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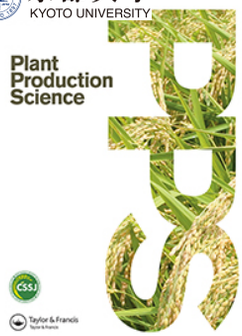

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REPORT

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Effects of *Phytophthora sojae* inoculation under flooded conditions on growth of soybean seedlings

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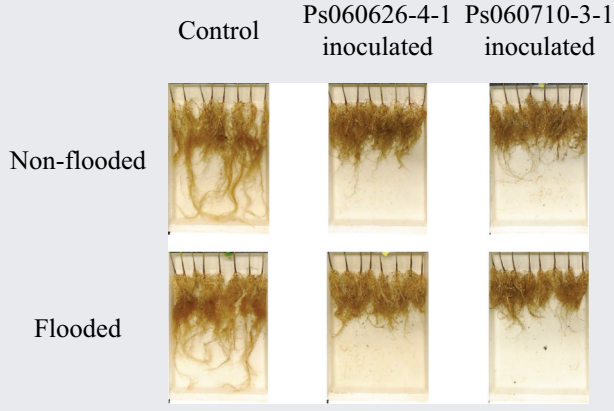
ABSTRACT

Phytophthora root and stem rot due to *Phytophthora sojae* is a major constraint of soybean production. This study evaluated the combined effect of *P. sojae* inoculation and flooding on growth of soybean seedlings. The soybean cultivar 'Enrei' was grown in a greenhouse in pots containing vermiculite and containers of field soil. The plants were inoculated with two *P. sojae* isolates and subjected to flooding. The ratio of dead to live plants ranged from 0 to 0.32 across all treatments. Pathogen inoculation caused a significantly shorter maximum root length (MRL) in all the three experiments. MRL and shoot and root dry weight were affected by interaction between inoculation and flooding in one experiment with vermiculite media. Flooding affected the growth parameters only in the experiment with soil media. The results indicated that root of the soybean seedlings that survived from *P. sojae* infection grew less well than the non-inoculated plants.



Soybean seedlings grew more poorly especially in root length, when the plants survived from *P. sojae* attack compared with non-inoculated plants.

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Flooded conditions; *glycine max*; *phytophthora sojae*; soil-borne; disease; soybean

Introduction

Phytophthora root and stem rot (PRSR), caused by an oomyceteous fungus-like protist *Phytophthora sojae* (Kaufmann & Gerdemann, 1958), is one of the most serious and widespread soil-borne diseases of soybean [*Glycine max* (L.) Merr.], with annual yield losses estimated at over one billion dollars worldwide (Tyler, 2007). Consequently, it is desirable to develop effective disease management strategies as an urgent initiative (Sugimoto et al., 2012). Several studies have reported that the use of resistant cultivars and chemicals results in the reduction of PRSR damage in crops. However, it is not good to rely only on resistant cultivars because there is a possibility of resistance breakdown, as reported in the Azuki bean (Fujita, 2013). Mefenoxam and metalaxyl are currently two of the most effective chemicals for preventing PRSR. In recent years, seed coating chemical

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Supplemental data for this article can be accessed here.

mixes containing metalaxyl for prevention of PRSR have been developed and widely used in Japan; however, the development of resistance to these chemicals has been reported in a few *Phytophthora* species (Parra & Ristaino, 2001).

One of the most sustainable approaches for controlling PRSR damage in crops is the adoption of appropriate cultivation methods or controls. There is a paucity of research publications on the effects of cultivation methods and/or environmental factors on PRSR, yet it is known that damage can be severe in poorly drained soils which facilitate the production of motile zoospores (Erwin & Ribeiro, 1996; Schmitthenner, 1985). In particular, there is limited experimental evidence about the interaction between *P. sojae* and varied water conditions on the occurrence of PRSR, and these observations are mainly qualitative.

Repeated experiments were carried out to evaluate the interaction effect of *P. sojae* infection and flooding on the mortality and growth of surviving seedlings, particularly in relation to root dry weight (RDW), shoot dry weight (SDW), and maximum root length (MRL).

