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The Effects of Inorganic Elements on the Reduction of Phytophthora Stem Rot Disease of Soybean, the Growth Rate and Zoospore Release of *Phytophthora sojae*

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Abstract

The effects of several inorganic elements contained in B5 medium on Phytophthora stem rot disease reduction of *Glycine max* (L.) Merr. cv. Chusei-Hikarikuro, fungal growth of *Phytophthora sojae* isolate and zoospore release were investigated. Application of B5 solution and macro inorganic nutrients in the B5 medium prior to inoculation significantly inhibited infection, compared with controls. Various concentrations of KNO_3, $(NH_4)_2SO_4$, $MgSO_4$, $CaCl_2$ and NaH_2PO_4 in the presence of macro inorganic nutrients were investigated in an effort to determine the elements most effective in suppressing the incidence of disease. A concentration of 2.47–24.7 mM KNO_3 and 0.1–10.2 mM $CaCl_2$ greatly inhibited infection. Although mycelium growth of the isolate was affected by the potassium and calcium concentration, no significant relationship was observed between inhibition of the growth rate and disease reduction at 2.47 mM KNO_3 and 0.1–5.1 mM $CaCl_2$ application. Disease suppression recorded in laboratory experiments using pathogen mycelium was due to the response of plant tissues rather than a direct inhibition of pathogen fungal growth by the application of potassium or calcium. The extent of disease reduction was related to an increased potassium and calcium uptake by plants, suggesting that the effective elements in reducing Phytophthora stem rot were potassium and calcium. The presence of 2.47–247 mM KNO_3 and 5.1–10.2 mM $CaCl_2$ decreased the release of zoospores, although 0.1–2.5 mM $CaCl_2$ significantly induced zoospore release. These results suggest that applying a solution containing more than 2.47 mM of potassium and 5.1 mM of calcium can decrease the incidence of disease in agricultural fields by the inhibition of zoospore release.

Introduction

Glycine max (L.) Merr. cv. Chusei-Hikarikuro is one of the most famous commercial and traditional black soybean cultivars in Japan. It fetches a higher market price than other yellow soybeans in Japan. The black soybeans are reported to have many positive effects on the human body and health (Kohama et al., 2005; Takahashi et al., 2005). Despite these desirable characteristics, cv. Chusei-Hikarikuro is susceptible to many pathogens (Tsuchiya et al., 1978).

One of the most important diseases on soybeans, Phytophthora stem rot on soybeans [*G. max* (L.) Merr.] caused by *Phytophthora sojae* Kaufmann and Gerdemann (1958), was first noted in Indiana and Ohio in 1951. When soybeans are infected, the infection may result in wilting and the death of plants. This disease was first observed in 1977 on Hokkaido, a northern island of Japan (Tsuchiya et al., 1978), after which it has spread to other part of Japan (Sugimoto et al., 2006). This disease also remains a serious problem in other soybean-producing areas, such as Argentina, Australia, Brazil, Canada, The People's Republic of China, Hungary, Italy, the former Soviet Union and the United States (Schmitthenner, 1999). The yields of soybean and the income of soybean producers have been sharply decreasing. Disease management strategies require immediate attention and implementation.

This disease has been controlled with chemical fungicides, the development of resistant or tolerant cultivars of soybeans, soil drainage, tillage practices for over 40 years. However, it was reported that the commercial use of the fungicide metalaxyl (Anderson and Buzzell, 1982), one of acylalanine fungicides developed by Ciba-Geigy (Tom River, NJ, USA), might lead to the loss of disease management (Hunger et al., 1982; Schmitthenner, 1999). Recently, *P. sojae* has evolved into new races that rapidly overcome the commonly used *Rps* genes (Jackson et al., 2004). Even more races of *P. sojae* may develop, rendering the *Rps* genes currently used ineffective (Sugimoto et al., 2006). Alternative disease management strategies are therefore needed, which may include physical control, chemicals, induction of resistance and the breeding of new resistant cultivars.

