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e-Xtra*

The Effect of Cold Stress on Damping-Off of Soybean Caused by *Pythium sylvaticum*

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Abstract

To improve our understanding of the timing of cold stress and its effect on Pythium damping-off, we performed a factorial experiment with two cold stress temperatures (4 and 10°C); exposure to 96 h of cold stress at 0, 1, 2, 4, 6, and 8 days after planting; and inoculation with *Pythium sylvaticum*-infested millet or control. Increased susceptibility to damping-off resulting in reduced emergence was found in inoculated plants when the cold stress period began 2 or 4 days after planting. In the noninoculated controls, no effect of cold stress on emergence was observed. Slower seedling growth was observed during the cold stress period and in inoculated plants after exposure to cold stress. Seed exudation, mycelial growth, and sporangia germination of *P. sylvaticum* was evaluated at 4, 10, and 18°C. The greatest seed exudation was observed at 4°C. Low temperatures delayed mycelial growth of *P. sylvaticum*, although the pathogen was still able to grow at 4°C. Sporangia incubated for 3 h at 18°C in the presence of seed exudates had higher germination in comparison with sporangia incubated at 10 or 4°C. Moreover, more sporangia germinated in response to seed exudates that were previously collected from seed imbibed for 24 h at low temperatures (4°C). These results suggest that cold stress 2 to 4 days after planting increases soybean susceptibility to damping-off, presumably because of increased seed exudation and delayed seedling growth.

Soybean (*Glycine max* (L.) Merr.) is an important oilseed crop in Iowa and in the United States. In total, 83.4 million acres were planted in the United States in 2016. In Iowa, 9.5 million acres were planted to soybean in 2016 and the crop value was estimated at $5.4 billion (USDA-NASS 2016).

Once a soybean field is planted, seedling disease may affect soybean production by reducing stands. Although reduced stands do not always result in yield loss, a farmer may need to replant the field. Later plantings may have reduced yield potential (De Bruin and Pedersen 2008). Seedling disease resulted in losses of up to 55 million bushels annually between 2010 and 2014, which was equivalent to $1.6 billion for the 4-year period, and was ranked as the second to fourth most damaging disease of soybean in 12 states of northern the United States from 2010 to 2014 (Allen et al. 2017). *Pythium* spp. are the predominant pathogens causing seedling disease in Iowa and the north-central region of the United States (Murillo-Williams and Pedersen 2008; Rizvi and Yang 1996; Rojas et al. 2017). *Pythium* spp. are oomycetes that may prevent germination, causing a seed rot, or may also be associated with death of the developing seedling, which is known as "damping-off" (Hartman et al. 2015). Symptoms on affected seedlings may include general discoloration of the plant, yellow to brown lesions on the roots, root rot, and seed rot (Hartman et al. 2015), resulting in reduced stand and potential yield loss, which often require replanting of entire fields (Yang 1997, 1999).

Cool and wet soils are frequently associated with a higher occurrence of damping-off caused by *Pythium* spp., presumably because low temperatures at planting keep seedlings at a susceptible stage for a longer period of time, providing greater chances for seedling infection (Martin and Loper 1999). Moreover, field observations have suggested a role of cold stress in increasing the occurrence of seedling diseases and poor plant stand (Bradley 2008; Robertson and Munkvold 2012). In controlled environments, Thomson et al. (1971) demonstrated that periods of cold stress (4°C) at planting increased soybean susceptibility to damping-off. Similarly, in preliminary research, we observed that cold (4°C) stress that occurred 1 day after planting increased the susceptibility of soybean to *Pythium sylvaticum* and reduced emergence by up to 70% (Serrano and Robertson 2016).

Planting soybean is not recommended if soil temperatures are below 13°C (Pedersen et al. 2004). Consequently, in Iowa, soybean crops are usually planted in late April through May, when soil temperatures are 13°C and warming. However, it is not uncommon for cold fronts to pass through the state and for soil temperatures to drop below 10°C for a few days (Serrano et al. 2015). It is unknown how cold stress that occurs a few to several days after planting affects the susceptibility of soybean to damping-off.

Cold stress has a detrimental effect on soybean germination and growth. For example, 50% of soybean seed germinated after 24 h at 23°C whereas, at 10°C, 120 h were required for 50% of seed to germinate (Duke et al. 1977). Once seed has germinated, hypocotyl elongation is extremely slow at or below 10°C (Hatfield and Egli 1974). In addition, when seed is planted at temperatures below 15°C, there is a high risk of chilling injury during imbibition, and there is more seed exudation than at higher temperatures (Bramlage et al. 1978; Leopold 1980). The diffusion of those seed exudates is increased in wet soils and, consequently, more sporangia are stimulated to germinate (Stanghellini and Hancock 1971a). Seed exudates contain several compounds, including sugars, amino acids, volatile compounds, secondary metabolites, and unsaturated fatty acids, that stimulated sporangia germination within a few hours (Nelson 1987; Nelson and Craft 1989). High seed exudation of soluble carbohydrates resulted in more damping-off of soybean caused by *Pythium* spp. (Keeling 1974). Therefore, it is possible that, when cold stress occurs, greater seed exudation increases *Pythium* sporangia germination and, consequently, risk of infection.

