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Effects of Symbiotic Fungi on Sugars and Soil Fertility and Structure-Mediated Changes in Plant Growth of *Vicia villosa*

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Abstract: Many terrestrial plants form reciprocal symbioses with beneficial fungi in roots; however, it is not clear whether *Vicia villosa*, an important forage and green manure crop, can co-exist with these fungi and how such symbiosis affects plant growth and soil properties. The aim of this study is to analyze the effects of inoculation with three arbuscular mycorrhizal fungi (AMF) such as *Diversispora spurca*, *Funneliformis mosseae*, and *Rhizophagus intraradices* and an endophytic fungus *Serendipita indica* on plant growth, root morphology, chlorophyll and sugar levels, soil nutrients, and aggregate size distribution and stability in *V. villosa* plants. After 63 days of inoculation, the beneficial fungi colonized the roots with colonization rates of 12% to 92%, and also improved plant growth performance and root morphology to varying degrees, accompanied by the most significant promoted effects after *R. intraradices* inoculation. All AMF significantly raised chlorophylls *a* and *b*, carotenoids and total chlorophyll concentrations, along with a significant increase in leaf sucrose, which consequently formed a significantly higher accumulation of glucose and fructose in roots providing carbon sources for the symbionts. Root fungal colonization was significantly ($p < 0.01$) positively correlated with chlorophyll compositions, leaf sucrose, and root glucose. In addition, inoculation with symbiotic fungi appeared to trigger a significant decrease in soil Olsen-P and available K and a significant increase in NH_4-N, NO_3-N, and glomalin-related soil protein levels, plus a significant increase in the proportion of water-stable aggregates at the size of 0.5–4 mm as well as aggregate stability. This improvement in soil aggregates was significantly ($p < 0.01$) positively correlated with root fungal colonization rate and glomalin-related soil protein concentrations. The study concludes that symbiotic fungi, especially *R. intraradices*, improve the growth of *V. villosa*, which is associated with fungal modulation of sugars, soil fertility and root structural improvement.

Keywords: aggregate stability; endophyte; glomalin; mycorrhiza; soil nutrient

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1. Introduction

Vicia villosa Roth is a leguminous herb widely cultivated in the world, rich in protein and mineral contents [1,2], which can be used as animal feed [3]. *V. villosa* is also used as a green manure cover crop because of its nitrogen fixation capacity [4,5]. However, intensified land degradation, food and feed competition, continued climate changes, and the increasing demand for animal feed limit the growth and yield of *V. villosa* [1].

Soil arbuscular mycorrhizal fungi (AMF) can establish reciprocal associations with over 80% of terrestrial plants [6,7]. Inoculation of AMF improves plant growth performance, along with increased root architecture and chlorophyll concentrations [8–11]. After AMF helps host plants to obtain water and nutrients, host plants provide photosynthetic products for AMF [7]. Because AMF requires the supply of carbon, large amounts of sugars

are transported to the roots, which are conducive to further strengthening the role of arbuscular mycorrhizae on plants [12]. In addition, AMF (e.g., *Rhizophagus irregularis*) elevates soil fertility, as well as soil structure [13], which is good for the growth performance of host plants (e.g., garlic) [13–15]. AMF-produced glomalin-associated soil protein (GRSP) promotes the formation of soil water-stable aggregate (WSA) [16,17], which assumes an improved role in soil health and quality. Nonetheless, whether AMF produces beneficial effects on *V. villosa* plants remains unclear.

AMF relies on host plants for their propagation, which limits its large-scale application [18]. In contrast to AMF, *Serendipita indica* (Sav. Verma, Aj. Varma, Rexer, G. Kost and P. Franken) M. Weiß, Waller, A. Zuccaro and Selosse (*Si*), a mycorrhiza-like endophytic fungus, can be cultured *in vitro* in a short timeframe, which greatly increases its application. *Si* has many positive functions, including promoted plant growth [19,20] and increased chlorophyll levels [21]. However, whether and how this fungus improves the growth of *V. villosa*, as well as soil physicochemical properties, is unclear. In addition, few studies have combined AMF and endophytic fungi to compare their effects and advantages on host plants, especially legumes, although a better improved effect on plant growth was observed under *Funneliformis mosseae* (T.H. Nicolson and Gerd.) C. Walker and A. Schüßler (*Fm*) (an arbuscular mycorrhizal fungus) than under endophytic fungus *Si* on *Poncirus trifoliata* (a mycorrhiza-dependent plant) [22].

The objective of this study was to assess the impacts of inoculation with three AMF species and the endophytic fungus *Si* on plant growth, chlorophyll, and sugar concentrations of *V. villosa* plants, as well as soil nutrients and structure.