One possible method of disease management is the amendment of inorganic elements. It was recently reported that inorganic elements had a suppressive effect on *Phytophthora* spp. (Byrt et al., 1982; Slade and Pegg, 1993; von Broembsen and Deacon, 1997; Toppe and Thinggaard, 1998), although there are few practical research works available for the control of *P. sojae* with the exception of calcium application (Sugimoto et al., 2005). Further investigation is required to discover effective inorganic elements for disease management.

The objectives of this study were to investigate the effects of inorganic elements in B5 solution (Gamborg et al., 1968) on disease reduction of cv. Chusei-Hikarikuro (black soybean), the growth rate and zoospore release of *P. sojae*, and to measure concentrations of effective inorganic elements in soybean seedlings. This is the first report, concerning the relationship between various inorganic nutrients and Phytophthora stem rot disease on soybean.

Materials and Methods

Pathogen and soybean used in this study

In 2004, 23 isolates of *P. sojae* were identified from 23 infected soybean plants [*G. max* (L.) Merr.] obtained from several soybean-producing fields in Japan. These isolates were tested using the virulence test on *G. max* cv. Chusei-Hikarikuro (black soybean), provided from the Tokachi Federation of Agricultural Cooperatives in Hokkaido, Japan. Virulence evaluations of isolates were performed using the agar medium inoculation method, and conducted in a test bottle (width = 8 cm, height = 20 cm) using mycelium and not zoospore (Sugimoto et al., 2003, 2006). One isolate chosen was PJ-H30. This isolate showed strong virulence test result with cv. Chusei-Hikarikuro (97.6%, PJ-H30). Cultivar Chusei-Hikarikuro was therefore inoculated with PJ-H30.

Effects of several inorganic elements in B5 solution on disease caused by *P. sojae*

Soybean seed (cv. Chusei-Hikarikuro) surfaces were first sterilized with 0.7% NaOCl for 7 min, rinsed three times with 100 ml of sterilized distilled water (SDW), placed on an autoclaved 0.7% agar medium (130 ml of total volume) containing 1.0% (w/v) of sugar and normal concentration of B5 liquid solution (Table 1), where the pH was adjusted to 5.8 with 0.1 M NaOH, in a test bottle. An agar medium containing only 1.0% of sugar was also used as a control.

B5 solution was placed into the following four groups: (A) macro inorganic nutrients, (B) micro inorganic nutrients, (C) Fe-EDTA solution and (D) organic elements (Table 1). The following four media were tested: (i) A + B + C (ABC), (ii) A + B + D (ABD), (iii) A + C + D (ACD) and (iv) B + C + D (BCD). An agar medium containing B5 solution was used as a control. The agar medium containing varied concentrations [0%, 10%, 100% (normal level), 250% and 500%] of A–D group was applied to evaluate disease reduction. To detect the most effective nutrients in inhibiting infection, the different agar medium containing various levels [0%, 10%, 100% (normal concentration), 250%, 500% and 1000%] of each nutrient in the A group was used for the final evaluation.

Soybean seeds, which were put on several agar media, were incubated at 23°C. After the first primary leaf appeared, approximately 10 days after the sowing of seeds, the stem of the soybean near ground level was covered with two 3 mm diameter plugs of 20-day-old mycelium of isolate PJ-H30 (onto cv. Chusei-Hikarikuro) cultured on potato dextrose agar (PDA; Nissui, Tokyo, Japan). The eight soybean seedlings of cv. Chusei-Hikarikuro in each treatment were then inoculated with PJ-H30. The inoculated plants were

Table 1
Several concentrations of inorganic and organic elements contained in B5 solution used in this study

