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SHORT REPORT

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Response of soybean plants to two inoculation methods with arbuscular mycorrhizal fungus of *Glomus* sp. strain R-10 under field condition

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ABSTRACT

Inoculation of arbuscular mycorrhizal (AM) fungi has a great potential to reduce input of phosphorus fertilizer. In this study, we tested the hypothesis that transplanting of pre-inoculated plants (pre-inoculation) with AM fungal inoculum *Glomus* sp. strain R-10 (R-10) is more effective for increasing AM fungal colonization and soybean yield than placing R-10 inoculum into field soil (direct inoculation). We cultivated pre-inoculated and direct inoculated plants with and without R-10 in the same field. On the contrary to the hypothesis, hyphal colonization was increased by direct inoculation, but decreased by pre-inoculation in an early growth stage. Shoot phosphorus concentration, shoot dry weight, and yield also showed the same trend as the hyphal colonization. These results indicated that pre-inoculation with R-10 would be less effective for increasing AM fungal colonization and yield than direct inoculation. It may be due to a colonization strategy of R-10 and short duration for establishment of seedling.

Abbreviations: AM: arbuscular mycorrhizal; AMF: arbuscular mycorrhizal fungus; G: Glomus; Gi: Gigaspora; P: phosphorus.

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Colonization; direct inoculation; *Glycine max* (L.) Merrill; phosphorus; pre-inoculation; transplanting; yield

Introduction

Phosphorus (P) is one of the most important nutrients for crops. However, a natural reservoir of P is decreasing in the world, leading to increase in fertilizer price in the last decades (Cordell et al., 2009). Arbuscular mycorrhizal (AM) fungi increase nutrient uptake efficiency of crop hosts, especially phosphate, by an extensive belowground hyphal network in exchanging for photosynthetic products (Smith & Read, 2008). Inoculation of AM fungi has a great potential to reduce input of P fertilizer, but high cost for producing inoculum is limiting the use of inoculum for agricultural production. Thus, it is necessary to develop the effective inoculation method to improve yield as well as reduce input of AM fungal inoculum.

Methods of field inoculation can be mainly divided into two categories: transplanting pre-inoculated plants from a greenhouse to a field (pre-inoculation) and placing inoculum material into field soil (direct inoculation). In field conditions, a success of the fungal inoculant is mostly influenced by competition with indigenous mycorrhizal community (Niwa et al., 2018; Verbruggen et al., 2013). In our previous study, we conducted direct inoculation trails in three fields close to a field used in this study, and demonstrated that the more the indigenous fungal population was, the less effective the direct inoculation became (Niwa et al., 2018). Pre-inoculation using sterilized soil is thought to overcome this situation, if strong colonization of inoculated AM fungus (AMF) can be established before transplanting (Douds et al., 2016; Maltz & Treseder, 2015). A meta-analysis using 28 field-based restoration trials showed that pre-inoculation was more common and slightly effective than the direct inoculation, though each trial only adopted one of the two methods (Maltz & Treseder, 2015). In addition, pre-inoculation can save a large amount of inoculum compared to direct inoculation. However, no studies have compared the effectiveness of these two methods in the same field condition.

Soybean (*Glycine max* (L.) Merrill.) is the most widely grown legume in the world and has a high potential as a source of protein and oil. In our previous study, we showed the soybean yield increase by direct inoculation (Niwa et al., 2018). In Japan, the transplanting method of soybean has been developed to improve seedling establishment, which is hindered by bird and flooding damage (Kudo & Kato, 1979). Further, Matsumoto and Yoshikawa (2001) conducted pre-inoculation trails using *Gigaspora margarita* in five fields, and showed the yield increase of

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pre-inoculated soybean plants with *Gi. margarita* compared to non-inoculated ones. Thus, the comparison of the effects of the two inoculation methods with AM fungus on soybean plants is an essential step for successful application of AM fungi in agricultural production. AM fungal inoculum *Glomus* sp. strain R-10 (R-10) has been patented and commercialized in Japan by Idemitsu Kosan Co., Ltd. Earlier studies using this inoculum have shown that it has a positive impact on growth and nutrition of a wide range of plant species including soybean, when added to sterilized soil in pots (Isobe et al., 2002; Matsubara & Hosokawa, 1999; Matsubara et al., 2000) and non-sterilized fields (Niwa et al., 2017; Tawaraya et al., 2012). In this study, we tested the hypothesis that pre-inoculation with R-10 is more effective for increasing AM fungal colonization and soybean yield than direct inoculation. To test it, we compared the hyphal colonization, P uptake, growth, yield, and yield component of pre-inoculated and direct inoculated plants with and without R-10 in the same field condition.