2. Materials and Methods

2.1. Experimental Design

This experiment consisted of five inoculations, including inoculation with *Diversispora spurca* (C.M. Pfeiff., C. Walker, and Bloss) C. Walker and A. Schüßler (*Ds*), *Fm*, *Si*, *Rhizophagus intraradices* (N.C. Schenck and G.S. Sm.) C. Walker and A. Schüßler (*Ri*), and non-fungi treatment (control). Each treatment was replicated five times, with four plants per pot, for a total of 25 pots.

2.2. Fungal Agents

Three AMF species, including *Ri*, *Ds*, and *Fm*, were trapped through white clover under potting conditions for 12 weeks. After harvesting, the shoots of the plant were removed, and both fungus-colonized root segments and growth substrates were gathered as AMF inoculum, in which the spore number was 15 spores/g. *Si* proliferation was carried out as per the procedure depicted by Sun et al. [23], using spore suspensions as the inoculant at a concentration of 2.97×10^8 CFU/mL. In addition, AMF inoculum was stored at 4 °C for no more than 3 months after harvest and the spore suspension of *Si* was used immediately. There was no difference in the spore germination rate among these fungi used here.

2.3. Plant Culture

Seeds of *V. villosa* provided by Hubei Academy of Forestry were sterilized with 70% alcohol for 20 min, rinsed with distilled water, and placed in a petri dish with sterile water for 12 h. On 16 September 2021, germinated seeds were sown in plastic pots (16 cm × 10.5 cm × 15 cm), in which 2.4 kg autoclaved substrates of soil and river sand (3:1, *v*/*v*) were pre-filled. The soil is the Ferralsol (FAO system), whose characteristics were described by Liu et al. [24]. At the time of sowing, fungal inoculations were performed, where each AMF-inoculated treatment received 100 g of corresponding mycorrhizal inoculum per pot and *Si* was 40 mL of spore suspension per pot. The 100 g of mixture of autoclaved inoculum of *Ri*, *Ds*, and *Fm* in equal quantities and 40 mL of autoclaved spore suspension of *Si* was applied together to the uninoculated treatment as the control. All treated pots were placed in a greenhouse at the West Campus of Yangtze University with

no extra temperature controls and in natural light conditions. Soil moisture was controlled to 70% of the field's maximum capacity by weighing, and the lost water was replenished at 6:30 pm each day. After 10 days, the seedlings were diminished to four plants for each pot and harvested following 63 days.

2.4. Determination of Root Fungal Colonization Rate and Plant Growth

At the time of harvest, height, diameter, and leaf number were measured for each treatment. The plants were collected from the pots, washed with water, and divided into roots, stems, and leaves, whose biomass was weighed. Harvested roots were scanned with a scanner (J221A, EPSON, Jakarta Selatan, Indonesia) and analyzed using a root analyzer, a WinRhizo (2007b, Regent Instruments Inc., Quebec City, QC, Canada), for root morphological parameters. Six 1 cm root segments from each plant were subjected to trypan blue staining [25] and the root fungal colonization rate was calculated according to the method of Sun et al. [23].

2.5. Determination of Chlorophyll and Carbohydrate Concentrations

Leaves were ground with 80% acetone solutions, filtered, and their absorbance value was recorded at 470 nm, 646 nm, and 663 nm by a spectrophotometer (UV-5900, METASH, Shanghai, China). The chlorophyll concentration was calculated according to the equation described by Arnon [26]. Dry samples of leaves and roots were ground into powder, passed through a 4 mm size sieve, and collected for the colorimetric determination of fructose, glucose, and sucrose concentrations [27].

2.6. Determination of Soil Nutrients, Aggregate Size Distribution, and Aggregate Stability

After the soil was natural air-dried for 7 days, easily extractable GRSP (EE-GRSP) and difficult-to-extract GRSP (DE-GRSP) were extracted using the method of Yang et al. [22] and assayed according to the protocol of Bradford [28]. The 1 g soil sample was extracted with 8 mL of 20 mM citrate buffers (pH 7.0) at 103 kPa and 121 °C for 0.5 h, and centrifuged at 10,000× *g* for 3 min. The supernatant was collected for examination of EE-GRSP. The centrifugal residue was mixed with 8 mL of 50 mM sodium citrate buffers (pH 8), extracted at 103 kPa and 121 °C for 1 h, and centrifuged at 10,000× *g* for 3 min. This supernatant was chosen for analysis of DE-GRSP. Total GRSP (T-GRSP) was the sum of EE-GRSP and DE-GRSP.

The protocol described by Muneer et al. [29] was used for the determination of WSA distribution at different sizes. Mean weight diameter (MWD) of WSA at different sizes was used to calculate aggregate stability, with reference to the equation of Muneer et al. [29]. Concentrations of NH_4-N, NO_3-N, Olsen-P, and available K in soil were assayed using a soil available-nutrient analyzer (HM-TYD, Shandong Hengmei Electronic Technology Co., Ltd., Weifang, China) as per the user's manual.