Materials and methods

Plant materials and cultivation conditions

Three experiments (Exps. 1, 2, and 3) were conducted in a greenhouse from 2018 to 2019 (Table 1). The soybean cultivar 'Enrei' (maturity group V) was grown in small pots for Exps. 1 and 2 (14.5 cm in diameter, 17.2 cm in height, and 1.9 L in volume) and in large containers for Exp. 3 (86 cm in width, 66 cm in depth, 34 cm in height, and 120 L in volume). In Exp. 1, four plants per pot and eight pots per plot were used. Seven plants per pot and 10 pots per plot were used in Exp. 2. In Exp. 3, around 40 plants (83.3–87.5 plants m^{-2}) per container and one container per plot were used. The sowing dates were July 2 and September 15, 2018, and July 31, 2019; the soybean plants were cultivated for 28–35 days. Two different types of plant culture media were used: vermiculite in the pot experiments (Exps. 1 and 2) and field soil in the container experiment (Exp. 3). The field soil (a sandy loam, Fluvic Endoaquept) was collected from a field at the experimental farm of the Graduate School of Agriculture, Kyoto University. A nutrient solution with a 250 mL per pot dilution of 1/2000 Hyponex (N: P_2O_5: K_2O, 5: 10: 6, HYPONeX Japan Co., Ltd., Osaka, Japan) was supplied two weeks after sowing to the pot experiments only.

Table 1. Cultural conditions of three experiments that investigated the effects of flooding and inoculation with two *Phytophthora sojae* isolates (Ps060626-4-1 and Ps060710-3-1) on soybean survival and growth.

Exp.	Pot/ Container	Medium	Sowing date	Period of plant culture
1	Pot	Vermiculite	2 July 2018	28
2	Pot	Vermiculite	15 September 2018	35
3	Container	Field Soil	31 July 2019	29

Pathogen culture and inoculation

The *P. sojae* isolates Ps060626-4-1 and Ps060710-3-1 used in this study were originally isolated from diseased plants in Miyagi and Niigata, Japan, and maintained as stock cultures in the Hokuriku Research Center, Central Region Agricultural Research Center, National Agriculture and Food Research Organization (NARO) (Takahashi et al., 2019). Both isolates are reported to be virulent to the soybean cultivar 'Enrei' (Takahashi et al., 2019). These isolates were cultured in plastic petri dishes (90 mm in diameter by 15 mm in height) on a V8 juice (Campbell Soup Company, Camden, NJ, U.S.A.) agar medium (200 mL V8 juice, 3.0 g $CaCO_3$, 15 g agar, and 800 mL H_2O) for 10–14 days at 25°C in dark conditions.

The inoculation of the soybean seedlings with the pathogen was performed according to the method described by Akamatsu et al. (2019) and Mukobata and Sekihara (2006), with some modifications. Each culture plate was mixed with 200 mL of distilled or tap water for 40 s using a mixer (Siroca Crossline; Siroca Inc., Tokyo, Japan). One week after sowing, when the length of unifoliolate leaves was approximately 1 cm, one plate per pot of the suspension was applied to the treatment group pots in Exps. 1 and 2. For Exp. 3, the suspensions from nine plates were applied to the container which served as the treatment group. In Exps. 1 and 2, water only was added to the non-inoculated (control) plants whereas a mixed pure V8–agar suspension was used for the control in Exp. 3.

Flooding treatment

The flooding treatment was carried out immediately after inoculation of the plants and, in all treatment groups, the water level was maintained at 2 cm above the medium surface for 72 h. After flooding, the water level was maintained at 10–12 cm below the medium surface in the pot experiments whereas in the container experiment, the water was allowed to drain naturally. For the non-flooded control groups, the water level was maintained at 10–12 cm below the medium surface in the pots in Exps. 1 and 2, and, in Exp. 3, the containers were watered enough to keep the surface of the soil moist.

Measurements

At the end of each experiment, the plants were carefully removed from the media to avoid breaking the roots. Whole-plant images were obtained for plants in both the treatment and control groups in Exps. 1 and 2. All plants were observed for signs and symptoms of disease, with reference to the descriptions by Erwin and Ribeiro (1996) and Nakagawa (2012), and the number of dead plants was determined. For the surviving plants, the maximum root length (the length between the cotyledonary node and the farthest tip of the root, MRL) was measured. The surviving plants were divided into shoot and root parts at the cotyledonary node, and each part was dried at 80° C for 48 h and then weighed for dry weight analysis.