Group	Number of nutrients	Elements	Concentration of several elements (mg/l)				
			×10%	×100%[a]	×250%	×500%	×1000%
(A) Macro inorganic elements	1	KNO_3	250	2500	6250	12500	25000
	2	$(NH_4)_2SO_4$	13.4	134	335	670	1340
	3	$Mg_4SO_4{\cdot}7H_2O$	25	250	625	1250	2500
	4	$CaCl_2{\cdot}2H_2O$	15	150	375	750	1500
	5	$NaH_2PO_4{\cdot}H_2O$	15	150	375	750	1500
(B) Micro inorganic elements	1	$MnSO_4{\cdot}4H_2O$	1.0	10.0	25.0	50.0	–
	2	$ZnSO_4{\cdot}7H_2O$	0.2	2.0	5.0	10.0	–
	3	H_3BO_3	0.3	3.0	7.5	15.0	–
	4	KI	0.083	0.83	2.075	0.415	–
	5	$CuSO_4{\cdot}5H_2O$	0.0025	0.025	0.0625	0.125	–
	6	$CoCl_2{\cdot}6H_2O$	0.0025	0.025	0.0635	0.125	–
(C) Fe-EDTA	1	$Na_2{\cdot}EDTA$	1.86	18.6	46.50	93.0	–
	2	$FeSO_4{\cdot}7H_2O$	1.39	13.9	34.75	69.5	–
(D) Organic elements	1	Myo-inositol	10.0	100	250	500	–
	2	Nicotinic acid	0.1	1	2.5	5	
	3	Pyridoxine hydrochloride	0.1	1	2.5	5	–
	4	Vitamin B1 hydrochloride	1.0	10	25	50	–

[a]A concentration of 100% nutrient indicates a normal content for each solution established for soybean cell culture by Gamborg *et al.* (1968).

incubated in a growth chamber at 23°C for 16 h daily, under fluorescent light (light intensity: 150 per $\mu Em^2/s$; Sanyo Growth Cabinet MLR-350, Osaka, Japan). The number of infected surviving plants in each bottle was recorded daily for the 16-day experiment. The disease incidence was measured after the seventh day (when the first evaluation was recorded; equivalent to the time of evaluation for growth rate of PJ-H30 isolate in the next experiments) and the 16th day (when the final evaluation was recorded) following inoculation. It was calculated as the ratio of infected plants to the initial eight seedlings in each bottle to evaluate the effect of inorganic elements.

Growth rate of PJ-H30 isolate on PDA containing several nutrients

Isolate of PJ-H30 was grown on 15 ml of PDA at 23°C for 20 days. The root tips of the hyphae (3 mm diameter plugs) were transferred to another 15 ml of PDA containing B5 solution, normal concentration (100%) of four media [A + B + C (ABC), A + B + D (ABD), A + C + D (ACD) and B + C + D (BCD)], where the pH was adjusted to 5.8. Several levels (0%, 10%, 100%, 250% and 500%) of A–D group and various concentrations (0%, 10%, 100%, 250%, 500% and 1000%) of KNO_3, $(NH_4)_2SO_4$, $MgSO_4$, $CaCl_2$ and NaH_2PO_4 in the macro inorganic nutrients (A group) were also tested. Seven days after incubation at 23°C, the diameter of the isolate was measured on each medium.

Measurement of potassium and calcium contents in soybean seedlings

Eight non-inoculated seedlings subjected to both potassium and calcium combinations (0%, 10%, 100%, 250%, 500% and 1000% of KNO_3 and $CaCl_2$; 2.47, 24.7, 61.75, 123.5 and 247 mM KNO_3 and 0.1, 1.0, 2.5, 5.1, and 10.2 mM of $CaCl_2$, respectively) were used to measure potassium and calcium. Each fresh seedling in each bottle was sampled 10 days after sowing (equivalent to the time of inoculation in the previous disease experiments); the shoot was separated from the root and both components were weighed. All samples were dried in an oven at 80°C for 2 days and then weighed. Dried samples were ground into a powder using a Wiley mill (Yoshida Seisakusho, Tokyo, Japan). A quantity of 200 mg of each dried sample was placed in a 100 ml flask with 50 ml of 1 M HCl. The samples were shaken for 2 h at 25°C and then filtered. Filtrates of 10 ml were collected and poured into a 100 ml flask with 40 ml of SDW. The potassium concentration of each sample was measured with a flame photometer (model LF-32, EKO-Seiki, Tokyo, Japan) in accordance with the instrument's instructions. Filtrates of 10 ml were poured into a 100 ml flask with 37.5 ml of 1 M HCl and 2.5 ml of $SrCl_2$ (20 000 mg/l), then the calcium concentration of each sample was analysed with an atomic absorption spectrometer (model AA-670, Shimazu, Tokyo, Japan).