Materials and methods

Experimental design

A field is situated in Miyakonojo Research Station, Kyushu Okinawa Agricultural Research Center, National Agricultural and Food Research Organization, Miyakonojo Miyazaki, Japan (31°45′05″N 131°00′46″E). Monthly average temperature in July, August, September, October, and November 2016 was 27, 28, 26, 22, and 14°C, respectively. Total accumulated precipitation in July, August, September, October, and November 2016 was 648, 117, 531, 263, and 113 mm, respectively. These climatic data were obtained at Miyakonojo observatory, Japan Meteorological Agency, which is located approximately 9 km from the experimental field. The soil of the field belongs to the order Andosol that is loam in texture, volcanic ash, deep percolating, and well drained. Chemical properties of the soil were as follows: pH (H_2O), 6.38; P_2O_5 absorption coefficient, 1823; available P_2O_5, 162 mg kg^{-1}; NH_4^+-N, 2.68 mg kg^{-1}; NO_3^-N, 2.98 mg kg^{-1}; K_2O, 108 mg kg^{-1}. P fertilizer was not applied. Ammonium sulphate and potassium chloride were applied to the field before sowing at 40 kg N ha^{-1} and 120 kg K_2O ha^{-1}, respectively.

A randomized complete block design with four replications was used. In each block, two inoculation methods (direct inoculation and pre-inoculation) were applied with and without mycorrhizal inoculation. An AM fungal inoculum R-10 or an R-10 free carrier (Idemitsu Kosan Co., Ltd., Tokyo) was used for the experiment. The inoculum consists of spores, extraradical hyphae, and chopped colonized roots with a crystalline-silica carrier (14 propagules g^{-1}). The replicate plot size was 3.9 × 1.4 m, in which six rows were arranged with distance of 0.65 m between rows and 0.2 m between hills. Two plants were grown in each hill. In direct inoculated plots, five grams of either the R-10 inoculum or an R-10 free carrier was placed at a depth of 80 mm and buried, and then three seeds of *Glycine max* cv. Fukuyutaka were sown at a depth of 30 mm on 27 July 2016. The plants were thinned to two after the first trifoliate appeared. In pre-inoculated plots, seedlings were grown according to Kudo et al. (1980) and Matsumoto and Yoshikawa (2001). The R-10 inoculum or the R-10 free carrier was mixed with autoclaved artificial soil mainly consisted of vermiculite and peatmoss (Napura soil mixes, Yanmar Co., Japan) at the rate of 200 g kg^{-1} soil. To enhance germination of spores and growth of hyphae, inoculated potting media were pre-incubated 7 days prior to sowing according to Douds et al. (2016). 128-cell plastic nursery trays (size of cell, 30 × 30 × 45 mm depth) were filled with 1 kg of an inoculated potting mix or a non-inoculated one on 20 July 2016 and watered as needed in the greenhouse until 27 July. On 27 July, two seeds of *Glycine max* were sown to each cell, and then the trays were covered with 200 g of each potting mix. The plants were grown in the greenhouse and watered as needed until 5 August. On 5 August, when the plants expanded cotyledons, two plants were uprooted with a potting mix from each cell, placed at a depth of 65 mm, and covered with field soil. Shoot dry weight with and without R-10 at the transplanting (n = 8) was 0.24 and 0.22 g $plant^{-1}$, respectively. The hyphal colonization with and without R-10 at the transplanting (n = 8) was 0.07 and 0%, respectively. Roots of pre-inoculated plants were insufficiently colonized by R-10. Only one of the eight plants formed arbuscules. Weeds were controlled manually. Intertillage and ridging were conducted on 25 August.