2.7. Data Analysis

All variables were measured in four replicates, and the other replicate was saved for subsequent molecular analysis. Levine's test was used for checking the homogeneity of variance, and the Kolmogorov–Smirnov test was used for checking data normality before performing data analysis. Data (means ± SD, $n = 4$) were statistically analyzed for the analysis of variance, and the Duncan's multiple range test was used to compare significant ($p < 0.05$) differences among treatments. All statistical analysis was performed by SAS software (9.1.3v) (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Changes in Root Fungal Colonization Rate

No signs of fungal colonization were observed in roots of *V. villosa* plants not inoculated with any fungi, while roots inoculated with symbiotic fungi showed signs of fungal colonization (Figure 1a–e), with the root fungal colonization rate ranging from

12% to 92%. A significantly higher root fungal colonization rate was listed as the order of *Ri* > *Ds* > *Fm* > *Si* (Figure 1f).

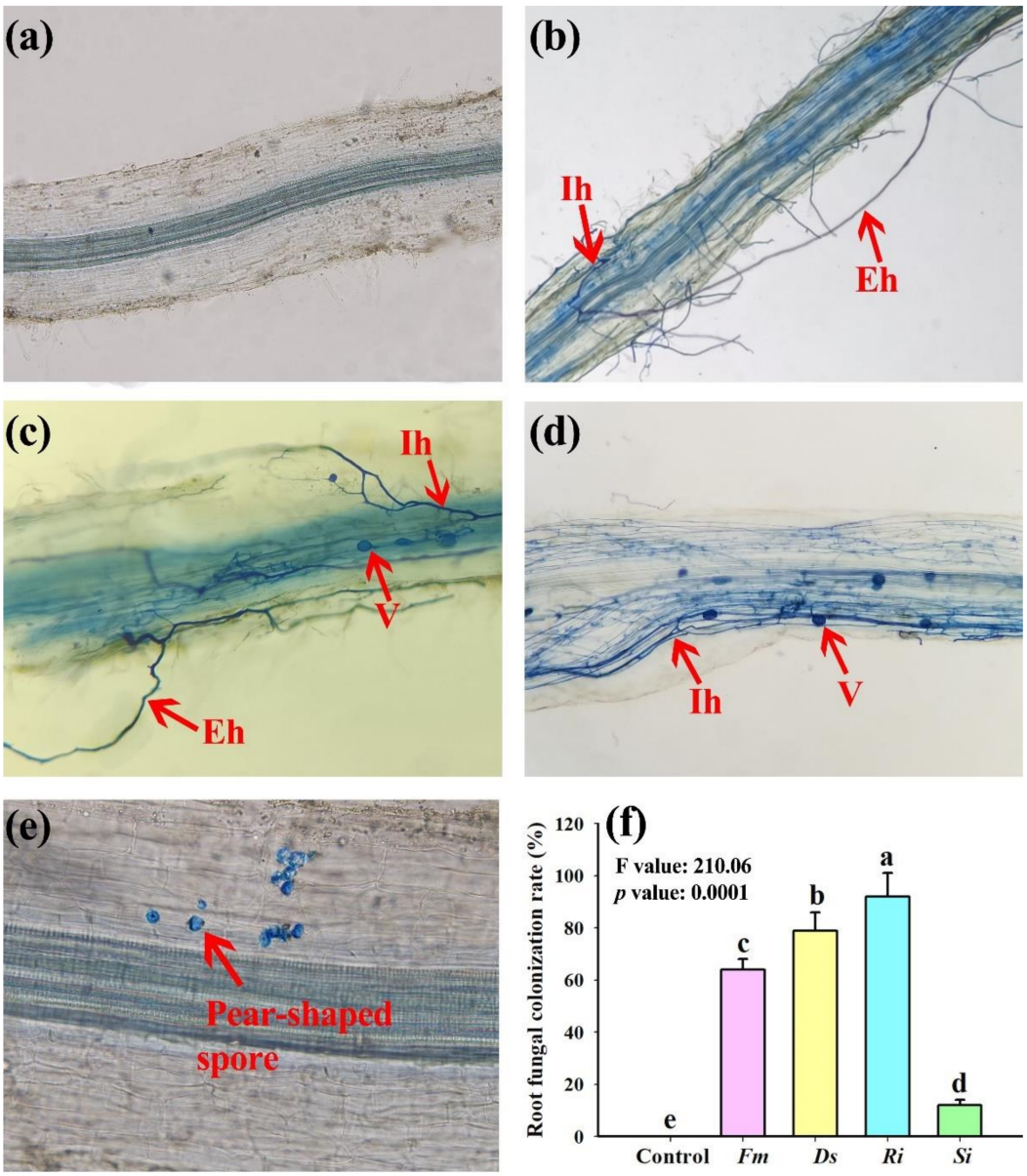

Figure 1. Root fungal colonization of *Vicia villosa* plants and changes in root fungal colonization rate after inoculation with four symbiotic fungi. Data (means ± SD, $n = 4$) followed by different letters above the bars indicate significant ($p < 0.05$) differences. (**a**): no fungal colonization in the control roots; (**b**): fungal colonization in *Fm*-inoculated roots; (**c**): fungal colonization in *Ds*-inoculated roots; (**d**): fungal colonization in *Ri*-inoculated roots; (**e**) fungal colonization in *Si*-inoculated roots; (**f**) changes in root fungal colonization rate. Abbreviations: control, no-fungi inoculation; *Ds*, *Diversispora spurca*; Eh, extraradical hyphae; *Fm*, *Funneliformis mosseae*; Ih, intraradical hyphae; *Si*, *Serendipita indica*; *Ri*, *Rhizophagus intraradices*; V, vesicle.

3.2. Changes in Growth Performance

Inoculation with symbiotic fungi improved the growth performance of *V. villosa* plants to different degrees (Figure 2). Compared with the control, height, diameter, leaf number, and leaf and stem biomass (except *Si*) were significantly improved by fungal inoculations, followed by the trend of *Ri* > *Ds* > *Fm* > *Si* (Table 1). Among them, *Ri* inoculation represented

the most prominent effect on height, diameter, leaf number, and leaf and stem biomass by 103%, 46%, 126%, 238%, and 244%, respectively.