Effect of inorganic elements on zoospore release from *P. sojae*

Isolate of PJ-H30 was grown on 15 ml of PDA or Lima Bean agar (Becton-Dickinson and Company, Sparks, MD, USA) at 21°C for 6 days. Four pieces of agar plug (each 3 mm in diameter) were rinsed three times with 10 ml of SDW, and then transferred to a Petri dish (5 cm diameter). Five millilitres of 0%, 10%, 100% (normal), 250%, 500% and 1000% solution selected as an effective element was poured into the Petri dish and incubated at 21°C in darkness to induce zoospore release. The zoospore suspension was collected 12 h after incubation. The number of zoospores in the suspension was recorded with a hemacytometer (Sugimoto et al., 2005).

Statistical analysis

The bioassays were replicated four times with three bottles at each different concentration. The growth rate of the isolate was replicated three times with four dishes. The measurements of inorganic element in the shoots and roots were replicated four times with five bottles at each concentration. Zoospore release from *P. sojae* was counted five times with three dishes. All results were analysed using ANOVA and the Microsoft Excel for MACINTOSH software program.

Results

Suppressive effects of B5 solution and several inorganic elements on Phytophthora stem rot of soybean

The result of the 16-day experiment evaluating the effect of B5 solution on the incidence of disease in cv. Chusei-Hikarikuro is illustrated in Fig. 1. The application of B5 solution showed a notable suppression at the initial stage (until 4 days after inoculation) of infection. The incidence of disease displayed by control soybean plants of cv. Chusei-Hikarikuro was 61.9% (7 days after inoculation) and 97.6% (16 days after inoculation). In the presence of B5 solution, the incidence of disease on cv. Chusei-Hikarikuro was 23.0% (7 days after inoculation) and 40.0% (16 days after inoculation).

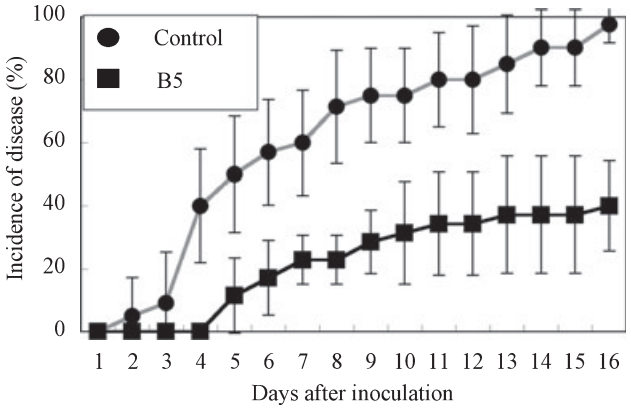

Fig. 1 Sixteen-day experiment evaluating the effect of B5 solution on the incidence of disease in *Glycine max* cv. Chusei-Hikarikuro. Eight seedlings of cv. Chusei-Hikarikuro were inoculated with PJ-H30. Bioassays were replicated four times with three bottles at each trial. Bars indicate standard error of the mean

When the three media (ABC, ABD and ACD) containing macro inorganic elements (A group) were applied, the incidence of disease significantly decreased 7 and 16 days after inoculation ($P < 0.01$); it was 17.1, 10.3 and 20.7% (7 days after inoculation), and 25.7%, 34.9% and 34.6% (16 days after inoculation; Fig. 2). However, the incidence of disease was 54.0% (7 days after inoculation), and 95.0% (16 days after inoculation) in the presence of medium BCD, without A group.