Field sampling

At the time of fifth unrolled trifoliolate leaf expanding (V5) stage (22 August) and full blooming (R2) stage (8 September), roots and shoots were collected from eight plants in each plot and combined. The roots were washed gently with tap water. Middle portions (10–20 mm) of roots were cut off, cut into <10-mm segments, randomized in water, and then about 1 g of lateral and tap roots were collected. The roots were cleared in 10% potassium hydroxide and stained with Trypan blue by a procedure from Phillips and Hayman (1970). Hyphal colonization was determined by the grid line intersect method (Giovannetti

& Mosse, 1980). The shoots were dried at 80°C for 72 h, weighed, ground with a mill, and digested with a mixture of nitric acid/perchloric acid. P concentration in the digests was determined with the vanadomolybdate-yellow assay (Olsen & Sommers, 1982).

At the time of full maturity (R8) stage (2 November), shoots were harvested from 24 plants grown within 1.56 m^2 (1.95 × 0.8 m) in each plot. Eight plants were selected for measuring main stem length and the number of branches, nodes, and pods. Then, seed yield and 100 seeds weight of 24 plants were recorded.

Statistical analysis

All statistical analyses were performed with statistical software (JMP 12.2, SAS Institute Inc., U.S.A). Percentage of hyphal colonization was arcsine transformed. Plant growth parameters and nutrient uptake were analysed by analysis of variance at significant level of $p < 0.001$, 0.01, 0.05, and 0.1 and the Tukey test (HSD) at significant level of $p < 0.05$.

Table 1. Hyphal colonization, shoot phosphorus (P) concentration, and shoot dry weight of direct inoculated and pre-inoculated soybean plants with and without R-10 at the time of V5 and R2 stage.

Treatment		Hyphal colonization (%)		Shoot P concentration (mg P g^{-1})		Shoot dry weight (g $plant^{-1}$)	
Method	Inoculum	V5	R2	V5	R2	V5	R2
Direct inoculation	Control	58.7 ^{ab}	77.6	3.95	3.72	3.43	18.5
	R-10	75.2 ^{a}	75.1	4.08	3.77	3.59	19.5
Pre-inoculation	Control	60.1 ^{ab}	77.5	2.89	3.96	1.91	13.9
	R-10	44.3 ^{b}	72.4	2.79	4.08	1.80	12.7
Method		*	ns	***	*	***	**
Inoculum		Ns	ns	ns	ns	ns	ns
Method × Inoculum		**	ns	ns	ns	ns	ns

Values are means ($n = 4$). Hyphal colonization was arcsine transformed for statistical analysis. The different letters indicate significant differences among the treatments (Tukey HSD test, $p < 0.05$). ANOVA: ns: not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Simple linear regression models were applied to analyse correlations among parameters at significant level of $p < 0.001$, 0.01, 0.05.

Results and discussion

There was a significant interaction between method and inoculation in hyphal colonization (Table 1). On the contrary to the hypothesis, the hyphal colonization was increased by direct inoculation, but decreased by pre-inoculation at the V5 stage. Shoot P concentration at the V5 stage and shoot dry weight at both two stages were followed the same trend as the hyphal colonization at the V5 stage. There was a significantly positive correlation between the hyphal colonization and the shoot P concentration at the V5 stage (Table 2). Additionally, the shoot P concentration at the V5 stage was significantly correlated with the shoot dry weight at the both stages. These results indicated that the significant difference in hyphal colonization resulted in the difference in the growth of soybean plants at the both stages through P uptake. At harvest, there was a significant interaction between method and inoculation at 10% in yield (Table 3). The yield of direct inoculated plants was significantly higher than that of transplanted plants. Moreover, yield was significantly correlated with hyphal colonization and shoot P concentration at the V5 stage, and shoot dry weight at the both stages (Table 2). From these results, we concluded that the pre-inoculation with R-10 would be less effective for increasing AM fungal colonization and soybean yield than the direct inoculation.

