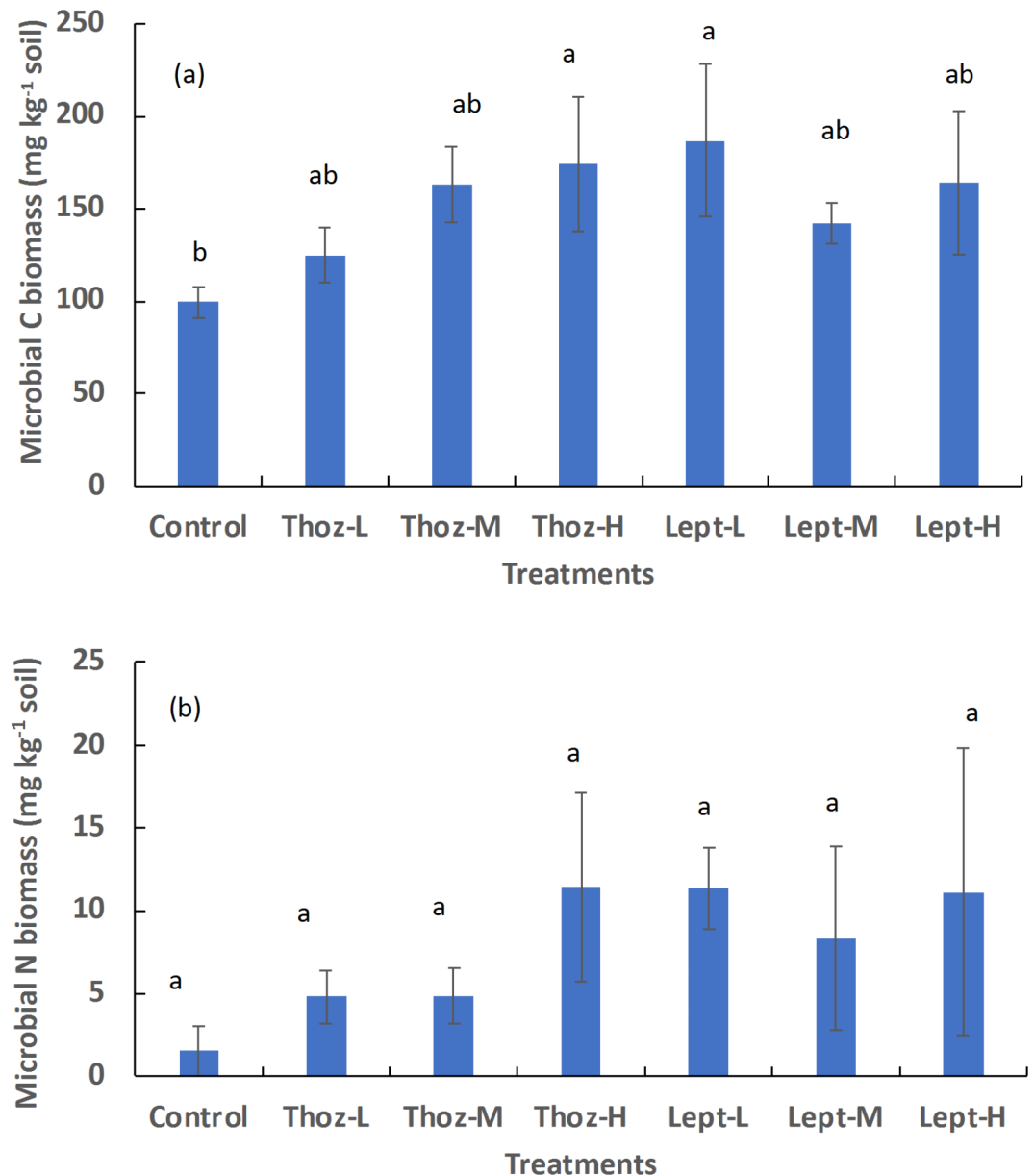
### Effect of fungal endophytes on soybean growth and grain yield

This study showed how certain inoculants, and their specific dosages could significantly enhance soybean plant growth and grain yield. Our findings indicated the general role of fungal endophytes in mediating root and shoot growth, as demonstrated in previous studies<sup>6,10,29,44–47</sup>. Fungal inoculants can enhance plant growth directly through hormone production, improved nutrient acquisition, and stress tolerance mechanisms, but they also indirectly influence biological processes and interactions within the soil and rhizosphere<sup>48</sup>. These interactions are critical for nutrient mineralisation, solubilisation, uptake, and translocation to the root, mediated by a dynamic microbial population in the rhizosphere, including arbuscular mycorrhizal fungi (AMF)<sup>48–51</sup>.

In this study, the fungal Inoculant *Thozetella* sp. at medium (hand-harvested) and *Leptodontidium* sp. at a low dosage (machine-harvested) were able to outperform the control in soybean grain yield under field conditions. The higher grain yield observed in hand-harvested samples compared to machine-harvested samples could be attributed to the nature of the harvesting methods. Hand harvesting involved selectively picking plants, often focusing on average-looking plants from the middle two rows of the plant bed. This selective process inherently reduced variability in plant health and density, resulting in higher yield estimates. In contrast, machine harvesting encompassed all rows, except two border rows of the plant bed, spanning from mid-furrow to mid-furrow. This results in inclusion of a broader range of plant conditions, including potentially less vigorous or damaged plants. Additionally, machine-harvesting could have introduced mechanical factors leading to grain loss due to shattering during the harvesting process. These differences highlight why hand-harvested values may be higher and why machine-harvested yields, while representing a more comprehensive picture of field-scale variability, might underestimate yield due to these operational losses. Nevertheless, supporting our observations, research by Zhang et al.<sup>44</sup> and Russo et al.<sup>45</sup> also highlighted the positive impact of inoculation on soybean plant growth and yield under field conditions.