Figure 2. Plant growth response of *Vicia villosa* seedlings by inoculation with arbuscular mycorrhizal fungi and *Serendipita indica* after 63 days. For abbreviations see Figure 1.

Table 1. Effects of inoculation with symbiotic fungi on plant growth of *Vicia villosa*.

Treatments	Height (cm)	Diameter (mm)	Leaf Number (num./Plant)	Biomass (g/Plant)		
				Leaf	Stem	Root
Control	13.6 ± 1.5 d	0.474 ± 0.048 c	15.9 ± 2.1 c	0.248 ± 0.042 d	0.116 ± 0.017 b	0.550 ± 0.035 a
Fm	24.1 ± 1.9 b	0.641 ± 0.074 ab	31.8 ± 3.1 a	0.698 ± 0.120 b	0.345 ± 0.048 a	0.663 ± 0.078 a
Ds	25.8 ± 2.9 ab	0.698 ± 0.035 a	33.6 ± 4.9 a	0.705 ± 0.133 b	0.362 ± 0.059 a	0.634 ± 0.078 a
Ri	27.6 ± 1.9 a	0.694 ± 0.051 a	36.0 ± 2.4 a	0.838 ± 0.090 a	0.378 ± 0.071 a	0.678 ± 0.052 a
Si	16.6 ± 1.5 c	0.584 ± 0.025 b	22.0 ± 1.3 b	0.404 ± 0.059 c	0.168 ± 0.032 b	0.614 ± 0.065 a
F value	46.96	17.62	40.08	32.59	30.89	2.52
p value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0739

Data (means ± SD, $n = 4$) followed by different letters in the column indicate significant differences at $p < 0.05$. For abbreviations see Figure 1.

3.3. Changes in Root Morphological Variables

The root morphology of *V. villosa* plants inoculated with symbiotic fungi was superior to that of non-inoculated plants (Figure 2; Table 2). Compared with the control, *Fm*-inoculation significantly promoted root length, surface area, diameter, and volume by 36%, 18%, 15%, and 189%, respectively; *Ds* only significantly raised root length and surface area by 30% and 14%, respectively; *Ri* dramatically increased root length, diameter, and volume by 32%, 14%, and 185%, respectively; *Si* distinctly elevated root length and surface area by 39% and 16%, respectively. Of the four inoculations, *Fm* and *Ri* had the most prominent effect on improved root morphology.

Table 2. Effect of inoculation with different symbiotic fungi on root system architecture of *Vicia villosa*.

Treatments	Total Length (cm)	Projected Area (cm^2)	Surface Area (cm^2)	Diameter (mm)	Volume (cm^3)
Control	131.2 ± 25.7 b	9.4 ± 1.0 a	13.9 ± 1.5 b	0.461 ± 0.026 b	0.47 ± 0.11 b
Fm	178.2 ± 9.3 a	10.9 ± 1.0 a	16.4 ± 0.8 a	0.530 ± 0.049 a	1.36 ± 0.24 a
Ds	171.0 ± 28.3 a	10.8 ± 1.3 a	15.8 ± 1.0 a	0.465 ± 0.025 b	0.78 ± 0.23 b
Ri	172.6 ± 6.1 a	11.2 ± 0.4 a	15.4 ± 1.1 ab	0.526 ± 0.059 a	1.34 ± 0.31 a
Si	183.0 ± 18.1 a	11.4 ± 1.5 a	16.1 ± 0.7 a	0.438 ± 0.011 b	0.59 ± 0.18 b
F value	4.49	2.02	3.54	4.70	14.11
p value	0.0139	0.1438	0.0317	0.0117	<0.0001

Data (means ± SD, $n = 4$) followed by different letters in the column indicate significant differences at $p < 0.05$. For abbreviations see Figure 1.