Data analysis

Each of Exps. 1 and 2 included six plots based on pathogen inoculation (non-inoculated, Ps060626-4-1 inoculated, and Ps060710-3-1 inoculated) and flooding treatment (non-flooded and flooded). Only four plots were included for Exp. 3 because the Ps060626-4-1 inoculated plots were excluded from analysis as a consequence of failure to control the water conditions.

The effect of inoculation and flooding on the mortality ratio was determined using a Generalized Linear Model with binomial error distribution and the logit link function, regarding each experiment as the experimental unit. Since the Exp. 3 Ps060626-4-1 data were excluded, this statistical analysis was carried out separately for the two pathogen isolates. The Ps060626-4-1 effect was determined using results from Exps. 1 and 2, and the Ps060710-3-1 effect, using data from all three experiments. Explanatory variables were considered at the 5% significance level determined by the likelihood ratio test. To determine the main effects and interaction effect on each growth measure (MRL, SDW, and RDW) of the seedlings that survived, two-way ANOVA (*P. sojae* inoculation and flooding treatment) was conducted, regarding each plant as the experimental unit. Since each surviving plant was a replicate, the error variance was calculated with at least 30 replicates per experiment. The means were compared using Tukey's HSD test in case an interaction between inoculation and flooding, or not such interaction but only inoculation effect was observed. All statistical analyses were carried out using R software (R Development Core Team, 2019). In the current study, the results by inoculation of the two pathogen isolates, Ps060626-4-1 and Ps060710-3-1, were inconsistent. But, since the difference between their effects was not major concern of this study, the effect of inoculation was regarded as to be detected if any one isolate of the showed significant influence on soybean seedling growth.

Results and discussion

No symptoms were observed in any plants without pathogen inoculation. Of the 10 plots with inoculation, in all three experiments, five did not show mortality in any plants, and the mortality ratios ranged from 0.06 to 0.32 for the remaining five plots (Table 2). The results of likelihood ratio test indicated that inoculation significantly affected the mortality ratio for Ps06026-4-1 isolate (Table 2). The effect of flooding on mortality was not evident. An interaction effect between inoculation and flooding was not observed (Table 2).

The mortality ratios were low for all treatments, indicating that the virulence of the two isolates was not considerably different. Although the mortality ratio of plants inoculated with the Ps060710-3-1 isolate without flooding was higher than for the other treatments, it was not clear why this was the case, especially in relation to the lack of flooding. Further research is needed to understand this phenomenon. Overall, there was no clear interaction effect of pathogen inoculation and flooding on the mortality ratio of the seedlings.

The seedlings that survived from infection included plants with and without disease symptoms. Since most of these plants had no evident symptoms, they were all regarded as surviving plants and statistical analysis was carried out accordingly.

The ANOVA results indicated that pathogen inoculation affected root growth of the surviving plants in terms of both length and dry weight (Table 3). MRL of plants in all treatment groups was significantly affected by inoculation with *P. sojae* where the inoculated plants had significantly shorter roots than the non-inoculated plants (Supplemental Fig.). RDW was also partially affected by

Table 2. Mortality ratios of soybean seedlings affected by flooding and inoculation with two *Phytophthora sojae* isolates. Results of GLM and likelihood ratio test of plots with each of the two isolates were compared with non-inoculated plots. N.S., **, and *** indicate $P \geq 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

		Non-inoculated		Ps060626-4-1		Ps060710-3-1	
Exp.	Flooding	Count	Ratio	Count	Ratio	Count	Ratio
1	Non-flooded	0/32	0.00	0/32	0.00	0/32	0.00
	Flooded	0/32	0.00	0/32	0.00	0/32	0.00
2	Non-flooded	0/61	0.00	4/62	0.06	21/64	0.32
	Flooded	1/65	0.02	5/60	0.08	7/55	0.13
3	Non-flooded	0/42	0.00			0/40	0.00
	Flooded	9/40	0.23			4/40	0.10
Inoculation [I]				$P \geq 0.01$		n/a	
Flooding [F]				$P \geq 0.05$		n/a	
I × F				$P \geq 0.05$		n/a	

"Note: n/a means that the test was not completed due to overdispersion according to the Pearson chi-square test.