The results of the tests using several concentrations (0%, 10%, 100%, 250% and 500%) of A–D group contained in B5 medium are illustrated in Fig. 3. Although 10% of A group did not show the effect of disease suppression 16 days after inoculation (disease incidence = 88.6%), a concentration of 100% and 250% of A group was effective with an incidence of disease of 7.1% and 16.4% (7 days after inoculation), and 17.9% and 54.6% (16 days after inoculation). Soybean plants branched poorly when a concentration of 500% of A group was applied. In the presence of 10%, 100%, 250%, and 500% of B group, the incidence of disease was 42.9%, 59.3%, 36.1% and 35.1%

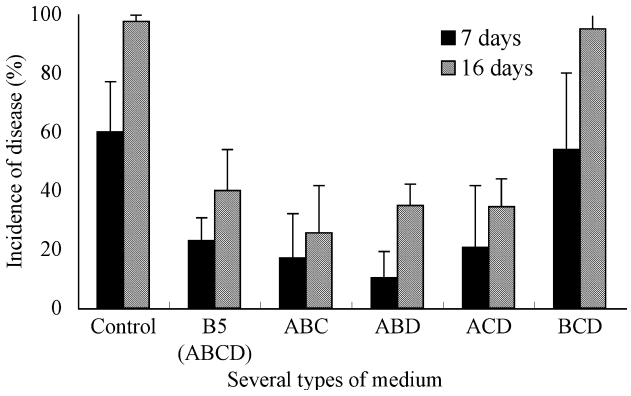

Fig. 2 Effect of four media (ABC, ABD, ACD and BCD) on the incidence of disease in *Glycine max* cv. Chusei-Hikarikuro 7 and 16 days after inoculation. Eight seedlings of cv. Chusei-Hikarikuro were inoculated with PJ-H30. Bioassays were replicated four times with three bottles at each trial. The medium ABC, ABD, ACD and BCD consists of normal concentration levels of (A) macro inorganic nutrients + (B) micro inorganic nutrients + (C) Fe-EDTA solution, A + B + (D) organic elements, A + C + D, and B + C + D, respectively. Black and shaded parts of the bar graph relate to the incidence of disease 7 and 16 days after inoculation. Bars indicate standard error of the mean