3.4. Changes in Leaf Chlorophyll Concentrations

All three AMF inoculations dramatically raised leaf chlorophylls *a* and *b*, and total chlorophyll and carotenoid concentrations, accompanied by a significant increase only in total chlorophyll after *Si* inoculation (Figure 3). Compared to the control, chlorophylls *a* and *b*, and total chlorophyll and carotenoid were increased by 93%, 110%, 100%, and 50% in *Fm*, 131%, 120%, 128%, and 88% in *Ds*, and 272%, 230%, 262%, and 138% in *Ri*, respectively.

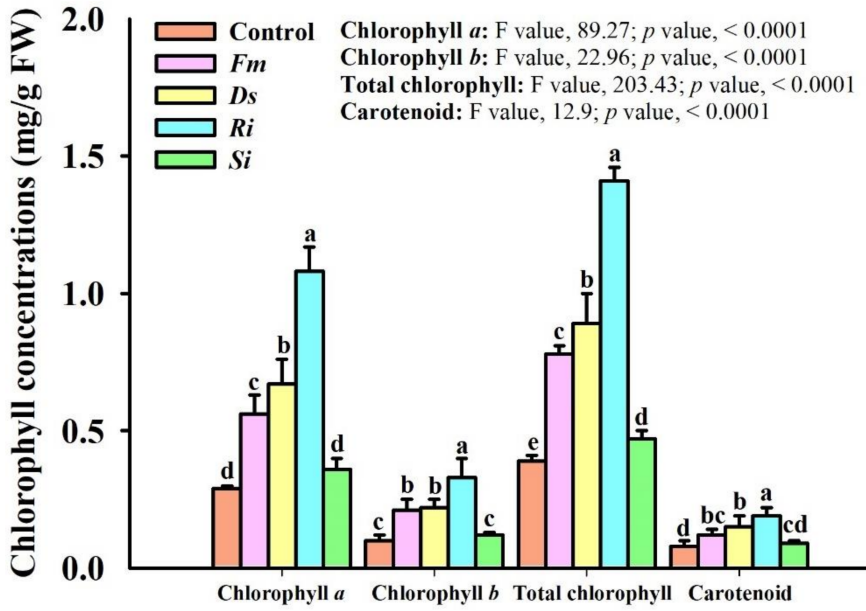

Figure 3. Effects of arbuscular mycorrhizal fungi and *Serendipita indica* on leaf chlorophyll fractions concentrations of *Vicia villosa* seedlings. Data (means ± SD, $n = 4$) followed by different letters above the bars indicate significant ($p < 0.05$) differences. For abbreviations see Figure 1.

3.5. Changes in Leaf and Root Sugar Concentrations

Inoculation with symbiotic fungi had different effects on sucrose, fructose, and glucose concentrations in leaves and roots (Table 3). Inoculations with *Fm*, *Ds*, *Ri*, and *Si* significantly increased leaf sucrose concentrations by 42%, 55%, 77%, and 30%, respectively. However, in roots, inoculation with *Fm* significantly increased their sucrose concentrations by 21%, along with no change after *Ds* and *Si* inoculation and a 25% reduction after *Ri* inoculation. Similarly, inoculation of *Fm*, *Ds*, *Ri*, and *Si* significantly increased leaf fructose concentrations by 38%, 35%, 42%, and 21%, respectively, compared with the control; inoculation of *Fm*, *Ds*, and *Ri* significantly increased root fructose concentrations by 58%, 38%, and 35%, respectively, coupled with no change after inoculation of *Si*. In the four inoculations, only the *Ds* and *Si* inoculations significantly increased leaf glucose concentrations by 31% and 29%, respectively, compared with the control, accompanied by

25%, 23%, and 26% significantly higher root glucose concentrations after *Fm*, *Ds*, and *Ri* inoculation, respectively.

Table 3. Effects of inoculation with symbiotic fungi on leaf and root sugar fractions of *Vicia villosa*.