### Role of root growth and its relationship with SOC

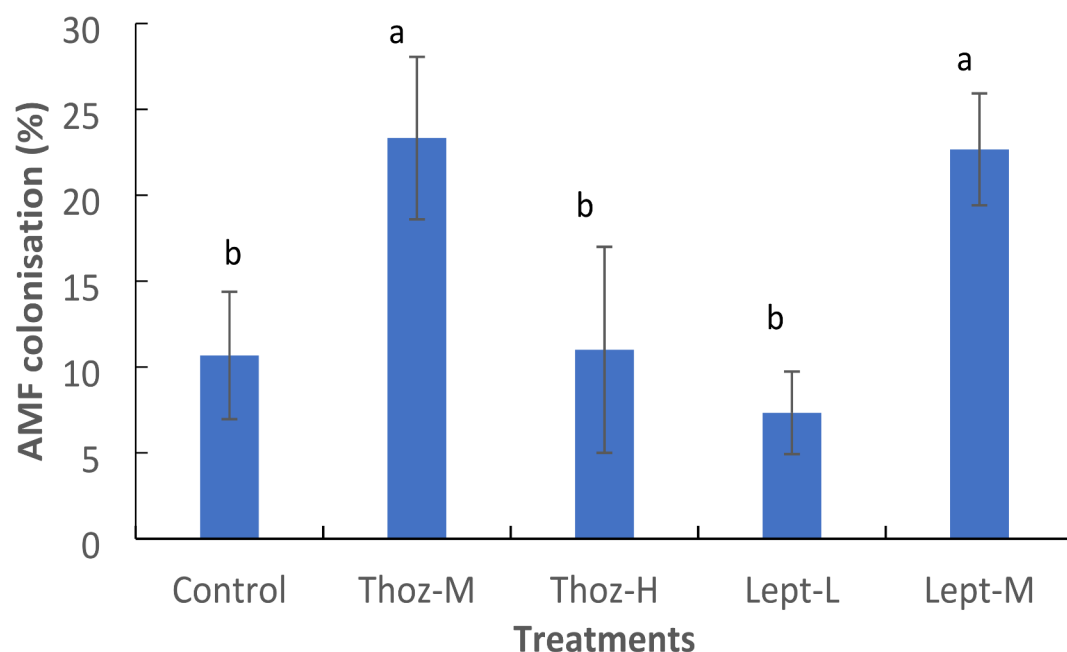
Root development plays a crucial role in carbon inputs to SOC pools, particularly through root exudation, turnover, and decomposition processes. In our study, inoculated treatments not only enhanced aboveground biomass and grain yield but also stimulated root biomass production, which in turn had direct implications for SOC sequestration and soil aggregation. The increase in root biomass with specific fungal inoculants is consistent with previous findings<sup>52–54</sup>, suggesting that root-derived carbon inputs contribute to enhancing particulate organic matter (POM) fractions in soil and promoting microbial-mediated stabilisation of organic matter.



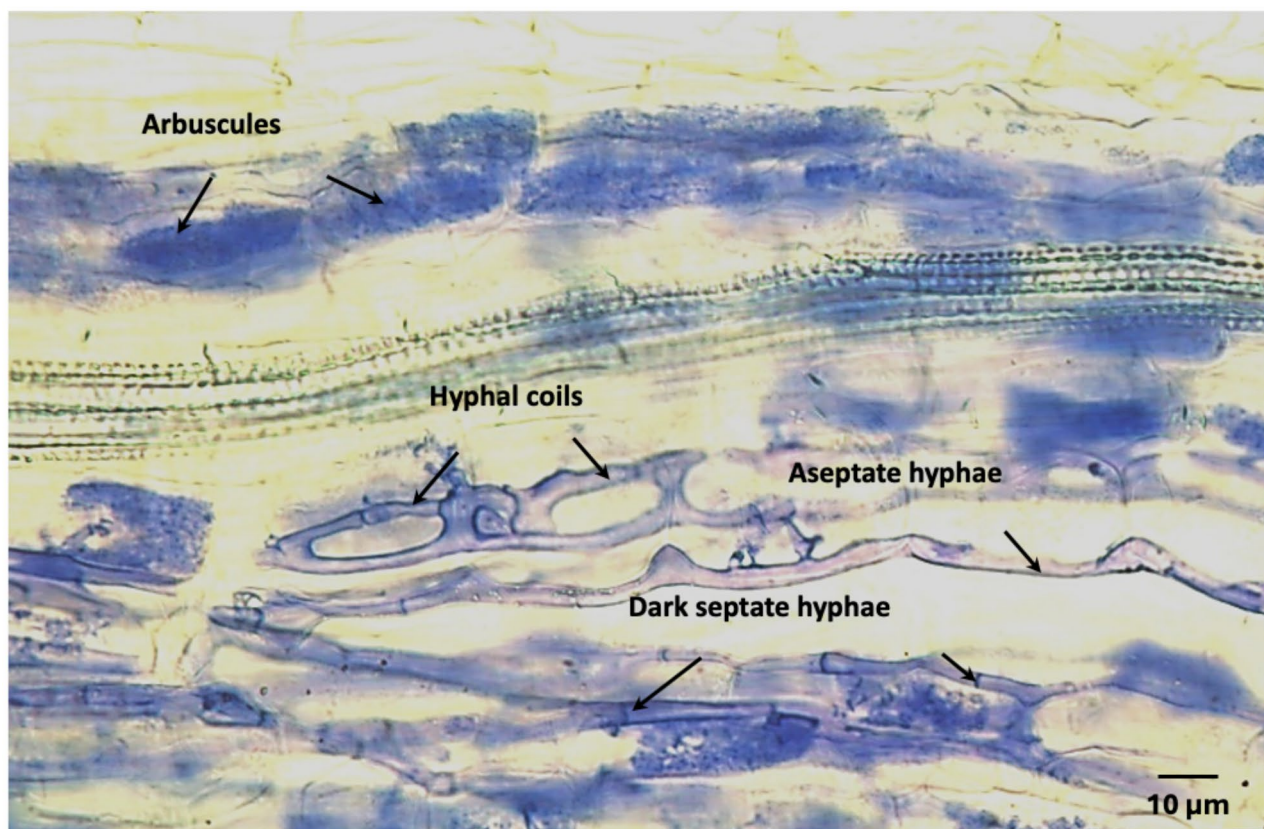
**Fig. 5.** Effect of fungal inoculant treatments on (a) microbial biomass C and (b) microbial biomass N at 0–15 cm depth. Where Thoz-L, M and H are low, medium, and high dosages of inoculant *Thozetella* sp., and Lept-L, M and H are low, medium, and high dosages of inoculant *Leptodontidium* sp. Bars represent standard error of the means (SE,  $n = 3$ ). Mean values with letters not in common differ significantly at  $P < 0.05$ .

Our study also found that root biomass was significantly correlated with oPOM-C stock and oPOM-C proportion (Table 2). This indicates that greater root growth likely contributed to increased organic matter inputs, which were subsequently incorporated into aggregate-occluded soil organic matter fractions. This relationship is particularly important for Vertisols, where soil aggregation plays a key role in carbon stabilisation<sup>43</sup>. The increased oPOM fractions in specific inoculated treatments suggest that fungal endophytes may have indirectly facilitated soil carbon sequestration by enhancing root biomass production and exudation. While our study showed significant correlations between root biomass and oPOM fractions, no significant correlations were observed between root biomass and total SOC stock or MAOM fractions. This suggested that root proliferation primarily contributed to the formation and stabilisation of oPOM rather than directly affecting total SOC stock.