Table 3. Growth of surviving soybean seedlings affected by flooding and inoculation with two *Phytophthora sojae* isolates. MRL, SDW, and RDW indicate the maximum root length, shoot dry weight, and root dry weight, respectively. Two-way ANOVA and Tukey's HSD test were conducted locating the two *P. sojae* isolates individually in the same factor of pathogen isolate. N.S., *, **, and *** indicate $P \geq 0.05$, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively for the two-way ANOVA. The same letters next to the values indicate no significant difference $P < 0.05$ for Tukey's HSD test.

		Exp. 1			Exp. 2			Exp. 3		
Flooding	Pathogen solate	MRL (cm)	SDW (g)	RDW (g)	MRL (cm)	SDW (g)	RDW (g)	MRL (cm)	SDW (g)	RDW (g)
Non-flooded	Non-inoculated	47.1 a	1.17 b	0.56 b	25.0 b	0.96 bc	0.23 b	32.6 a	0.99	0.32 a
	Ps060626-4-1	31.3 b	1.21 ab	0.63 ab	24.5 b	1.10 ab	0.24 ab			
	Ps060710-3-1	30.8 b	1.18 b	0.62 a	25.3 b	1.12 ab	0.24 ab	28.2 b	1.01	0.25 b
Flooded	Non-inoculated	49.1 a	1.32 a	0.62 b	28.1 a	1.15 a	0.28 a	27.3 b	0.74	0.29 ab
	Ps060626-4-1	32.3 b	1.18 b	0.64 ab	23.3 bc	0.99 abc	0.25 ab			
	Ps060710-3-1	28.4 b	1.20 ab	0.67 a	21.9 c	0.86 c	0.22 b	27.1 b	0.81	0.33 a
Inoculation [I]		***	N.S.	*	***	N.S.	*	**	N.S.	N.S.
Flooding [F]		N.S.	N.S.	*	N.S.	N.S.	*	**	***	N.S.
I × F		N.S.	*	N.S.	***	***	**	*	N.S.	**

pathogen infection. However, there was no significant effect on SDW in any of the experiments (Table 3).

The effect of flooding on root and shoot growth of soybean seedlings was less evident than that of *P. sojae* inoculation. Flooding affected SDW, to the greatest extent in Exp. 3 where the plants were grown in field soil (Table 3), while flooding showed a rather positive effect on RDW in Exps. 1 and 2 with vermiculite. The fact that flooding did not negatively affect the growth of seedlings in vermiculite could be attributed to a difference in the stability of the two substrates. In comparison with field soil, the physical environment in vermiculite is more stable due to a greater porosity, which may account for why flooding did not affect seedling growth in this medium (Motomura, 1970). In addition, the substrate used in Exp. 3 contains soil organic carbon with microorganisms, and this may cause deoxidization and reduction of the soil environment. Besides, there may happen various damages by flooding (Kokubun, 2013), which may lead to the greater effect of the flooding treatment on soybean growth in Exp. 3.

The interaction between *P. sojae* inoculation and flooding was significant or insignificant for root and shoot growth of soybean seedlings depending on measurement items and experiments. (Table 3). In the pot experiments (Exps. 1 and 2), the growth of the surviving plants inoculated with each pathogen isolate was inhibited more in combination with flooding, as evidenced by the results of ANOVA for SDW (Exps. 1 and 2), MRL (Exp. 2) and RDW (Exp. 2). In Exp.1, SDW was similar between inoculated and non-inoculated plants under non-flooded conditions, whereas under flooded conditions SDW of inoculated plants was 11% (Ps060626-4-1) and 9% (Ps060710-3-1) lower than for the non-inoculated plants (Figure 1). In Exp. 2., SDW of the plants inoculated with Ps060626-4-1 and Ps060710-3-1 was 15% and 17% greater than that of the non-inoculated plants, respectively, under non-flooded conditions, whereas under flooded conditions SDW of the inoculated plants was 14% (Ps060626-4-1) and 25% (Ps060710-3-1) lower than that of the non-inoculated plants. Root growth (MRL and RDW) of the plants in Exp. 2 showed the same tendency as the shoot growth (Figure 1).