Sporangia play a crucial role in the survival of some *Pythium* spp. in the soil (Nelson 2004; Stanghellini and Hancock 1971b). In addition, sporangia serve as primary inoculum that germinate in response to seed and root exudates (Martin and Loper 1999; Nelson 2004; Stanghellini and Hancock 1971a).

P. sylvaticum, first described by Campbell and Hendrix (1967), was one of the most frequently isolated species recovered from diseased soybean seedlings in recent surveys in Iowa and the north-central

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region of the United States (Matthiesen et al. 2016; Rojas et al. 2017). *P. sylvaticum* produces intercalary or terminal globose sporangia (hyphal swellings) that germinate directly in response to seed exudates and volatile compounds (Campbell and Hendrix 1967; Nelson 1987; Van der Plaats-Niterink 1981).

P. sylvaticum may cause more disease at lower temperatures. Rojas et al. (2017) found greater seed rot at 13 than at 20°C. Although mycelial growth is optimal at 25°C, the pathogen can grow at temperatures below 5°C (Van der Plaats-Niterink 1981). An improved understanding of how low temperatures affect the soybean–*P. sylvaticum* interaction may help to explain the higher occurrence of damping-off observed after periods of cold stress.

The objectives of this study were to (i) determine how cold stress imposed at different times after planting affects the susceptibility of soybean to damping-off, (ii) measure the effect of cold stress on mycelial growth and sporangia germination of *P. sylvaticum*, (iii) evaluate the effect of cold stress on soybean seed exudation during imbibition, and (iv) compare sporangia germination in response to seed exudates from seed imbibed at different temperatures. We hypothesized that, because *P. sylvaticum* is able to grow at low temperatures and soybean germination and growth is slowed during periods of cold stress, exudates released from the germinating seed stimulate sporangia germination that results in infection of the soybean seedling and, consequently, increased damping-off. The data from this study will improve our understanding of how periods of cold stress that occur soon after planting increase the occurrence of damping-off and this, in turn, will help to improve disease management recommendations.

Table 1. Effect of different variables on probability of emergence of soybean (IA 2094) in the growth chamber

Effect	*P* value, probability of emergence
Run	0.4769
Temperature	0.0006
Timing	<0.0001
Pathogen	<0.0001
Temperature × timing	0.3128
Temperature × pathogen	0.0073
Timing × pathogen	<0.0001
Temperature × timing × pathogen	0.0066

Materials and Methods

Plant material and *P. sylvaticum* culture. All experiments were done using soybean cultivar IA 2094, which has a relative maturity of 2.4 and was susceptible to Pythium damping-off in previous experiments (Serrano and Robertson 2016; Weidenbenner et al. 2014). *P. sylvaticum* isolate IASO 2-8.18 (Matthiesen et al. 2016) was used for inoculum production, mycelial growth, and sporangia germination experiments. *P. sylvaticum* was grown on 4% V8 juice media plus neomycin sulfate (0.05 g/liter) and chloramphenicol (0.01 g/liter) ($dV8^{++}$) (Matthiesen et al. 2016).

Cold stress growth chamber assay. *P. sylvaticum*-infested millet was used as a source of inoculum. Autoclavable bags with a filter patch (Myco Supply Company Inc., Pittsburgh) were filled with approximately 600 ml of millet seed that had been soaked overnight and autoclaved twice, with 24 h between each cycle. *P. sylvaticum* was grown on $dV8^{++}$ (Matthiesen et al. 2016) in the dark at room temperature (20°C). Each bag was inoculated with 20 pieces (1 cm^2) of media colonized with 3-day-old mycelium of *P. sylvaticum*. The inoculated bags were kept in the dark at room temperature (20°C) for 10 days. The bags were gently shaken once per day to ensure colonization of all millet seed.

Vermiculite (90 ml/cup) was added to polystyrene cups (237 ml) with three 2-mm-diameter holes punched through the base of the cup. A layer of pathogen-infested or sterile millet (5 ml/cup) was placed on top of the vermiculite and a second layer of 40 ml of vermiculite covered the millet. Ten soybean seeds per cup were placed on the second layer of vermiculite, before a final layer of vermiculite (90 ml) was used to cover the seed. The cups were placed in a growth chamber at 18°C and periods of 12 h of light and 12 h of darkness. The cups were watered with tap water until runoff at planting and then watered with 50 ml of tap water every other day.