Higher MBC in specific fungal-inoculated treatments suggested that root-derived carbon inputs were actively utilised by soil microbial communities. This could have further stimulated organic matter turnover and stabilisation in soil aggregates. These findings are supported by previous studies emphasising the role of root-derived carbon in fostering microbial activity and enhancing soil aggregation processes<sup>55–57</sup>. However, it is important to note that root biomass was measured at post flowering, which may not fully capture its contribution to SOM formation throughout the growing season, an area that warrants further research.



**Fig. 6.** Effect of selected fungal inoculant treatments on arbuscular mycorrhizal fungi (AMF) colonisation. Where Thoz-M and H are medium and high dosages of inoculant *Thozetella* sp., and Lept-L and M are low and medium dosages of inoculant *Leptodontidium* sp. Bars represent standard error of the means (SE,  $n = 3$ ). Mean values with letters not in common differ significantly at  $P < 0.05$ .



**Fig. 7.** Microphotograph showing arbuscular mycorrhizal fungi (AMF) colonisation (Arbuscules and Aseptate hyphae), along with the Dark septate hyphae. Photos were taken at  $400\times$  magnification and the scale bar =  $10\ \mu\text{m}$ .

In addition to MBC analysis, preliminary anatomical studies on soybean plants from the experiment revealed increased AMF colonisation in root sections from specific inoculant treatments (Thoz-M and Lept-M). These observations provide direct evidence of interactions between fungal endophytes and plant roots. However, to gain a more comprehensive understanding of these fungal-plant interactions under field conditions, future research should incorporate detailed anatomical and molecular assessments.

### Dosage-dependent effects of fungal endophyte inoculation on soil carbon and nitrogen dynamics

The differential responses of *Thozetella* and *Leptodontidium* to increasing inoculant dosages can be explained by distinct mechanistic processes governing plant-microbe-soil interactions. For *Thozetella*, the progressive increases in grain yield, SOC and TN stocks, oPOM and MAOM-C and N stocks, and microbial biomass with higher dosages suggest a positive feedback loop driven by multiple factors. First, moderate to high fungal colonisation likely enhanced plant signalling and symbiotic efficiency, allowing plants to maximise benefits without triggering desensitisation of fungal receptors<sup>39</sup>. Second, *Thozetella* may have facilitated improved carbon allocation, where plants increased exudate production and root-derived C inputs, supporting greater microbial biomass and soil carbon sequestration<sup>40</sup>. This aligns with findings that moderate to high fungal colonisation can stimulate soil aggregate formation, enhancing organic matter protection and SOC stability<sup>52</sup>. Additionally, *Thozetella* may have promoted a balanced microbial community structure that favoured soil aggregation and slow carbon turnover, leading to progressive increases in soil carbon pools<sup>41</sup>. The enhanced microbial activity at higher *Thozetella* dosages likely contributed to more efficient nutrient cycling and nitrogen retention in the soil, supporting plant productivity and sustaining the observed yield increases<sup>38</sup>.

Conversely, *Leptodontidium* exhibited a progressive decline in plant and soil responses with increasing dosages, likely due to excessive fungal colonisation triggering a plant-mediated downregulation of symbiotic investment<sup>39,42</sup>. At higher inoculant levels, the plant may have perceived *Leptodontidium* as overly costly, reducing carbon allocation towards fungal interactions in favour of direct nutrient acquisition via root growth<sup>38,52,58–61</sup>. This shift likely disrupted carbon fluxes in the rhizosphere, leading to lower microbial biomass and decreased soil aggregation, ultimately reducing SOC and TN stocks<sup>40,52</sup>. Additionally, the higher dosages of *Leptodontidium* may have fostered rapid microbial turnover, increasing carbon mineralisation rather than stabilisation, thereby exacerbating SOC losses<sup>41</sup>. The observed decline in grain yield with increasing *Leptodontidium* dosages further supports the hypothesis that excessive fungal loads imposed a metabolic burden on the plant, resulting in reduced nutrient uptake efficiency and lower productivity.

These contrasting responses highlight the importance of optimising fungal inoculant dosage to balance plant-microbe interactions, ensuring that beneficial fungi enhance rather than disrupt plant and soil health. Future studies should further explore these dosage-dependent relationships to optimise the application of fungal inoculants for sustainable soil management and improved crop productivity in different environments and soil types.

### Effect of fungal inoculants on SOM and SOM fractions

The fractionation of SOC into fPOM, oPOM, and MAOM, provided valuable insights into the dynamics of C sequestration and stabilisation in soil systems<sup>25,47,57</sup>. The proportions of these fractions varied significantly between the topsoil layer (0–15 cm) and the subsoil layer (15–30 cm), reflecting differences in organic matter input, decomposition rates, and microbial activity across soil depths. Our findings indicated that fungal endophyte inoculation influenced the proportions of all three SOC fractions, particularly in the topsoil layer. The fPOM fraction, which represents the most labile SOC pool, was highest in the *Thozetella* sp. (low dosage) treatment. This fraction is composed of fresh plant residues and microbial biomass, including fungal hyphae, and is crucial for short-term carbon dynamics<sup>52</sup>.

The oPOM fraction, which is a more stable SOC pool protected within soil aggregates, showed significant increases in treatments inoculated with high-dosage *Thozetella* sp., and low and medium dosages of *Leptodontidium* sp., compared to the control (Table 1, Supplementary Table S1). This is consistent with previous studies by Franzluebbers and Stuedemann<sup>53</sup> and Handayani et al.<sup>54</sup>. They demonstrated that endophyte-infected fescue plants showed higher C concentrations in oPOM fractions, which was attributed to increased root biomass and rhizodeposition.

Conversely, minimal changes were observed in MAOM fractions across treatments, suggesting that MAOM remained relatively stable. This stability may be due to a shift in carbon allocation toward aggregate-protected oPOM fractions rather than increasing long-term mineral-associated carbon storage. Furthermore, the potential incorporation of MAOM into oPOM aggregates could explain the limited variation observed in MAOM fractions. These findings highlight the dynamic nature of soil carbon stabilisation processes and emphasize the role of fungal endophytes in modifying soil structure. This is particularly relevant in Vertisols, where MAOM constitutes the dominant SOM pool, and enhanced aggregation has important implications for soil health and carbon sequestration<sup>43</sup>.