In the container experiment (Exp. 3), on the other hand, root growth (MRL and RDW) of inoculated plants was inhibited only under non-flooded conditions (Table 3). MRL of the inoculated plants in Exp. 3 was 13% shorter than the non-inoculated plants under non-flooded conditions, whereas there was no difference in MRL under flooded conditions in response to inoculation with the Ps060710-3-1 isolate. RDW in the container experiment was 20% lower in inoculated than in non-inoculated plants under non-flooded conditions, whereas it was higher in inoculated plants than non-inoculated plants under flooded conditions (Figure 2). Although the more severe effects of inoculation were detected in the pot experiments (Exps. 1and 2), they were inconsistent. For example, an interaction effect on root growth (MRL and RDW) was not detected in Exp. 1 (Table 3). While determination of the factors that differentiate the interaction between inoculation and flooding remains an important issue for further study, results of this study indicate that flooding does not necessarily make the influence of *P. sojae* on plant mortality and/or growth more severe.

The evidence of reduced growth of the surviving plants suggests that soybean seedling growth is potentially inhibited by *P. sojae* even when most plants appeared to be unaffected by the pathogen. Although it has been reported that excess water leads to a poor growth rate in soybean plants (Kokubun, 2013), the reduction in growth observed in this study could be

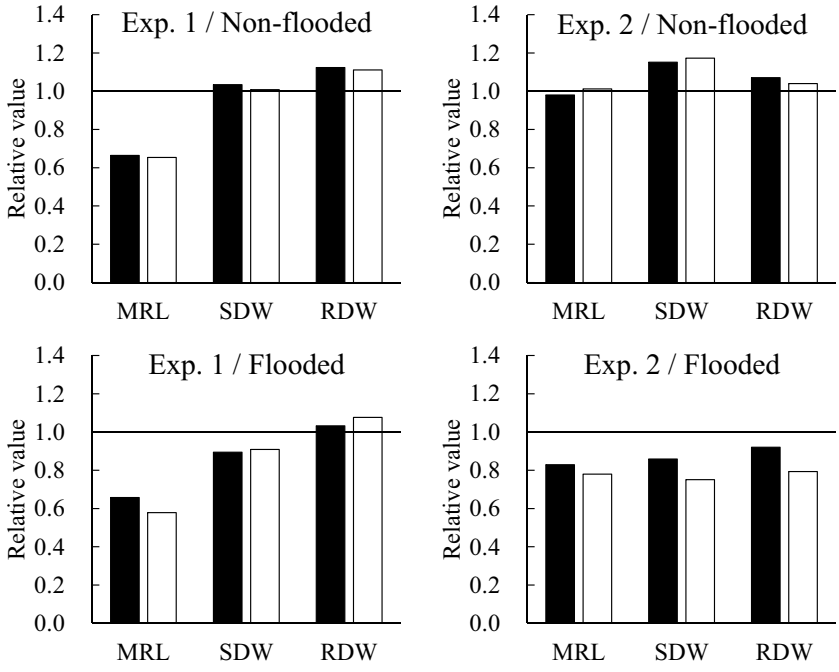

Figure 1. Effects of *Phytophthora sojae* inoculation on the relative growth performance of soybean seedlings to non-inoculated ones grown in 1.9 L pots of vermiculite under non-flooded and flooded conditions (Experiments 1 and 2). The black and white bars indicate the values of the Ps060626-4-1 and Ps060710-3-1 isolate inoculation plots, respectively. MRL, SDW, and RDW indicate maximum root length, shoot dry weight, and root dry weight, respectively.