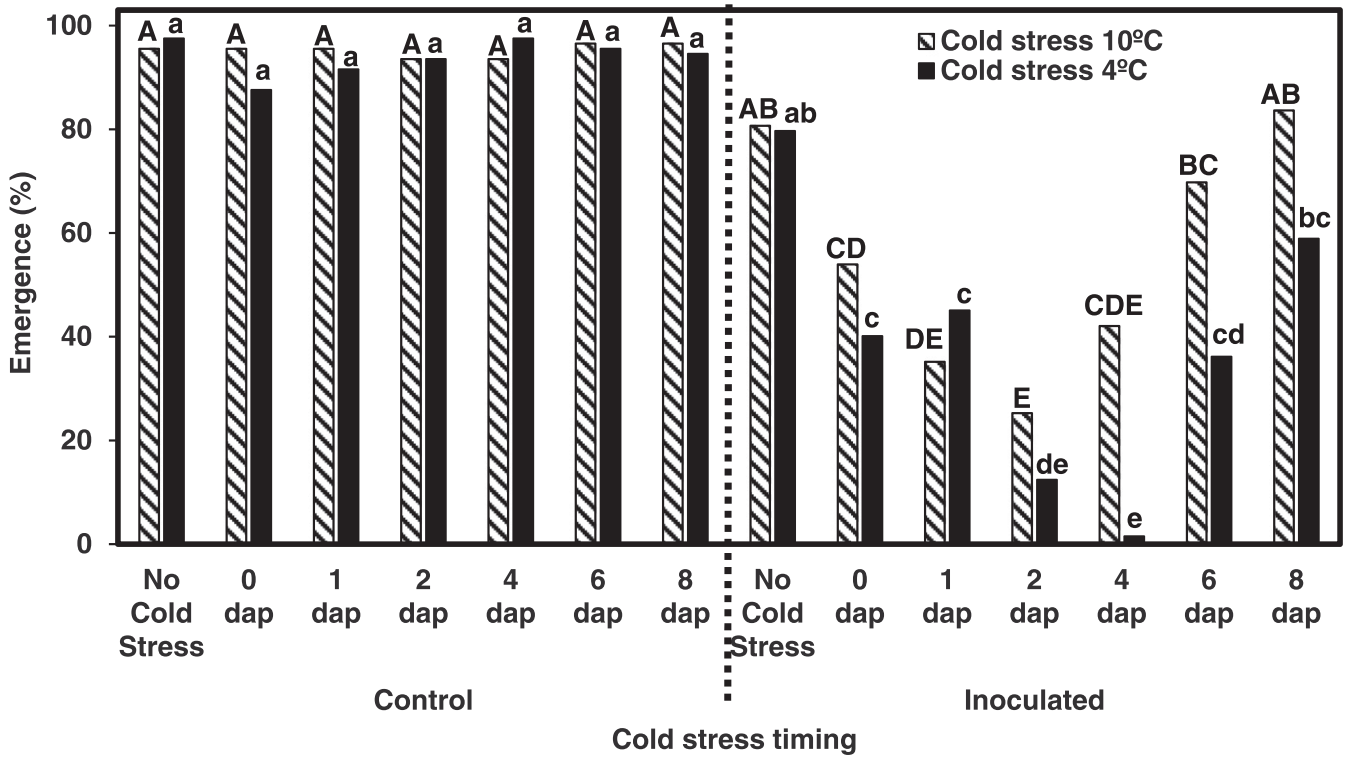

Fig. 1. Emergence (%) of soybean IA 2094 noninoculated (control) and inoculated with *Pythium sylvaticum* subjected to 96 h of cold stress (4 or 10°C) at different days after planting (dap). Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$); uppercase letters for cold stress at 10°C and lowercase letters for cold stress at 4°C.

The experimental design was a three-way (two by seven by two) factorial of cold stress temperature, timing of cold stress, and inoculation, respectively, with five replications. Two cold stress temperatures (4 or 10°C, for 96 h) were used. Cold stress timing was imposed at 0, 1, 2, 4, 6, and 8 days after planting (dap) and a no-cold-stress control. Cups were inoculated with *P. sylvaticum*-infested millet or sterile millet (noninoculated control). Final emergence data were taken 21 dap. Emergence was assessed 3, 6, 7, 8, 10, 13, 16, 18, and 21 dap by counting the number of seedlings with cotyledons completely emerged from the vermiculite.

Three additional replications of the above treatments were planted for use in measuring seedling length at each time. Seedling length was measured from the proximal end of the cotyledon to the root tip, as described by Ellis et al. (2011). Seedling growth was measured at the beginning and at the end of the 96-h period at 4, 10, and 18°C for each treatment. Also, to determine whether cold stress reduced seedling growth once the plants were returned to optimal temperatures for growth, seedling growth was measured 72 h after returning the plants to 18°C. All experiments were repeated once.

Growth of *P. sylvaticum* at different temperatures. One plug (3 mm in diameter) of *P. sylvaticum*-colonized dV8^{++} media was placed 2 mm from the edge of a 90-mm-diameter petri dish plate filled with 20 ml of dV8^{++} media, and incubated in the dark at 4, 10, or 18°C. Mycelial growth from the edge of the plug to the furthest edge of the colony was measured 48, 72, and 96 h after inoculation.