The role of microbial biomass in these transformations is also evident from the significant increases in MBC in high dosage of inoculant *Thozetella* sp., and low dosage of inoculant *Leptodontidium* sp. treatments, which also showed higher SOC stocks and oPOM fractions. These findings suggested that microbial activity, stimulated by fungal inoculation, could have contributed to organic matter stabilisation by promoting root exudate utilisation and microbial necromass accumulation in SOM fractions.

The effects of fungal endophyte inoculation on SOC fractions were more pronounced in the topsoil layer than in the subsoil. The proportion of oPOM-C in the subsoil was approximately half of that observed in the topsoil, likely due to lower root biomass, reduced microbial activity, and diminished organic carbon inputs at deeper depths. Franzluebbers et al.<sup>55</sup> also reported greater particulate organic matter (POM) C and N in the top

layer with higher endophyte infection compared to lower endophyte infection levels. They suggested that higher POM-C and POM-N at this depth could indicate increased root activity, which was typically associated with greater endophyte infection<sup>56</sup>. In this context, Franzluebbers et al.<sup>55</sup> and Franzluebbers and Stuedemann<sup>53</sup> also reported that presence of higher microbial C in endophyte-infected soils, suggesting increased biological activity but lower mineralisable C, which was indicative of enhanced organic matter stabilisation. These results highlight the sensitivity of microbial and mineralisable C fractions to fungal endophyte inoculation and emphasise the need for further research into how endophytes influence SOM stabilisation across different soil layers.

### SOM and SOM fractions affect and are affected by soybean crop

This study has highlighted the key relationships between grain yield and the SOM C and N in the soil, alongside their ratios and distribution within soil fractions. Consistent with the global observations across various crops and soils, we found that grain yield is positively associated with the concentrations of C and N in the soil. Conversely, a higher C:N ratio tends to correlate negatively with grain yield, indicating that an optimal balance of these elements is crucial for net N mineralisation and availability and crop productivity.

A novel finding from our research is the distinct impact of different soil organic C and N fractions on grain yield, and the effect of soybean crop growth on SOM C and N and its fractions. Specifically, the relationships observed between grain yield and the different fractions of SOM provide insights into how various forms of organic matter influence soil fertility and crop productivity. Fungal endophyte-affected rhizosphere contributes to both the oPOM and MAOM stocks, which are positively correlated with grain yield, indicating that these fractions contribute significantly to nutrient dynamics in the soil. However, the stocks of oPOM (C and N) were more closely and positively associated with grain yield than the stocks of MAOM (C and N). oPOM generally has a faster turnover rate, providing readily accessible nutrients to plants, whereas MAOM is more stable, decomposing slowly and releasing nutrients over a longer time frame. Despite its slower turnover, MAOM is the largest source of nitrogen in the soil and plays a critical role in meeting the long-term nitrogen requirements of plants<sup>19,35,36,62</sup>.

To understand the effect of grain yield on SOM fractions oPOM (C and N stocks) and MAOM (C and N stocks), relationships were drawn between grain yield and the proportions of MAOM-C and N and oPOM-C and N to total stocks of SOC and soil N, respectively (Supplementary Figure, S4). In this study, increasing grain yield and biomass resulted in increased proportion of oPOM-C stock (Supplementary Figure, S4a), suggesting significant SOC sequestration in the form of oPOM-C. Grain yields and biomass are often associated with better nutrient supply and soil structure. This can lead to the formation of more soil aggregates, within which oPOM is occluded. The process of aggregation protects oPOM from rapid decomposition, enriching the soil with C within these aggregates. This would increase the proportion of oPOM C, as organic matter is continuously cycled and incorporated into these protected forms. Cotrufo et al.<sup>52</sup> also reported that oPOM has lower N demand and can potentially accumulate more C indefinitely. The increase in the proportion of oPOM-C to the total SOC with increasing grain yield suggests that soils under high-yielding crops benefit from greater root activity and exudation, which contribute to the incorporation and protection of organic matter within soil aggregates. As a legume crop, soybean roots release substantial exudates during growth, which may enhance rhizosphere microbial activity, enhance favourable C and N stoichiometry and aggregation processes despite relatively lower root biomass compared to other crops. This not only enhances soil fertility by maintaining a pool of readily decomposable organic matter but also improves soil physical properties, such as porosity and water retention, that are critical for long-term soil health and productivity<sup>63,64</sup>.

The observed relationships between grain yield and the proportions of oPOM and MAOM suggest a dynamic interaction between crop productivity and SOM fractions. Specifically, the proportion of oPOM-C increased with grain yield, while the proportions of MAOM-C and N decreased (Supplementary Figure S4c, d). This suggests that higher grain yields may enhance MAOM turnover, contributing to the soil nitrogen cycling. Studies have shown that root exudates can destabilise and mineralise MAOM-N in the short term<sup>57,62,65</sup>. By turning over MAOM, the soil provides a steady supply of nutrients, particularly during critical growth stages like grain filling, contributing to higher yields within a crop cycle. Microbial activity in biologically active soils, supported by favourable moisture and temperature conditions, has been shown to rapidly respond to crop N demands<sup>66,67</sup>. This includes release of plant-available N from MAOM during periods of high demand<sup>57</sup>. In this context, Villarino et al.<sup>62</sup> reported that, contrary to expectations, the MAOM fraction consistently supplied more N than POM during SOM mineralisation across different land uses (forest, pasture, and crop lands). They suggested that MAOM's contributions to short-term N mineralisation and supply to plants have been undervalued. These findings support our observation that increasing grain yield is associated with potentially higher MAOM turnover.

Despite the decreasing proportions of MAOM-C and N with increasing grain yield, the absolute amounts of MAOM-C and N might remain stable because the total input of organic matter and the overall SOM pool increase with higher grain yields. This is demonstrated in Supplementary Figure S5, where the ratios of fPOM and oPOM stocks to the total SOM stocks increase with their absolute amounts, indicating net increases in these fractions. In contrast, MAOM ratios remain stable, suggesting no net decrease in MAOM stocks, despite their declining proportions, which could be attributed to concurrent net increases in fPOM and oPOM stocks. In particular, stocks of oPOM (C and N) increased around fivefold, whereas stocks of MAOM (C and N) increased around twofold with increasing grain yields (Fig. 4a,c). This complex interaction between crop productivity and SOM dynamics highlights the importance of understanding how different SOM fractions respond to agricultural practices.