Treatments	Sucrose (mg/g DW)		Fructose (mg/g DW)		Glucose (mg/g DW)	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
Control	26.55 ± 4.91 c	84.64 ± 9.92 b	32.60 ± 1.74 c	62.89 ± 5.47 c	87.05 ± 5.63 b	102.87 ± 4.58 b
Fm	37.66 ± 1.91 ab	102.55 ± 8.79 a	44.93 ± 4.17 ab	99.53 ± 5.02 a	85.42 ± 8.93 b	128.51 ± 9.79 a
Ds	41.27 ± 8.14 ab	80.59 ± 11.05 b	44.10 ± 3.50 ab	86.68 ± 6.87 b	114.33 ± 7.59 a	126.67 ± 7.81 a
Ri	47.06 ± 7.72 a	63.81 ± 3.93 c	46.34 ± 6.15 a	85.17 ± 4.85 b	86.36 ± 1.31 b	129.45 ± 3.19 a
Si	34.58 ± 6.54 bc	80.23 ± 7.81 b	39.31 ± 1.86 b	69.10 ± 5.72 c	112.00 ± 6.69 a	106.76 ± 16.27 b
F value	5.7	10.22	8.52	27.19	20.22	7.39
p value	0.0054	0.0003	0.0009	<0.0001	<0.0001	0.0017

Data (means ± SD, $n = 4$) followed by different letters in the column indicate significant differences at $p < 0.05$. For abbreviations see Figure 1.

3.6. Changes in Soil Nutrients

Inoculation of *Fm* and *Ds* significantly increased soil NH_4-N concentrations by 25% and 123%, respectively, plus no change after inoculation of *Ri* and *Si* (Table 4). Inoculation of *Fm*, *Ds*, *Ri*, and *Si* all significantly increased soil NO_3^--N concentrations by 167%, 793%, 953%, and 573%, respectively. However, inoculation of *Fm*, *Ds*, *Ri*, and *Si* collectively reduced Olsen-P concentrations by 31%, 35%, 30%, and 29%, respectively. Inoculation of *Fm*, *Ds*, and *Ri* decreased available K concentrations by 16%, 16%, and 11%, respectively, accompanied by no change after *Si* inoculation.

Table 4. Effect of inoculation with symbiotic fungi on soil chemical properties of *Vicia villosa*.

Treatments	NH_4-N (mg/kg)	NO_3-N (mg/kg)	Olsen-P (mg/kg)	Available K (mg/kg)
Control	23.3 ± 3.9 c	1.5 ± 0.3 e	254.8 ± 32.7 a	29.9 ± 1.7 a
Fm	29.2 ± 4.1 b	4.0 ± 0.8 d	176.4 ± 17.1 b	25.2 ± 1.2 c
Ds	51.9 ± 1.1 a	13.4 ± 2.0 b	164.5 ± 7.6 b	25.1 ± 1.5 c
Ri	22.5 ± 2.1 c	15.8 ± 0.6 a	177.2 ± 6.9 b	26.7 ± 2.0 bc
Si	27.1 ± 4.0 bc	10.1 ± 0.8 c	180.8 ± 10.2 b	28.3 ± 0.6 ab
F value	55.27	126.08	16.79	7.45
p value	<0.0001	<0.0001	<0.0001	0.0016

Data (means ± SD, $n = 4$) followed by different letters in the column indicate significant differences at $p < 0.05$. For abbreviations see Figure 1.

3.7. Changes in Soil GRSP Levels

In comparison with the control, inoculation of *Fm*, *Ds*, *Ri*, and *Si* significantly increased soil GRSP levels, with EE-GRSP levels being increased by 95%, 120%, 195%, and 85%, DE-GRSP levels by 53%, 38%, 34%, and 27%, and T-GRSP levels by 61%, 54%, 66%, and 38%, respectively (Figure 4).

3.8. Changes in Soil WSA Distribution and Aggregate Stability

Inoculation of *Fm*, *Ds*, and *Ri* significantly increased $WSA_{2–4\ mm}$ by 160%, 81%, and 243%, respectively, and also increased $WSA_{1–2\ mm}$ by 222%, 231%, and 244%, respectively, compared with the control (Table 5). Inoculation of *Fm*, *Ds*, *Ri*, and *Si* all significantly increased $WSA_{0.5–1\ mm}$ by 149%, 136%, 186%, and 39%, respectively. All fungal inoculations did not affect $WSA_{0.25–0.5\ mm}$, compared with the control. In contrast with the control, inoculation with symbiotic fungi significantly increased soil MWD by 34−116%, and the differences among the four fungal treatments were also significant, showing the increased trend of *Ri* > *Fm* > *Ds* > *Si*.