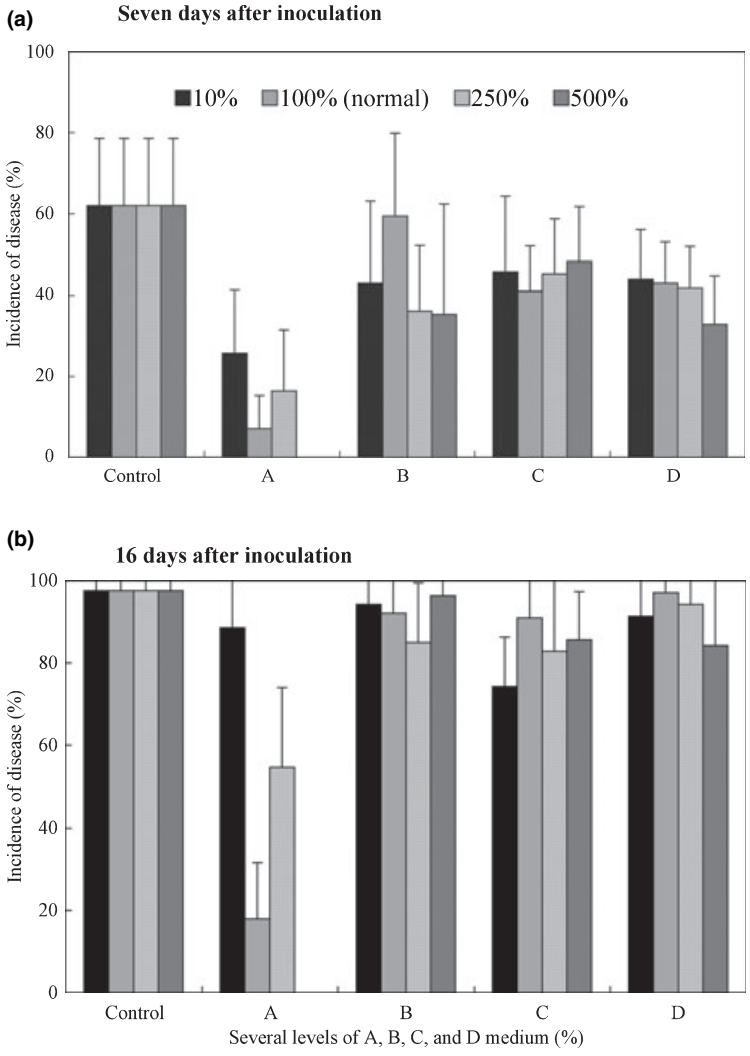

Fig. 3 Effect of several levels (0%, 10%, 100%, 250% and 500%) of (A) macro inorganic nutrients, (B) micro inorganic nutrients, (C) Fe-EDTA solution and (D) organic elements on the incidence of disease in *Glycine max* cv. Chusei-Hikarikuro 7 and 16 days after inoculation. Eight seedlings of cv. Chusei-Hikarikuro were inoculated with PJ-H30 in each bottle. Bioassays were replicated four times with three bottles at each concentration. The macro inorganic nutrients, micro inorganic nutrients, Fe-EDTA solution and organic elements were abbreviated as (A), (B), (C), and (D) in the figure, respectively. A concentration of 500% macro inorganic elements was phytotoxic to soybean plants. Bars indicate standard error of the mean

(7 days after inoculation), and 94.3%, 92.1%, 85.0% and 96.4% (16 days after inoculation). When several concentrations of C and D groups were applied, the disease was estimated at almost the same level as that of the controls; it ranged from 32.9% to 48.2% (7 days after inoculation), and from 74.3% to 97.1% (16 days after inoculation). These results indicate that macro inorganic elements were effective for disease suppression 16 days after inoculation.

Six concentrations (0%, 10%, 100%, 250%, 500% and 1000%) of KNO_3, $(NH_4)_2SO_4$, $MgSO_4$, $CaCl_2$ and NaH_2PO_4 contained in the macro inorganic elements group were assessed. A concentration of 10–250% (2.47–61.75 mM) of KNO_3 was effective in inhibiting infection; the incidence of disease was 8.6%, 0% and 36.1% (7 days after inoculation), and 25.7%, 9.7% and 55.4% (16 days after inoculation) at 10%, 100% and 250% (2.47, 24.7 and 61.75 mM) KNO_3, respectively. A concentration of 10%, 100%, 250%, 500% and 1000% (0.1, 1.0, 2.5, 5.1 and 10.2 mM) $CaCl_2$ strongly inhibited infection ($P < 0.01$); the incidence of disease was 23.8%, 0%, 0%, 5.6% and 14.3% (7 days after inoculation), and 42.1%, 38.9%, 9.5%, 5.5% and 14.3% (16 days after inoculation; Fig. 4). The concentrations of 10–500% of $(NH_4)_2SO_4$, $MgSO_4$ and NaH_2PO_4 increased infection at almost the same rate as that of the control plants. The 100% of $MgSO_4$ induced an infection (6–15 days after inoculation), compared with the control plants. Although 1000% $(NH_4)_2SO_4$ and NaH_2PO_4 induced slight resistance to the disease, plant growth was suppressed by the higher concentration level.