In this context, Dalal et al.<sup>68</sup> demonstrated that long-term land use change in Australia, specifically transitioning from native forest to cropping, led to a reduction in all fractions of SOC in Vertisols. Notably, this decline was not observed in sown pasture systems, consistent with the findings from a global meta-analysis

by Kopittke et al.<sup>69</sup>. The study emphasised that biomass C inputs from crops, particularly root biomass, were insufficient to reverse or slow down the rate of SOC decline in these soils. Dalal and Mayer<sup>70,71</sup> further reported that rates of SOC decline in cropping soils were positively correlated with enzyme activity (urease) and negatively correlated with soil aggregation. This suggested that microbial enzyme accessibility to SOC plays a critical role in its turnover, a notion supported by subsequent studies<sup>72,73</sup>. Interestingly, in our study, significant negative relationship with proportions of MAOM stocks (C and N) were observed mainly with the grain yields at harvest, and to a lesser extent with the total plant biomass at flowering or maturity (data not shown). This suggests that podding and grain formation in crops may influence the microbial enzyme accessibility through the dynamics of root exudates and microbial activities. Subsequently, leading to potential mineralisation of MAOM to access N rather than that from oPOM (the relationship between ratio of oPOM and grain yield was positive). This new understanding may further contribute to why grain cropping is more deleterious to SOC compared with the pasture cropping in Australian soils<sup>68,74–77</sup>, which does not remove N in grains, hence maintains the stable MAOM fraction. These findings call for further investigation to determine if these phenomena observed with Vertisol and soybean crops are consistent across other soil types and grain crops.

It should also be highlighted here that the increased occlusion within soil aggregates, resulting in higher levels of oPOM, occurred during four months of the soybean cropping cycle in this study. This rapid formation of oPOM indicates that organic matter inputs from soybean residues and root exudates quickly contribute to soil aggregation, where they are physically protected from rapid decomposition. A rapid occlusion within soil aggregates was also observed for annual grasslands<sup>68</sup>. Over time, these occluded organic materials undergo further transformation and stabilisation processes, gradually converting into more stable forms of soil organic matter, such as MAOM. This transformation enhances long-term carbon sequestration and soil health by increasing the proportion of C that is relatively resistant to microbial decomposition<sup>63,78</sup>.

### Nitrogen needs of soybean

While soybean, as a legume, relies heavily on biological nitrogen fixation (BNF) to meet its N requirements, high-yielding varieties may exceed the capacity of BNF alone to fulfill their total N demand. For example, soybean crops producing 5 Mg grain ha<sup>-1</sup> require approximately 350 kg N ha<sup>-1</sup>, with additional N retained in stubble and roots<sup>79</sup>. In such systems, mineralisable N from SOM complements BNF during periods of peak N demand.

In our study, we were unable to quantify BNF, nodule biomass, or plant nitrogen content, but we acknowledge the importance of these measurements in better understanding the interactions between fungal endophytes and soybean N dynamics. Root nodules were visually observed in significant numbers during root sampling at the flowering (Supplementary figure, S6). Future studies should prioritise these measurements to provide a clearer picture of how fungal endophytes influence BNF and N cycling in soybean systems.

It is also important to highlight here that once N needs are met through BNF, soybean growth and grain yield become increasingly reliant on phosphorus (P) and potassium (K) availability. In particular, P is essential for nodule formation, nitrogenase enzyme activity, and ATP synthesis, all of which drive the energy-intensive process of N fixation in legumes<sup>80,81</sup>. Research has shown that P application often has a greater impact on soybean yield than direct N inputs, particularly in Australian cropping systems, highlighting the importance of P management in legume-based cropping systems<sup>82</sup>. Therefore, ensuring adequate P availability not only supports nodulation and nitrogen fixation but also contributes to enhanced root development, nutrient uptake, and overall soybean productivity in P-limited soils. In this study, the soil contained 162 mg kg<sup>-1</sup> soil of Colwell P, which was sufficient for the soybean crop (P deficiency < 20 mg P kg<sup>-1</sup> soil).

### Synergistic interaction between fungal endophytes and AMF

In our study, medium dosages of *Thozetella* sp., and *Leptodontidium* sp. showed significantly higher AMF colonisation, which corresponded with significant increases in total plant biomass compared to the control. This positive correlation provides direct evidence that fungal endophytes interacted with plant roots, potentially facilitating AMF colonisation and thereby enhancing plant growth. Studies have shown that AMF play an important role in enhancing root absorption area, facilitating phosphorus solubilisation, and improving soil structure through hyphal networks that contribute to soil aggregation<sup>42,83</sup>. Given that soybean relies on both BNF and soil nitrogen for its growth, the increased AMF colonisation observed in medium dosages of *Thozetella* sp., and *Leptodontidium* sp. might have also contributed to improved water and nutrient acquisition, either directly or indirectly, through interactions with the rhizosphere microbiome<sup>41</sup>, resulting in increased soybean plant biomass compared to other treatments. While our study demonstrated a significant increase in AMF colonisation in specific inoculant treatments and dosages, the underlying mechanisms driving this interaction remained unclear. The potential synergistic effects between fungal endophytes and AMF, particularly in enhancing nutrient acquisition, root exudation, and soil aggregation, warrant further investigation. Future research should explore whether specific fungal endophytes and dosages actively promote AMF colonisation or if this co-occurrence is an indirect result of improved plant health and root development. Understanding these interactions could provide valuable insights into optimising microbial inoculants and their dosages for sustainable crop production and soil carbon sequestration.

### Conclusions

Our comprehensive study demonstrated complex dynamics of the influence of fungal inoculation treatments on plant growth, grain yield, SOM and SOM fractions. The impact of fungal endophytes, on the changes in SOC and TN stocks and their fractions provided significant insights into potential C sequestration and stabilisation within the soil, particularly in Australian Vertisols. The distribution of SOM into fPOM, oPOM and MAOM fractions was important to understand the C and N dynamics. The recovery and proportion of these fractions highlighted the influence of organic matter input, decomposition rates, and microbial activity, with notable

differences between topsoil and subsoil layers. The role of fungal endophytes was highlighted by variations in SOC fractions, suggesting that high endophyte colonisation could enhance root proliferation and microbial activities, thereby increasing soil aggregation and protecting oPOM.

This study demonstrated that specific inoculants and their dosages significantly enhanced soybean growth and grain yield. The fungal endophytes inoculants *Thozetella* sp. at medium to high doses and *Leptodontidium* sp. at low to medium doses showed pronounced improvements over the control for various plant parameters, SOM and its fractions. This observation revealed a complex interaction between inoculation treatments, soil C and N dynamics, and plant productivity. Notably, the study found a positive relationship between biomass and grain yield and the stocks of oPOM (C and N) and MAOM (C and N). Furthermore, the proportion of oPOM-C stock to total SOC stock increased as biomass and grain yield significantly increased, leading to substantial carbon sequestration in oPOM. Conversely, the proportion of MAOM stock to total SOM significantly decreased, suggesting that MAOM stock build up was restricted due to N limitation, which was required for increasing grain yield. However, the absolute stocks of MAOM remained stable because the total input of organic matter increased resulting in the increase of the overall SOM stocks.