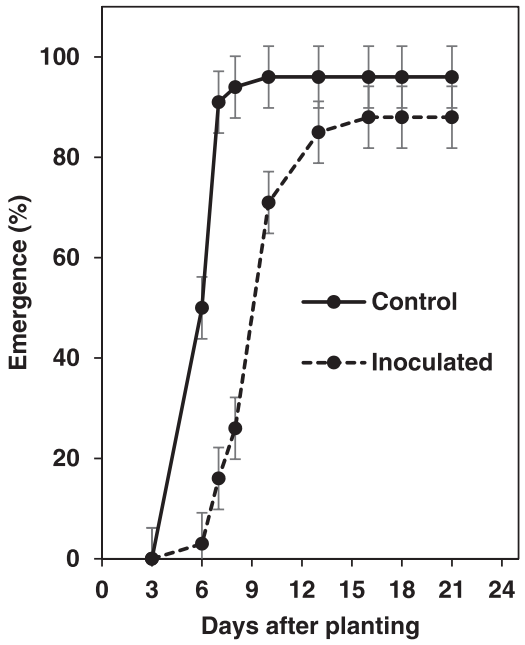

Fig. 2. Emergence (%) of soybean IA 2094 noninoculated (control) and inoculated with *Pythium sylvaticum* at different days after planting at 18°C. Error bars indicate confidence interval 95%. Area under the curve was significantly different between the curves ($P < 0.0001$). The area under the curve was 1,484.0%-days and 1,065.5%-days for noninoculated and inoculated plants, respectively.

The experimental design was completely randomized with five replicates. The experiment was repeated once.

Solute leakage after imbibition of seed at different temperatures. To estimate the amount of seed exudates released from seed imbibed at different temperatures, solute leakage was measured with the Electrical Conductivity Test modified from AOSA (2009). Soybean seed were inspected visually for damage to the seed coat and undamaged seed were selected for the test. Four reps of 50 seeds per treatment were weighed and then imbibed for 24 h in 75 ml of distilled sterile water at 4, 10, and 18°C. The water was equilibrated for at least 4 h in growth chambers at each temperature before imbibition. After imbibition, the seed were removed and the remaining solution was equilibrated at 25°C for at least 4 h. The electrical conductivity of each replication of each solution was measured with an Electrical Conductivity meter (Solution Analyzer, Model 4603; Amber Science Inc., San Diego, CA) that had been calibrated with a potassium chloride conductivity standard solution (718 μS/cm) at 25°C (Ricca Chemical Company, Arlington, TX). The conductivity of a water control (blank) was subtracted from each reading before calculations of electrical conductivity were done. Data for electrical conductivity were calculated per gram of seed weight for each replicate. The experiment was repeated once.

Production of sporangia for sporangia germination assays. *P. sylvaticum* was grown on dV8^{++} media at 24°C for 48 h in the dark. A method modified from Nelson and Craft (1989) was used for sporangia production. Briefly, colonized disks (3 mm in diameter) from 2-day-old cultures of the pathogen were placed in sterile petri dishes and submerged for two consecutive 10-min periods in approximately 20 ml of leaching buffer (pH 5.8) containing 0.01 M $Na(NO_3)_2 \cdot 4H_2O$, 0.004 M $MgSO_4 \cdot 7H_2O$, and 0.005 M KNO_3, followed by one 3-h submergence period. The buffer was replaced with fresh buffer after each 10-min period of submergence. Finally, the disks were rinsed with sterile distilled water and incubated in a sterile petri dish at 24°C for 48 h to facilitate sporangia production.

Effect of exudates from seed imbibed at cold temperatures on sporangia germination. A modification of the method described by Nelson and Craft (1989) was used to assess percent germination of

Table 3. Mean growth of soybean seedlings IA 2094 that were noninoculated (control) or inoculated with *Pythium sylvaticum* and placed at 4, 10, or 18°C for 96 h at various days after planting (dap)[z]

Cold stress period (dap)	Mean growth (mm)					
	Control (noninoculated)			*P. sylvaticum*-inoculated		
	18°C	10°C	4°C	18°C	10°C	4°C
0	48.6 c	1.7	0.1	28.1b	0.9	0.1
1	68.5 b	8.5	1.3	35.4 ab	7.1	1.0
2	78.3 ab	9.0	2.7	35.6 ab	4.0	0.7
4	91.5 a	8.2	1.6	40.2 ab	1.2	0.9
6	82.6 ab	4.0	0.0	50.9 a	2.4	0.1
8	50.7 c	5.8	2.9	42.8 ab	7.9	1.6
P value	<0.0001	0.0533	0.2556	0.0475	0.0866	0.3745

[z] Temperature of 18°C = no cold stress (control). Different letters in the same column indicate significant differences according to Tukey's test ($\alpha = 0.05$).