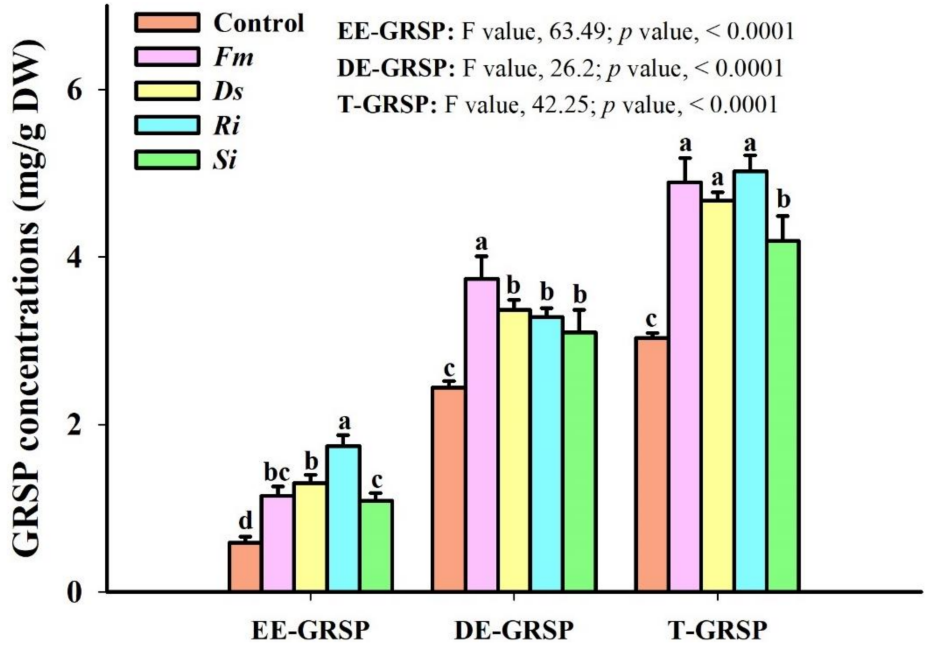

Figure 4. Effects of arbuscular mycorrhizal fungi and *Serendipita indica* on soil easily extractable glomalin-related soil protein (EE-GRSP), difficultly extractable glomalin-related soil protein (DE-GRSP), and total glomalin-related soil protein (T-GRSP) concentrations of *Vicia villosa* seedlings. Data (means ± SD, $n = 4$) followed by different letters above the bars indicate significant ($p < 0.05$) differences. For abbreviations see Figure 1.

Table 5. Effects of inoculation with different symbiotic fungi on the distribution of water-stable aggregates (WSAs) and mean weight diameter (MWD) of *Vicia villosa*.

Treatments	Distribution of WSA Fraction (%)				MWD (mm)
	2–4 mm	1–2 mm	0.5–1 mm	0.25–0.5 mm	
Control	0.42 ± 0.08 d	0.36 ± 0.07 b	1.38 ± 0.20 d	8.82 ± 1.21 a	0.061 ± 0.004 e
Fm	1.09 ± 0.18 b	1.16 ± 0.15 a	3.43 ± 0.35 ab	11.31 ± 2.38 a	0.118 ± 0.004 b
Ds	0.76 ± 0.07 c	1.19 ± 0.19 a	3.26 ± 0.43 b	10.81 ± 1.25 a	0.105 ± 0.011 c
Ri	1.44 ± 0.25 a	1.24 ± 0.16 a	3.94 ± 0.42 a	10.90 ± 1.86 a	0.132 ± 0.008 a
Si	0.53 ± 0.12 cd	0.35 ± 0.07 b	1.92 ± 0.34 c	12.38 ± 1.35 a	0.082 ± 0.003 d
F value	29.45	45.19	36.77	2.4	71.94
p value	<0.0001	<0.0001	<0.0001	0.0966	<0.0001

Data (means ± SD, $n = 4$) followed by different letters in the column indicate significant differences at $p < 0.05$. For abbreviations see Figure 1.

3.9. Correlationship Studies

There was a significant ($p < 0.01$) positive correlation between the root fungal colonization rate and soil EE-GRSP, DE-GRSP, and T-GRSP (Table 6). In addition, the root fungal colonization rate and three GRSP types were significantly ($p < 0.01$) positively correlated with soil WSA in the size of 2–4 mm, 1–2 mm, and 0.5–1 mm and MWD, and negatively correlated with Olsen-P ($p < 0.01$) and available K ($p < 0.05$ and 0.01) concentrations. In addition, root fungal colonization also significantly and positively correlated with chlorophylls a and b, total chlorophyll, carotenoid levels, leaf sucrose, and root glucose concentrations (Table 7).

Table 6. Pearson's correlation coefficient (*r*) between glomalin-related soil protein fractions and soil properties.

	Root Colonization	NH_4-N	NO_3-N	Olsen-P	Available K	Distribution of WSA Fraction				MWD
						2–4 mm	1–2 mm	0.5–1 mm	0.25–0.5 mm	
Root colonization	1	0.36	0.66	−0.64 **	−0.65 **	0.81 **	0.91 **	0.92 **	0.09	0.89 **
EE-GRSP	0.83 **	0.12	0.83	−0.69 **	−0.52 *	0.79 **	0.72 **	0.80 **	0.36	0.87 **
DE-GRSP	0.66 **	0.31	0.34	−0.74 **	−0.64 **	0.61 **	0.67 **	0.74 **	0.41	0.75 **
T-GRSP	0.82 **	0.25	0.63 **	−0.79 **	−0.65 **	0.77 **	0.77 **	0.85 **	0.43	0.91 **

*, $p < 0.05$; **, $p < 0.01$.