Additionally, microbial biomass and AMF colonisation measurements provided further evidence of the impact of fungal inoculation on soil microbial communities and potential synergistic relationship between fungal endophytes and AMF, which may enhance root-associated microbial interactions, nutrient acquisition, and soil aggregation. These findings highlight the importance of fungal endophyte inoculants as a promising strategy for improving crop productivity, enhancing microbial activity, and promoting soil carbon sequestration. Future studies should focus on exploring the mechanisms underlying microbial-root interactions, particularly the synergy between fungal endophytes and AMF, and their collective role in SOM stabilisation and nutrient cycling.

Materials and methods

Experimental field site

The experiment was conducted at the CSIRO Forest Hill Research Station (27°3'13" S, 152°2'10" E) at Gatton, Queensland, Australia. The site is situated on the flat alluvial plain of Lockyer Creek at an elevation of 93 m above mean sea level. The experimental field soil is classified as Black Vertosol (Australian Soil Classification<sup>84</sup>; World Reference Base for Soil Resources<sup>85</sup>) classification- Pellic Calcic Protosodic Vertisol (Aric, Hypereutric, Grumic, Magnesic). This soil shows high water-holding capacity, dark, self-mulching, cracking clay soil with a dark calcareous subsoil<sup>86</sup>. The experimental field had high indigenous available P concentration of 162.0 mg kg<sup>-1</sup> and exchangeable K concentration of 102.0 mg kg<sup>-1</sup>, along with moderate mineral N (NO<sub>3</sub>-N) concentration of 3.0 mg kg<sup>-1</sup>. Soil pH (water) is 7.85, and clay content is 50% (Table 3). The previous two cropping seasons included forage sorghum and wheat, which were turned into the soil for organic matter incorporation (brown manuring). The experimental field site represents conventional cropping with minimum tillage and no stubble burning in the previous season. No biological formulations were applied in the previous year, except for rhizobium inoculation. Moreover, there is no history for three previous years of using residual herbicides damaging to legumes and rhizobium inoculation, such as certain Group B (sulfonyleurea such as chlorsulfuron, metsulfuron) and Group I (Clopyralid) herbicides.

Crop establishment and management

Land preparation adhered to local industry standards, including weed management with two sprays of Glyphosate (Crucial, 600 g L<sup>-1</sup> Glyphosate) at a rate of 2.0 L ha<sup>-1</sup> and S-Metolachlor (DualGold, 960 g L<sup>-1</sup> S-Metolachlor) at a rate of 2.0 L ha<sup>-1</sup>. Although the field site follows a minimum tillage system, three cultivations were performed using Agrowplow (20–25 cm depth, surface loosening), Rotary hoe (10–15 cm depth, breaking up clods and incorporating residues), and Bed shaping (5–10 cm depth). Fertilisation involved the application of Superfect Pot 5&1 (N0:P7.3:K8.3:S9.2:Ca15.8) at a rate suitable for soil requirements. Soybean (cv. Hayman) was sown with sufficient seed to establish a plant population of 250,000 plants per hectare. The experimental units (plots) measured 6 m in length with seven rows spaced at 22 cm apart, resulting in a plot size of 9.24 m<sup>2</sup>. Seven

| Soil analysis   | Values |
|---|--------|
| Gravel (%)  | 0      |
| Clay content (%)  | 50     |
| Ammonium Nitrogen (mg kg <sup>-1</sup> )                                | 3.5    |
| Nitrate Nitrogen (mg kg <sup>-1</sup> )                                 | 3      |
| Phosphorus Colwell (mg kg <sup>-1</sup> ) -bicarbonate extractable form | 162    |
| Potassium Colwell (mg kg <sup>-1</sup> )                                | 102    |
| Sulfur (mg kg <sup>-1</sup> )   | 1.55   |
| Organic Carbon (%)  | 1.21   |
| Conductivity (dS m <sup>-1</sup> )                                      | 0.073  |
| pH (CaCl <sub>2</sub> )   | 6.5    |
| pH (H <sub>2</sub> O) (1:5)   | 7.85   |

Table 3. Soil characteristics (0–10 cm depth).

| Treatment                 | Dosages | Description  |
|---------------------------|---------|--|
| Control                   |         | Rhizobium + Rhizobium Sticker                                  |
| <i>Thozetella</i> sp.     | Low     | Powder seed coat + MCL sticker + Rhizobium + Rhizobium Sticker |
| <i>Thozetella</i> sp.     | Medium  | Powder seed coat + MCL sticker + Rhizobium + Rhizobium Sticker |
| <i>Thozetella</i> sp.     | High    | Powder seed coat + MCL sticker + Rhizobium + Rhizobium Sticker |
| <i>Leptodontidium</i> sp. | Low     | Powder seed coat + MCL sticker + Rhizobium + Rhizobium Sticker |
| <i>Leptodontidium</i> sp. | Medium  | Powder seed coat + MCL sticker + Rhizobium + Rhizobium Sticker |
| <i>Leptodontidium</i> sp. | High    | Powder seed coat + MCL sticker + Rhizobium + Rhizobium Sticker |

**Table 4.** Inoculant treatments and application dosages. Low, medium and high dosages of inoculants were applied at the rates of 0.21 g, 0.42 g and 0.84 g kg<sup>-1</sup> seed, respectively. Rhizobium was applied at a rate of 5 g kg<sup>-1</sup> seed. Methyl Cellulose (MCL sticker) was applied uniformly across all treatments at 6.25 mL kg<sup>-1</sup> seed.

treatments, including two inoculants x three dosages and one control, were arranged in a randomized complete block design with eight replications.

Two commercial fungal inoculants produced by Loam Bio, *Thozetella* sp. and *Leptodontidium* sp. (respectively identified as 2359 and 4873) specifically selected for soybean crops, were used for field testing. These fungal inoculants were originally isolated from surface-sterilised roots of multiple species of grasses and shrubs from diverse natural environments across south-eastern Australia and were screened for traits that may support plant growth and soil C storage by Loam Bio Pty Ltd (Orange, New South Wales, Australia). As a part of the commercial product development, several fungi and various crop types were extensively tested in greenhouse and field trials. In lab and greenhouse studies conducted from period 2020–2022 (data not shown), several fungi were tested for their suitability for soybeans. As a result, isolates 2359 (*Thozetella* sp.) and 4873 (*Leptodontidium* sp.) were identified as showing promising results and thus were progressed to larger trials to further understand the interactions and implications on soil carbon sequestration when used with soybeans.