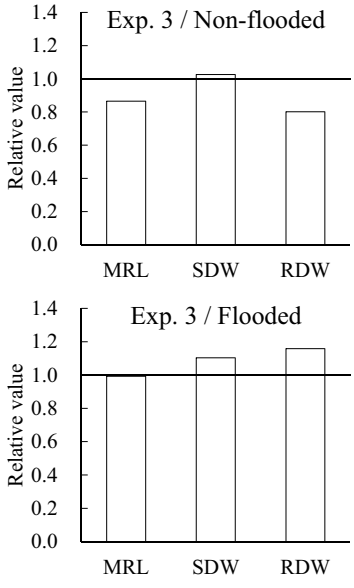

Figure 2. Effects of inoculation with the *Phytophthora sojae* isolate Ps060710-3-1 on the relative growth performance of soybean seedlings to non-inoculated ones grown in containers of field soil under non-flooded and flooded conditions (Experiment 3). MRL, SDW, and RDW indicate maximum root length, shoot dry weight, and root dry weight, respectively.

attributed to PRSR occurrence, which is more prevalent in flooded and poorly drained soils. In general, the effect of PRSR on soybeans has often been discussed with respect to host mortality because of its strong pathogenicity. However, it is thought that, under field conditions, soybean plants have a high rate of latent PRSR infection. The effects of latent infection were observed in this study through the measurements that reflected shoot and root growth.

Previous research indicates that *P. sojae* produces motile zoospores in flooded conditions, thus aggravating the damage caused by PRSR (Erwin & Ribeiro, 1996), which leads to a higher death rate of affected plants. However, in the current study, the majority (up to 68%) of soybean plants survived from inoculation with the pathogen, even under flooded conditions (Table 2). This discrepancy between the observed results in the current study and the expected results may be a consequence of a low *P. sojae* inoculum density and/or dry conditions after inoculation. Research reports on lima beans, a legume closely related to soybeans, suggests that a minimum population density of *Pythium* spp. is required to cause significant death of seedlings (Kendrick & Wilbur, 1965). If the same condition applies to soybeans, it may be that the inoculum density in the current study was not high enough to cause significant death of the soybean seedlings.

Another factor that contributes to death in plants with oomyceteous pathogen diseases is atmospheric humidity. Watanabe et al. (2007) reported that high relative humidity caused disease severity 7 days after inoculation of

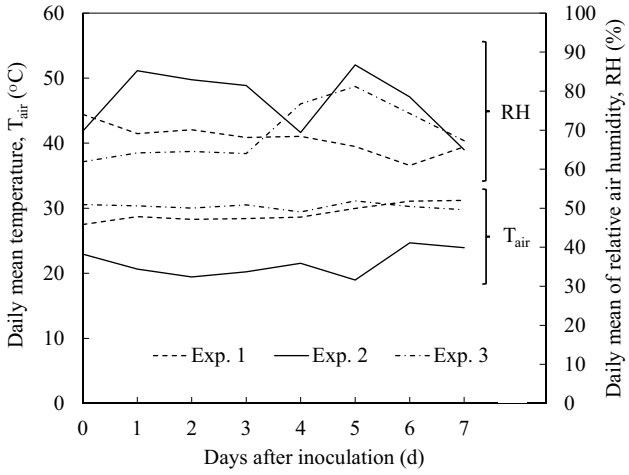

Figure 3. Air temperature and relative humidity immediately after inoculation in Experiments 1–3 that investigated the effects of flooding and inoculation with two *Phytophthora sojae* isolates on soybean survival and growth.

kalanchoe by *Pythium* pathogens. In the current study, higher mortality was observed in Exp. 2 than in the other experiments (Table 2). This observation could be attributed to the higher atmospheric humidity observed in Exp. 2 within three to 4 days of inoculation (Figure 3). In fact, the mortality ratio was greater than 0.3 in Exp. 2. Another contributing factor is temperature, and it has been reported that a temperature of approximately 20°C promotes the production of *P. sojae* zoospores (Eye et al., 1978).

Conclusions

In this study, the growth of soybean seedlings was quantitatively evaluated after *P. sojae* inoculation under flooded conditions. The mortality ratios were unexpectedly low, but based on analysis of growth of the surviving seedlings, it appears that plants inoculated with *P. sojae* can be negatively affected, even if symptoms of PRSR are not observed. This observation provides valuable information about the effect of latent PRSR infection on soybean growth. Reduction of MRL and seedling dry weights (SDW and RDW) of inoculated plants was shown only under flooded conditions in the experiment with vermiculite media. The physiological mechanisms governing the poor growth of soybean seedlings inoculated with *P. sojae* under flooded conditions need further investigation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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