Table 7. Pearson's correlation coefficient (*r*) between root fungal colonization and plant physiological variables.

	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll	Carotenoid	Leaf Sucrose	Root Sucrose	Leaf Fructose	Root Fructose	Leaf Glucose	Root Glucose
Root colonization	0.88 **	0.87 **	0.90 **	0.78 **	0.73 **	−0.21	−0.21	−0.21	−0.06	0.76 **

**, $p < 0.01$.

4. Discussion

AMF could be applied as a biofertilizer to promote plant growth [16,30]. The present study showed that four symbiotic fungi promoted the growth and biomass production of *V. villosa* plants, depending on the fungal strains, with the *Ri* being the most effective. Similar results were also reported for *Poncirus trifoliata* [22] and *Polygonum cuspidatum* [23]. This suggests that symbiotic fungi, especially *Ri*, have a potential role in promoting the growth of *V. villosa* plants, attributed to symbiotic fungi helping the plants to absorb soil water and nutrients [31]. Meanwhile, inoculation with symbiotic fungi also accelerated the accumulation of various chlorophyll components in *V. villosa* plants, and the promoted effect of AMF was higher than that of the endophytic fungus *Si*. In fact, AMF could accelerate nitrogen metabolism of host plants by increasing leaf chlorophyll and carotenoid concentrations and regulating activities of enzymes associated with N assimilation [32], allowing plants to accumulate more carbohydrates and thus increasing biomass production.

In this study, root architecture (especially total length) of *V. villosa* plants was improved to different degrees by symbiotic fungi, and the positive effect was most pronounced for *Fm* and *Ri* inoculations, indicating that symbiotic fungi act as a biostimulator to promote the establishment of root architecture, but it depends on the symbiotic fungi species used. This is consistent with the results of Huang et al. [33] on *Juglans regia* plants colonized by AMF and Feng et al. [34] on *Lycopersicum esculentum* plants inoculated with five AMF isolates. The improvement of root architecture by symbiotic fungi is related to the increase of auxins, polyamines, and dopamine concentrations in the host by the fungi [35,36].

After establishing a symbiosis with AMF, fungi receive 4–25% of the photosynthetic products from the host plant for maintaining the symbiosis [12,37–39]. Our results showed that AMF promoted chlorophyll accumulation and consequently sucrose production in leaves, which may facilitate the transport of sucrose through the phloem to the roots as a carbon source for mycorrhizae. Therefore, we found that the increased trend of leaf sucrose decreased in roots, accompanied by a significant increase in root glucose and fructose in the AMF-inoculated roots. The fungal colonization rate positively correlated with root glucose. AMF directly takes up hexoses, mainly glucose, from host plant roots [39]. It is the cleavage of sucrose in roots into fructose and glucose for fungal growth [38], thus establishing higher mycorrhizal colonization rate and better root architecture [40]. AMF-modulated changes in sugars did not appear after endophytic fungal *Si* inoculation, indicating a difference in the regulation of sucrose synthesis and cleavage between AMF and *Si*.

In the present study, inoculated plants showed significant changes in soil nutrients, as evidenced by decreased Olsen-P and available K concentrations, as well as increased NH_4-N and NO_3-N concentrations, with differences between fungal inoculations. This is

consistent with the results of Zhu et al. [41] in *Paris polyphylla* inoculated with *Gigaspora albida* because mycorrhizae accelerated the mineralization of soil P and K, thus allowing the roots to take up more P and K from the soil. However, *V. villosa* is a leguminous crop, and AMF can accelerate the effect on N fixation [32], thus increasing the levels of various soil N. In addition, concentrations of GRSP were found in the rhizosphere of uninoculated plants because the extracts of GRSP contained other non-AMF-derived proteins and impurities [42].

Our study also reported that these symbiotic fungi increased soil GRSP levels, which agrees with the results of Cheng et al. [43] inoculating *Ds*, *D. versiformis*, and *Si* on the Newhall navel orange in the field. Correlation analysis also revealed a significant correlation between the root fungal colonization rate and three GRSPs levels. Mycorrhizal hyphae and its GRSPs are essential agents for soil aggregate formation and soil structural improvement [17]. Our study showed that the symbiotic fungi distinctly increased WSA at 0.5–4 mm size, thus promoting aggregate stability, based on the change in MWD. Correlation analysis also showed that the root fungal colonization rate and GRSPs were involved in the improvement of WSAs. This indicates that GRSP released by fungi is important for improving soil WSA formation and stability and is also an important reason for the fungal improvement of plant growth.

5. Conclusions

In summary, inoculation with symbiotic fungi improved the soil structure and fertility of *V. villosa* plants, thus improving plant growth. Among the fungi used, *Ri* inoculation showed the most prominent positive effect. In this way, the application of symbiotic fungi, especially *Ri*, can improve growth and soil properties in *V. villosa*, thus facilitating economic benefits.

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