In addition, although the isolates originated from roots of grasses and shrubs, these fungi are categorised as root associated dark septate endophytes (DSE), and fungi of this group have been reported to associate with a very broad range of host plants as such. For example, a recently published study by Stuart et al.<sup>25</sup> has also demonstrated the interactions and performance of 2359 (*Thozetella* sp.) in wheat, thus showing that these fungi are capable of interacting with a wide range of hosts.

For this trial, each inoculant was formulated at three distinct dose concentrations (low, medium, and high), leading to a total of seven treatments. This includes six inoculant-based treatments (2 inoculants x 3 dosages) and one control (Table 4). The inoculants were applied as a powder seed coating using an MCL adhesive, complemented by a rhizobium application with a rhizobium-specific adhesive. Control treatment for soybean seeds involved the sole application of rhizobium culture with a rhizobium adhesive.

A total of 285 mm irrigation water was applied over the growing season as required, mostly near flowering and into pod fill stages, with a weekly application of 40 mm when necessary. For pest management, two in-crop sprays were conducted. The first spray involved the application of Dipel (*Bacillus thuringiensis* subsp. Kurstaki, Strain ABTS-351, fermentation solids and soluble) at a rate of 1 kg ha<sup>-1</sup>. The second spray utilized Vivus Max at a rate of 100 mL ha<sup>-1</sup> (5 x 10<sup>9</sup> POLYHEDRAL).

Sowing occurred on January 16, 2023, and harvesting took place on May 25, 2023. At maturity, a mechanical harvester (KEW) was used for grain yield assessment.

Plant sampling and measurements

Crop establishment/emergence was performed around 3 weeks post-sowing by recording the number of emerged plants per linear metre of row per plot. Two plant samplings were undertaken, one around flowering and the other around maturity just before harvest. Both times, plant height and plant vigour were visually assessed for each plot, and two plants representing the average visual plant height and vigour of the plot was selected for sampling. Leaf DM, stem DM, root DM, plant biomass and root shoot ratio and grain yield per plant were determined. Hand harvested grain yield at maturity (n = 16 plants per treatment) was converted into Mg ha<sup>-1</sup> based on plant population (around 250,000 plants ha<sup>-1</sup>). Grain yield was also determined for each plot by machine harvesting.

Soil sampling and analysis

Prior to sowing, one soil core (0–15 cm depth) using hand corer (internal diameter, 45 mm) was collected from each plot for analysis of physical and chemical properties. Post harvest soil sampling was performed five days after the soybean crop was harvested, a petrol percussion corer with an internal diameter of 45 mm was employed to gather intact soil cores, 0–30 cm deep. Soil sampling was undertaken at three distinct locations in each plot close to the root zone, along the plant line and removing plant roots and litter. Extracted soil cores were divided at depths of 0–15 cm and 15–30 cm. The soil was stored in esky in the field and then transferred to cold room near the field site to store soil at 4°C until processing. The three samples per plot for each depth were mixed uniformly and oven dried at 40°C in aluminium trays for 5 days. Following drying, the soil was first ground to 2 mm using Dayton (6XH81BA, USA). Samples were sieved to < 2 mm by hand.

### SOC fractionation

Soil fractionation was performed on 2 mm sieved samples whereas further grinding of the 2 mm soil samples was carried out with Retsch Mill (MM400, Germany), to get a fine size around 0.25 mm for the analysis of SOC and TN. Sieved soils (<2 mm) were processed for fPOM, oPOM and MAOM fraction separation. The SOC fractionation methodology employed in this study was adapted from the protocol outlined by Haddix et al.<sup>87</sup>. This method facilitates the isolation of three distinct soil C fractions: fPOM, oPOM, and MAOM. Briefly, the procedure is described as follows.

Soil samples were dried at 40°C, following drying, the soil samples were ground with Dayton Mill (6XH81BA, USA) and passed through a 2 mm sieve to remove litter and root material. For each sample, 10 g of soil was placed in 50 mL Falcon tubes in duplicates, and oven-dried at 60°C overnight. This step ensures the removal of moisture that could affect the density separation process. After drying, samples were allowed to cool for approximately 15 min and tubes were reweighed before initiating the density separation using sodium polytungstate (SPT) solution (density of 1.85 Mg m<sup>-3</sup>). Each tube was filled to 30 mL mark with SPT solution, capped, and gently inverted 20 times to ensure removal of entrapped air and uniform distribution of soil particles. The particles adhering to the sides of the tubes were rinsed with SPT and topped up to 40 mL mark. The mixture was then left to settle overnight to facilitate separation of the fPOM fraction from the denser soil components. On the following day, the samples were centrifuged at 3500 rpm for 60 min at 25°C. The fPOM fraction was collected by gently inverting the tube over a 53 µm sieve. This fraction was then washed with Milli-Q water until the electrical conductivity dropped below 5 µS, and subsequently dried in a low-airflow oven at 60°C for approximately 2 days.

The pellet remaining after fPOM removal underwent a series of washes to eliminate residual SPT. This included vigorous shaking using Vortex with Milli-Q water, 0.2 mL of 0.25% solution of CaCl<sub>2</sub> and MgCl<sub>2</sub>, and finally washed with Milli-Q water. Each washing step was followed by centrifugation and removal of the supernatant. The pellet tube was then filled with 40 mL of 0.5% sodium hexametaphosphate, mixed and was shaken vigorously using Vortex to disperse the soil/disrupt aggregates and placed the tube in the reciprocal shaker on low shaking for 18 h. The final pellet, representative of the oPOM and MAOM fractions, was subjected to further separation through sieving (53 µm sieve) and collecting > 53 µm the oPOM fraction and < 53 µm the MAOM fraction. Both the oPOM and MAOM fractions were dried in a 60 °C oven until completely dry. The recovery efficiency of each fraction was calculated by weighing the dried material. Samples falling outside the 95–105% recovery range were either re-dried or re-processed to ensure accurate fractionation (98–100%). Finally, the dried fractions were ground with Retsch Mill (MM400, Germany) and stored in glass vials for subsequent C and N analysis using the Leco combustion method (LECO 928 Series Carbon/Nitrogen Analyser, Model: 630–500–300, Leco Corporation, Michigan, USA) by oxidation combustion at 1250 °C and measuring released CO<sub>2</sub> and N<sub>2</sub> by non-dispersive infrared detection.