Kudo and Kato (1979) showed the reduction of number of nodes and pods of transplanted soybean plants compared to sowed ones. Similar results were obtained in this study. Yield and yield components except nodes number of branches were significantly decreased by transplanting (Table 3). Among yield components, the

Table 2. Bivariate correlation coefficient matrix between growth parameters and yield component.

Variables	HC-V5	HC-R2	SPC-V5	SPC-R2	SDW-V5	SDW-R2	MSL	NN-MS	NN-B	NN-T	PN-MS	PN-B	PN-T	100 SW	Yield
HC-V5	1.000														
HC-R2	0.119	1.000													
SPC-V5	0.594	0.176	1.000												
SPC-R2	−0.435	−0.065	−0.371	1.000											
SDW-V5	0.607	0.053	**0.867**	−0.395	1.000										
SDW-R2	0.424	−0.044	**0.784**	−0.497	0.782	1.000									
MSL	0.424	0.206	0.670	−0.070	0.639	0.687	1.000								
NN-MS	0.711	−0.027	**0.826**	−0.528	**0.916**	**0.837**	0.674	1.000							
NN-B	0.448	−0.545	0.205	0.000	0.262	0.334	0.231	0.344	1.000						
NN-T	0.610	−0.465	0.445	−0.174	0.522	0.556	0.416	0.618	0.951	1.000					
PN-MS	0.392	0.080	0.329	**−0.763**	0.361	0.330	0.149	0.457	−0.167	0.011	1.000				
PN-B	0.495	−0.339	0.626	−0.167	0.733	0.732	0.536	**0.770**	0.714	**0.852**	−0.048	1.000			
PN-T	0.608	−0.296	0.711	−0.424	**0.825**	**0.813**	0.563	**0.893**	0.624	**0.817**	0.301	0.938	1.000		
100 SW	0.472	−0.051	**0.754**	−0.472	**0.818**	**0.870**	0.585	**0.794**	0.208	0.437	0.511	0.629	**0.778**	1.000	
Yield	0.681	−0.204	0.661	−0.143	**0.797**	0.651	0.620	**0.802**	0.566	0.738	−0.020	**0.856**	**0.810**	0.621	1.000

HC: hyphal colonization; SPC: shoot P concentration; SDW: shoot dry weight; MSL: main stem length; NN nodes number; PN: pods number; SW: seed weight; MS: on main stem; B: on branch; T: total. Hyphal colonization was arcsine transformed for statistical analysis. ■t $p < 0.05$; ■, $p < 0.01$; ■,H$p < 0.001$.

Table 3. Yield and yield component of direct inoculated and pre-inoculated soybean plants with and without R-10 at the time of R8 stage.

Treatment		Yield	Main stem length	Nodes number			Pods number			100 seeds weight
Method	Inoculum	(g m^{-2})	(cm)	(no. $plant^{-1}$)	Branch	Total	Main stem	Branch	Total	(g)
Direct inoculation	Control	319 ^{ab}	46.5 ^{ab}	12.3 ^{b}	17.6	29.9 ^{b}	22.6	40.8	63.4	27.3
	R-10	352 ^{a}	49.0 ^{a}	12.7 ^{a}	20.3	33.1 ^{a}	21.1	46.9	68.0	27.1
Pre-inoculation	Control	303 ^{b}	46.3 ^{ab}	11.7 ^{c}	18.0	29.7 ^{b}	21.2	37.0	58.2	26.1
	R-10	295 ^{b}	43.5 ^{b}	11.2 ^{d}	18.8	29.9 ^{b}	19.4	38.8	58.2	25.8
Method		**	*	***	ns	**	*	**	***	***
Inoculum		ns	ns	ns	**	**	*	*	ns	ns
Method × Inoculum		†	*	***	ns	*	ns	ns	ns	ns

Values are means ($n = 4$). The different letters indicate significant differences among the treatments (Tukey HSD test, $p < 0.05$). ANOVA: ns: not significant; †, $p < 0.10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

significant interactions between method and inoculation were observed in yield, main stem length, nodes number on main stem, and total nodes number. These parameters were affected by R-10 inoculation as well as inoculation method. Especially, nodes number of main stem and total nodes number of direct inoculated plants were significantly higher than the other plants, and total pods number of direct inoculated plants was the highest. In contrast, nodes number of main stem of pre-inoculated plants was significantly lower than the other plants. The highest correlation coefficient of hyphal colonization at V5 stage was observed with nodes number on main stem (Table 2). However, hyphal colonization at V5 stage was not correlated with 100 seeds weight. These results indicated that R-10 colonization increased yield mainly through increment of nodes and pods, but not seed weight.