Table 2. Comparison of mean growth of soybean seedlings IA 2094 that were noninoculated (control) or inoculated with *Pythium sylvaticum* at 4, 10, or 18°C for 96 h[z]

Seedlings	Temperature (°C)	Seedling growth (mm)	Mean seedling growth (mm)
Control (noninoculated)	18	70.0 a	25.9 a
	10	6.2 c	…
	4	1.4 d	…
P. sylvaticum-inoculated	18	38.8 b	14.5 b
	10	3.9 cd	…
	4	0.7 d	…
P value	…	<0.0001	<0.0001

[z] Different letters in the same column indicate significant differences according to Tukey's test ($\alpha = 0.05$).

sporangia in response to seed exudates from seed imbibed at different temperatures. Prepared culture disks containing sporangia were placed approximately 10 mm apart on sterile glass slides (three culture disks per slide). Either seed exudate from seed imbibed at 4, 10, or 18°C (as described above) or sterile water (10 µl each) was pipetted carefully on top of each culture disk so that the disk was submerged in the solution. The slides of each culture disk were incubated for 2 and 3 h at 24°C in the dark; then, the culture disks were stained with 0.03% acid fuchsin in 85% lactic acid and examined microscopically (×200). The number of germinated and nongerminated sporangia within the whole culture disk was recorded. Each examined culture disk contained at least 30 sporangia, for a total of 270 to 540 sporangia counted by treatment. The experimental design was a randomized complete block with three imbibition-temperature treatments and three replicates. The replication unit was a slide with three culture disks. The experiment was repeated once.

Effect of cold temperatures on sporangia germination. To evaluate the effect of temperature on sporangia germination, soybean seed were imbibed at 18°C, as described above, and 10 µl of the seed exudate solution or sterile water was pipetted on top of culture disks as described above. The slides were incubated for 3 h at either 4, 10, or 18°C in the dark. The culture disks were stained and sporangia were counted as described above. The experimental design was a randomized complete block with three incubation-temperature treatments and three replicates. The examined culture disks contained at least 30 sporangia for a total of 270 to 540 sporangia counted by treatment. The replication unit was a slide with three culture disks. The experiment was repeated once.

Data analysis. All data were analyzed using SAS 9.3 (SAS Institute, Cary, NC). Emergence 21 dap was analyzed with PROC LOGISTIC to model the probability of emergence as a function of treatments testing temperature, timing, and pathogen as fixed factors, with all the interactions. Area under the emergence curve was calculated as described by Campbell and Madden (1990) and analyzed with PROC GLIMMIX testing the effect of treatment as a fixed factor. Seedling growth was analyzed with PROC GLIMMIX testing treatment, timing, and its interaction as fixed factors. Mycelial growth was analyzed with PROC GLIMMIX testing temperature, time, and temperature–time as fixed factors, and the random effect run–temperature–replication was included to account for the correlation between measurements on the same plate across three units of time. Seed exudation (solute leakage) was analyzed with PROC GLIMMIX testing temperature as a fixed factor. Sporangia germination data were analyzed with PROC GLIMMIX testing treatment as a fixed factor and block as a random factor. When a treatment effect was detected ($P < 0.05$), Tukey's test ($\alpha = 0.05$) was used for mean comparisons.

Results

Effect of cold stress at different times after planting on soybean growth and susceptibility to *P. sylvaticum*. Significant differences in emergence among treatments were detected for cold stress temperatures ($P = 0.0006$), cold stress timing ($P < 0.0001$), and presence of the pathogen ($P < 0.0001$). A temperature–pathogen interaction ($P = 0.0073$) and timing–pathogen ($P < 0.0001$) interaction was detected (Table 1). Overall, inoculated cups exposed to 4°C at 2 and 4 dap showed a reduction of 88 to 99% in emergence, followed by cups exposed to 10°C at 2 and 4 days that showed a reduction in emergence between 58 to 75% (Fig. 1). The reduction in emergence 2 and 4 dap was not observed in noninoculated cups subjected to cold stress at either 4 or 10°C. Furthermore, when cold stress was absent, no differences in emergence were observed between inoculated and noninoculated treatments (Fig. 1). However, when cold stress was absent, the area under emergence curve was reduced in inoculated cups in comparison with the noninoculated cups ($P < 0.0001$) (Fig. 2).

Seedlings inoculated with *P. sylvaticum* were shorter in comparison with noninoculated plants ($P < 0.0001$; Table 2). When the pathogen was absent, cold stress reduced seedling growth. Less seedling growth was observed at 4 and 10°C compared with 18°C ($P < 0.0001$; Table 2). In the inoculated cups at 18°C, *P. sylvaticum* reduced seedling growth in comparison with the noninoculated control. However, the presence of the pathogen did not further reduce seedling growth in plants subjected to cold stress at 4 and 10°C ($P > 0.05$; Table 2).

When the time of initiation of cold stress (cold stress timing) was compared, differences in seedling growth were observed at 18°C in both noninoculated and inoculated plants ($P < 0.0001$ and $P = 0.0475$, respectively; Table 3). At 18°C, the seedling growth was reduced the most by the 96-h cold stress period that started 0 dap in both inoculated and noninoculated plants. Seedling growth was also reduced by a cold stress period that started 8 dap only in the noninoculated plants. No effect of cold stress timing on seedling growth was observed at 4 and 10°C (Table 3).