### Carbon analysis and stocks equation

Sieved soil and dried fractions were ground to 0.25 mm for the analysis of total SOC and N concentrations (TN) using the LECO combustion method<sup>88</sup>. The concentrations were then used to determine total SOC or N stock of soil and fractions with the following formula:

$$\text{SOC or TN stock (Mg ha}^{-1}\text{)} = \text{element concentration (\%)} \times \text{bulk density (Mg m}^{-3}\text{)} \times \text{depth (m)} \times 10,000 \text{ m}^2 \text{ ha}^{-1}$$

### Soil microbial C and N biomass determination

Sieved soils (2 mm) were processed to determine MBC and N by chloroform fumigation<sup>43,89</sup>. Briefly, two sets of 10 g of sieved samples were placed in a 50 mL Falcon tubes, soil moisture adjusted to field capacity by adding distilled water (gravimetric soil moisture content of sample and its field capacity were determined earlier). Tubes were then incubated at room temperature (~25 °C) with lids loosely placed for 7 days. After incubation, one set of samples was used for fumigation with ethanol-free chloroform, using fumigation vessel (desiccator) in a fume cupboard. A damp paper towel and a vial of soda lime (to absorb CO<sub>2</sub>) were placed at the base of the desiccator. A glass jar, 100 mL half-full of chloroform and some boiling beads was prepared, and incubated Falcon tubes (no lids) placed inside the desiccator around chloroform jar. Silicone grease was smeared around the rim and lid of the desiccator, and the lid was placed on the desiccator. Desiccator was then attached to a vacuum hose and whole system (desiccator and hose) was placed under vacuum (by opening the tap of the desiccator and the vacuum tap). Once the pressure of the vacuum reached –20 in HG (–70 kPa) and the chloroform begins to boil, vacuum tap and desiccator were closed. Fumigation of samples conducted for 24 h. Once the fumigation was completed, the chloroform was removed by repeated evacuation. Finally, the desiccator was repressurized (by opening the tap on the desiccator) and sample tubes were removed. Microbial biomass (unfumigated and fumigated samples) was then extracted by adding 35 mL of 0.5 M K<sub>2</sub>SO<sub>4</sub>, and by shaking the tubes on a rotary/orbital shaker for 1 h. Tubes were then centrifuged at 4,500 rpm for 30 min. 20 mL of supernatant of liquid C and N was used for microbial C and N analyses using liquid C and N analyser. Calculations of microbial C and N<sup>90</sup>:

$$\text{Microbial C (mg kg}^{-1} \text{ soil)} = \frac{(\text{C fumigated} - \text{C unfumigated})}{0.45}$$

$$\text{Microbial N (mg kg}^{-1} \text{ soil)} = \frac{(\text{N fumigated} - \text{N unfumigated})}{0.57}$$

0.45 = C recovery factor, 0.57 = N recovery factor.

### AMF colonisation study

Roots were dug out during post flowering, and were cut at the hypocotyl, moistened, and put with the adhering soil into plastic bags. Harvested samples were stored in a cold room at the field station and were transferred to the laboratory in a cooler. Once in the laboratory, roots were carefully washed in tap water to remove adhering soil particles and organic matter. From each plant root sample, a representative sample of roots was taken from three different locations and fixed in 60% ethanol (v/v) and stored at 4 °C for subsequent analysis. For the observation, the stored roots were immersed in water to remove ethanol and cut into 1 cm segments with their side branches attached. Sixty to 100 root segments were cleared with 10% potassium hydroxide (KOH) (w/v) at 55 °C. Depending on the pigmentation of the samples, KOH was replaced and incubated back in the oven until roots were clear of pigmentation and eliminated intracellular constituents (KOH solution turns yellow when tannins have gone into the solution).

Subsequently, the roots were rinsed with water, acidified with 1% HCl, and stained with 0.05% cotton blue (w/v)<sup>91</sup>. Fifty segments of roots per seedling were observed under 200× magnification using a light microscope (Nikon Eclipse E600, Nikon instruments Inc., NY, USA). Details of DSE, AMF structures were documented with a microscopic digital camera (Nikon, NY, USA) and using Capture 2.1 V software at 400×1000 x magnifications.

The AMF colonisation was recorded for the presence of arbuscules, as this is the most accurate way to confirm the colonisation. The percentage of colonisation was expressed as the ratio of the number of colonised sections: total number of root sections observed \*100<sup>92</sup>. The presence of DSE was only qualitatively recorded.

### Data processing and statistical analysis

The initial step in our analysis involved a thorough screening of the dataset for accuracy and completeness, followed by the identification and removal of outliers using the Grubb's test. Only few outliers were noted in SOC fractions data due to leaks in the test tubes with soil samples. We assessed the normality of the data distribution for all morphometric parameters (pod, shoot, root, grain, and biomass) collected at crop maturity. The Shapiro–Wilk test, a powerful method for testing normality in small sample sizes, was employed. Contrary to the morphometric parameters, which demonstrated a normal distribution, the SOC and total soil N concentrations did not conform to normal distribution criteria; however, several data transformation techniques were explored, but none of the transformation techniques successfully normalized the data for SOC and total N concentrations, prompting the decision to proceed with analyses on the original datasets for these parameters. Analysis of Variance (ANOVA) was performed, using XLSTAT<sup>93</sup>, to identify significant effects by treatments and blocks. To further analyse the differences identified by the ANOVA, Fisher's Least Significant Difference (LSD) test at a 5% significance level was utilized as a post-hoc analysis. The standard error of the mean (SEM) was also calculated for all original datasets.

### Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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### Author contributions

V. Singh, R. C. Dalal, and A. Jones developed the research concepts. A. James and P. Van Drie designed the field experiment layout and conducted the field trials. N. Purushotham and A. Jones. selected the group of microbial inoculants and contributed to interpretation of their effects. V. Singh, H. Gamage, and H. Vera Wood collected the field data. V. Singh and R. C. Dalal conducted the data and statistical analyses, interpreted the data, and wrote the paper. A. James. and R. Oppenheimer contributed to interpretation of results. H. Gamage and V. Singh optimised the SOC fractionation methodology and performed the analysis. H Vera Wood and B. Bruning contributed to soil processing and laboratory analysis.

### Declarations

### Competing interests

The authors declare no competing interests.

### Ethical approval

The necessary permissions were obtained to conduct the field experiments at CSIRO Forest Hill Research Station, Gatton, Australia, ensuring compliance with institutional and regulatory requirements.

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-94982-3>.

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