In this study, the difference in hyphal colonization at the early growth stage resulted in the difference in yield through the P uptake. Though Matsumoto and Yoshikawa (2001) examined the effect of pre-inoculation of soybean plants with *Gi. margarita* and showed the yield increase compared to non-inoculated ones, we used *G*. sp. strain R-10 and showed the yield reduction by pre-inoculation. Maherali and Klironomos (2007) suggested that colonization of roots by specific AMF species may influence subsequent colonization by other AMF species. Further, Mummey et al. (2009) showed that pre-inoculation of *G*. spp. restricted colonization by other AMF species in field soil and decreased nutrient uptake and shoot dry weight of *Leucanthemum vulgare*, though *Gi*. spp. did not restrict colonization by other AMF species. In their study, the AM colonization of pre-inoculated plants at the transplanting was as insufficient as this study (Mummey et al., 2009). Though the majority of fungal biomass in the Gigasporaceae is found in the hyphae that are located outside the plant root, in contrast, the majority of fungal biomass in the Glomeraceae is found in hyphae growing inside the root (Hart & Reader, 2002; Maherali & Klironomos, 2007). Thus, the different results between this study and Matsumoto and Yoshikawa (2001) might be caused by the difference in the colonization strategy of AMF species. This speculation can be examined in detail using *Gi*. spp. and *G*. spp., the same experimental design as this study, and the plants whose hyphal colonization is different. It would be meaningful for understanding of the colonization strategy of each AMF species.

In contrast to soybean, Tawaraya et al. (2012) transplanted the pre-inoculated *Allium fistulosum* seedlings with the same inoculum as this study and showed significant increase in yield. However, the duration for the seedling establishment of soybean in this study (9 days) was quite shorter than *Allium fistulosum* (58 days) and the hyphal colonization at the transplanting of soybean in this study and *Allium fistulosum* was 0.07% and 94%, respectively (Tawaraya et al., 2012). In the transplanting of soybean plants, the older leaf age became, the lower yield became. The yield of soybean plants transplanted at second unrolled trifoliolate leaf expanding was 27% lower than that of ones transplanted at cotyledons expanding (Kudo & Kato, 1979). Hence, the extension of the nursery period for soybean seedling establishment would not be appropriate for agricultural production. Establishment of AM fungal colonization from spores will require more than nine days under optimal conditions: more than three days for spores to germinate, more than two days for contact to germ tube hyphae with the root, and more than four days after contact for intracellular development (Bécard & Fortin, 1988; Sward, 1981). Douds et al. (2016) succeeded to increase the mycorrhizal colonization of maize seedling roots from 3.3% to 10.5% by pre-incubating the inoculated potting media 7 days prior to sowing. In this study, we also pre-incubated the inoculated potting media in the greenhouse 7 days prior to sowing, but the hyphal colonization of pre-inoculated soybean plants at the transplanting reached only 0.07%. It indicated that the duration for the seedling establishment of soybean would be still short for the establishment of R-10 colonization, and thus pre-

inoculation with R-10 would not be suitable for agricultural production of soybean.

We concluded that the pre-inoculation with R-10 would be less effective for increasing AM fungal colonization and soybean yield than the direct inoculation to fields. In our previous study, we also succeeded to increase AM fungal colonization and soybean yield by direct inoculation with R-10 in a nearby field (Niwa et al., 2018). These results indicated that direct inoculation with R-10 would be effective for improving soybean yield. Thus, we recommend that future studies should examine the appropriate amount of R-10 inoculum for direct inoculation to develop feasible inoculation method.

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Disclosure statement

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