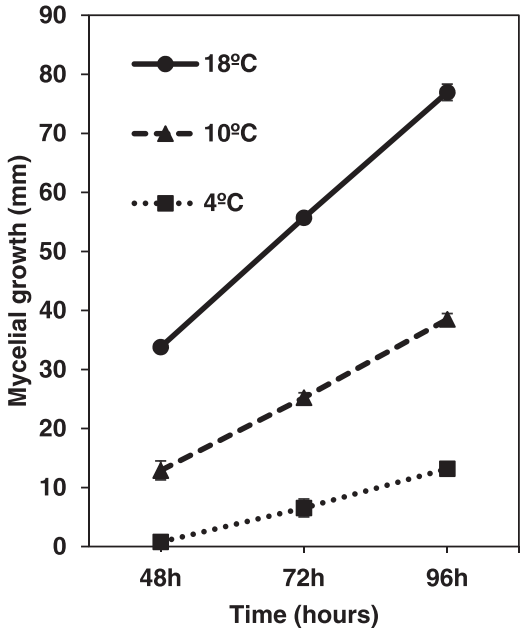

Fig. 3. Mycelial growth of *Pythium sylvaticum* at three temperatures on dilute V8 media plus antibiotics. Error bars indicate standard deviation.

Table 4. Comparison of the growth of soybean that were noninoculated (control) or inoculated with *Pythium sylvaticum*, subjected to a period of cold stress (96 h at 4 or 10°C), or kept at an optimum growth temperature (18°C) after a rewarming period (72 h at 18°C)[z]

Soybean	Previous temperature (°C)	Seedling growth (mm)	Mean seedling growth (mm)
Control (noninoculated)	18	45.3 ab	45.4 a
	10	46.7 a	…
	4	38.4 b	…
P. sylvaticum-inoculated	18	26.9 c	19.3 b
	10	19.3 d	…
	4	11.8 d	…
P value	…	0.0282	<0.0001

[z] Different letters in the same column indicate significant differences according to Tukey's test ($\alpha = 0.05$).

After the 96-h cold stress period at 4 or 10°C, the seedling growth in a subsequent period of 72 h at 18°C (rewarming period) was compared with the growth of seedlings that had been continually at 18°C. During the rewarming period, less seedling growth was observed in plants inoculated with *P. sylvaticum* in comparison with noninoculated plants ($P < 0.0001$; Table 4). Plants inoculated with *P. sylvaticum* and previously subjected to cold stress at 4 and 10°C were shorter in comparison with plants previously subjected to 18°C ($P < 0.0282$; Table 4).

When the effect of cold stress timing on seedling growth was compared in the subsequent rewarming period (18°C) of 72 h, significant differences were observed in noninoculated plants at 18, 10, and 4°C ($P < 0.0001$, $P < 0.0001$, and $P = 0.0023$, respectively) and in inoculated plants at 18 and 4°C ($P = 0.0015$ and $P = 0.0098$, respectively). Less seedling growth occurred when the cold stress timing occurred 8 dap in both noninoculated and inoculated plants at 18°C (Supplementary Table S1).

Effect of temperature on the growth of *P. sylvaticum*. There were significant differences in mycelial growth of *P. sylvaticum* ($P < 0.0001$) at different temperatures. Growth was greater at 18 compared with 10°C and at 18 compared with 4°C at each time point assessed (Fig. 3).

Effect of temperature on seed exudation during imbibition of soybean seed. Higher solute leakage (seed exudation) occurred when seed were imbibed at 4°C in comparison with 10 and 18°C ($P < 0.0001$; Table 5).

Effect of seed exudates from seed imbibed at cold temperatures on sporangia germination. *P. sylvaticum* sporangia germinated after exposure to seed exudate solutions but not when they were exposed to sterile water ($P < 0.0001$) (Table 6). After 3 h of exposure to seed exudates from seed imbibed at 4°C, more sporangia germinated compared with those exposed to a solution of seed exudates from seed imbibed at 10 and 18°C ($P < 0.0001$).

Sporangia germination after incubation at 4, 10, or 18°C. Differences in sporangia germination were detected between runs ($P = 0.0086$) (Table 7). In both runs, however, greater sporangial germination occurred at 18 than at 10 or 4°C ($P < 0.0001$). No sporangia germinated in sterile water.

Discussion

In this study, we demonstrated that periods of cold stress that occurred between 2 and 4 dap increased the susceptibility of soybean to damping-off under controlled conditions. To our knowledge, this study is the first to evaluate the effect of cold stress after planting on emergence. Previous studies evaluating the effect of suboptimal temperatures on germination of soybean have been done to study chilling injury during imbibition or when screening cold-tolerant varieties in field trials and laboratory experiments (Cabane et al. 1992; Hobbs and Obendorf 1972; Ismail et al. 1989; Littlejohns and Tanner 1976). The cold-stress treatments that we tested mimic what occurs in the field soon after planting in Iowa when cold fronts pass through the region for a few days, resulting in suboptimal soil temperatures for germination and seedling development. Previous research suggested that cold soils delay emergence and, thus, provide a greater chance for seedling infection (Martin and Loper 1999). In this study, we demonstrated that the timing at which cold temperatures are initiated can have substantial impact on the incidence of damping-off caused by *P. sylvaticum*.

Similarly, Thomson et al. (1971) reported that cold stress increased susceptibility to damping-off but, in their experiments, cold stress (4°C) was applied only at planting. They also observed reduced emergence in their noninoculated control that may have been a consequence of chilling injury during imbibition, which is common at temperatures below 15°C (Bramlage et al. 1978; Leopold 1980). In our study, we did not observe reduced emergence when cold stress was applied at planting, possibly because it took 3 to 4 h to equilibrate the temperature inside the cup to the growth chamber temperature (Supplementary Fig. S1). Seed imbibition is the first event in the germination process and high water uptake occurs within the first 35 min of planting the seed. During this time, seed are particularly susceptible to imbibitional chilling injury because rearrangement of cell membranes occurs during the first few minutes of imbibition (Bramlage et al. 1978; Parrish and Leopold 1977; Vertucci and Leopold 1983). In the first 30 min after planting in our study, the temperature of our cups was 19°C and it took 3 to 4 h for our cups to reach 4°C. Thus, the risk of imbibitional chilling injury in our experiments was likely low.

No significant reduction in emergence was observed in the cold-stress treatments when the pathogen was absent. High-vigor soybean seed usually germinates well in prolonged cold conditions. In the standard cold germination test, in which soybean seed are subjected to 7 days of cold stress at 10°C, seed lots with high vigor show high germination rates (AOSA 2009). Low-quality seed has greater seed exudation and, thus, stimulates soilborne pathogens to attack germinating seedlings (AOSA 2009; Hobbs and Obendorf 1972). The seed we used in this study was of high quality, with a 97% germination rate (data not shown); consequently, the effect of soilborne pathogens on emergence was not significantly confounded by characteristics of poor-quality seed.

Table 5. Seed exudation (solute leakage) of soybean IA 2094 seed after incubation in distilled water for 24 h at different temperatures

Temperature (°C)	Leakage (µS/cm g)[z]
4	65.9 a
10	59.6 b
18	56.9 b
P value	<0.0001

[z] Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

Table 6. Sporangia germination of *Pythium sylvaticum* after exposure to seed exudates from seed imbibed at different temperatures and incubation at 24°C for 2 and 3 h

	Germinated sporangia (%) at 24°C[y]	
Seed exudate[z]	2 h	3 h
Seed imbibed at 4°C	35.5 a	65.2 a
Seed imbibed at 10°C	32.0 a	42.9 b
Seed imbibed at 18°C	31.9 a	48.2 b
Sterile water	0.6 b	0.5 c
P value	<0.0001	<0.0001

[y] Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

[z] Three seed exudates were prepared by imbibition of soybean seed in sterile water at 4, 10, or 18°C for 24 h.

Table 7. Sporangia germination of *Pythium sylvaticum* after exposure to soybean seed exudate or sterile water and incubation for 3 h at different temperatures

	Germinated sporangia (%)[y]	
Incubation temperature (°C)[z]	Seed exudate	Sterile water
Run 1		
4	9.6 b	0.1
10	18.4 b	0.3
18	55.3 a	0.2
P value	<0.0001	0.8088
Run 2		
4	2.1 b	0.0
10	9.0 b	0.7
18	48.2 a	0.0
P value	<0.0001	0.2913

[y] Seed exudate was prepared by imbibition of soybean seed in sterile water at 18°C for 24 h. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$).

[z] Significant differences between runs were detected; therefore, results for both runs are presented.

Cold stress under the conditions we used favored damping-off when the pathogen was present. We hypothesized that higher susceptibility to damping-off would occur in seed subjected to cold stress at planting because of the risk of chilling injury and greater solute leakage that would attract the pathogen. Unexpectedly, soybean was more susceptible to damping-off caused by *P. sylvaticum* when cold stress occurred 2 and 4 dap, which is well after imbibition, when chilling injury usually occurs. Still, soybean seedlings may be particularly susceptible to cold stress and other stresses initiated several days after imbibition. Wuebker et al. (2001) reported that soybean susceptibility to flooding damage was highest at 3 dap, and suggested that soybean seedlings may be more susceptible to stress at this developmental stage. Interestingly, isoflavone levels in the hypocotyl undergo a programmed decrease between 2 and 4 dap (Graham 1991). Isoflavonoids are phenylpropanoid-derived metabolites that have been involved in resistance to oomycetes such as *P. aphanidermatum* and *Phytophthora sojae* (Avanzato and Rupe 2011; Bhattacharyya and Ward 1986; Morris et al. 1991). A change in isoflavonoid levels was found in the hypocotyl and roots of 3-day-old soybean seedlings subjected to several days of cold stress at 1°C and subsequently returned to 25°C for 3 days compared with seedlings that were not cold stressed (Posmyk et al. 2005). Further research is required to elucidate whether the increased susceptibility observed in our study when cold stress occurred at 2 to 4 dap is related to decreased levels of isoflavonoids, or other factors related to the structure of the root and other metabolic processes that may be impaired by cold stress at this specific stage.

In our study, we determined that soybean seedling growth is slower at 10°C and even more so at 4°C. These results agree with Hatfield and Egli (1974), who reported that hypocotyl elongation was minimal at temperatures below 10°C. Suboptimal temperatures for growth delay emergence and have been suggested to increase the chances for seedling infections caused by *Pythium* spp. (Martin and Loper 1999). However, the postchilling effect of cold stress on seedling growth has not been considered before in the occurrence of Pythium damping-off. In soybean seedlings subjected to 1°C for 2 days, Posmyk et al. (2005) observed postchilling stress in that seedling growth was slower over the following 3 days at 25°C. Similarly, we observed reduced seedling growth during the rewarming period in plants previously subjected to cold stress at both 4 and 10°C in noninoculated plants and more so in inoculated plants. Thus, the rewarming period might present another important opportunity for seedling infection. It is possible that cold stress not only results in increased risk of seedling infection but also may favor colonization of the seedling by the pathogen because growth of the plant is initially slower even when more favorable conditions for seedling growth resume.

For this study, we selected *Pythium sylvaticum* as the pathogen causing damping-off of soybean. Although *P. sylvaticum* is the most frequently recovered species in the United States, there is a great diversity of *Pythium* spp. causing soybean seed and root rot (Rojas et al. 2017). Moreover, the aggressiveness of some pathogenic *Pythium* spp. can be enhanced or reduced at low temperatures (Matthiesen et al. 2016; Rojas et al. 2017; Thomson et al. 1971). Further research is required to determine whether the results observed in this study differ for other *Pythium* spp.

Although *P. sylvaticum* prefers warmer soil temperatures, we observed that the pathogen was still able to grow at 4 and 10°C. Moreover, our data suggest that *P. sylvaticum* grows faster than soybean roots in the soil at these low temperatures. We also observed that seedling growth and emergence of soybean was delayed in the presence of the pathogen even at favorable temperatures (18°C) for soybean growth. Although we did not do root rot assessments, we propose that some infection had occurred and, consequently, seedling growth was reduced.

Sugars, amino acids, and unsaturated fatty acids that are present in seed and root exudates stimulate sporangia germination (Nelson 1990; Ruttledge and Nelson 1997). We observed sporangia germination only when sporangia were flooded with soybean seed exudate solution. Moreover, temperature played a role in sporangia germination. The optimal temperature for sporangia germination in our study was 18°C, which was the closest to the optimal temperature reported for mycelial growth (25°C) (Van der Plaats-Niterink 1981).

In this study, we detected greater solute leakage when soybean seed were imbibed at 4°C in comparison with 10 and 18°C. These data are similar to those reported by Bramlage et al. (1978), in which more solutes leaked from seed imbibed at 10°C and lower temperatures compared with seed imbibed at 20°C. In our study, we also observed greater germination of *P. sylvaticum* sporangia in the presence of seed exudates from seed imbibed at 4°C. Because sporangia germination is stimulated by seed exudates, it is possible that the quantity of the seed exudates varied at each temperature at which the seed was imbibed.

In our study, however, we measured exudation at 24 h after imbibition. Exudates are continually released from plant roots and their quantity and composition can vary over time depending on plant age, and can influence the composition and activity of microbial communities (Gransee and Wittenmayer 2000; Han et al. 2017). Moreover, cold stress may have affected exudation. Vančura (1967) analyzed exudates from maize and cucumber seedlings planted at 19°C and subjected to cold stress (5°C) 48 h after planting, and found that cold stress increased the quantity of exudates and substantially increased exudation of amino acids. Fructose, saccharose, and three other oligosaccharides were detected only when maize seedlings were subjected to cold stress. In our study, we did not specifically quantify the amount of exudates or determine the composition of exudates. Consequently, we do not know if the increased germination of sporangia in exudates from seed imbibed at 4°C for 24 h was due to a higher concentration of some specific exudates or a different composition of exudates. Further research is needed to determine whether the quantity and composition of soybean seed exudates produced at 2 and 4 dap differs, if cold stress affects the composition of exudates, and the effect of the exudates on sporangia germination.

In this study, we found that cold stress increased soybean susceptibility to damping-off caused by *P. sylvaticum*, particularly when cold stress occurred 2 and 4 dap. *P. sylvaticum* delayed seedling growth and emergence at 18°C. Also, *P. sylvaticum* reduced seedling growth in plants previously exposed to cold stress at 4 and 10°C. Our data suggest that the increase in damping-off that we observed after a period of cold stress could be due to several factors, including the apparent growth rate advantage of the pathogen at low temperatures, reduced seedling growth after a period of cold stress, and increased solute leakage that stimulates germination of sporangia of *P. sylvaticum*. We also hypothesize that susceptibility to damping-off may be increased by physiological and metabolic changes that occur in the soybean seedling during germination and seedling development. Further research is required to elucidate why soybean seedlings are more susceptible to damping-off when cold stress occurs 2 and 4 dap.

Acknowledgments

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