Effect of several nutrients in B5 solution on growth rate of PJ-H30 isolate on PDA

The growth rate of PJ-H30 on PDA was affected by B5 (ABCD) solution and four media (ABC, ABD, ACD and BCD), and several concentrations (0%, 10%, 100%, 250%, 500% and 1000%) of A–D group, as illustrated in Fig. 5. The diameter of the isolate was significantly suppressed in the presence of B5 solution, compared with the control ($P < 0.01$; Fig. 5a). The fungal growth was decreased significantly ($P < 0.01$) when three media (ABC, ABD and ACD) containing A group (macro inorganic nutrients) was applied. The mycelium growth was estimated at almost the same

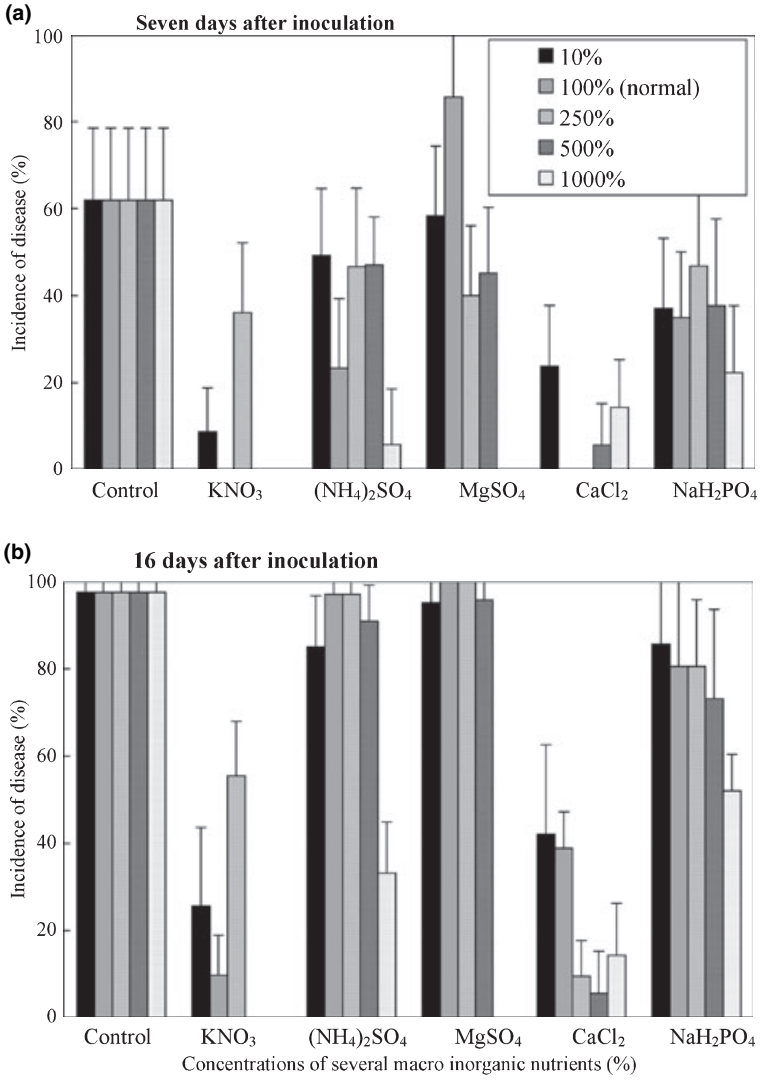

Fig. 4 Effect of several levels (0%, 10%, 100%, 250%, 500% and 1000%) of KNO_3, $(NH_4)_2SO_4$, $MgSO_4$, $CaCl_2$ and NaH_2PO_4 on the incidence of disease in *Glycine max* cv. Chusei-Hikarikuro 7 and 16 days after inoculation. Eight seedlings of cv. Chusei-Hikarikuro were inoculated with PJ-H30 in each bottle. Bioassays were replicated four times with three bottles at each concentration. A concentration of 500–1000% KNO_3 and 1000% $MgSO_4$ were phytotoxic to soybean plants. Bars indicate standard error of the mean

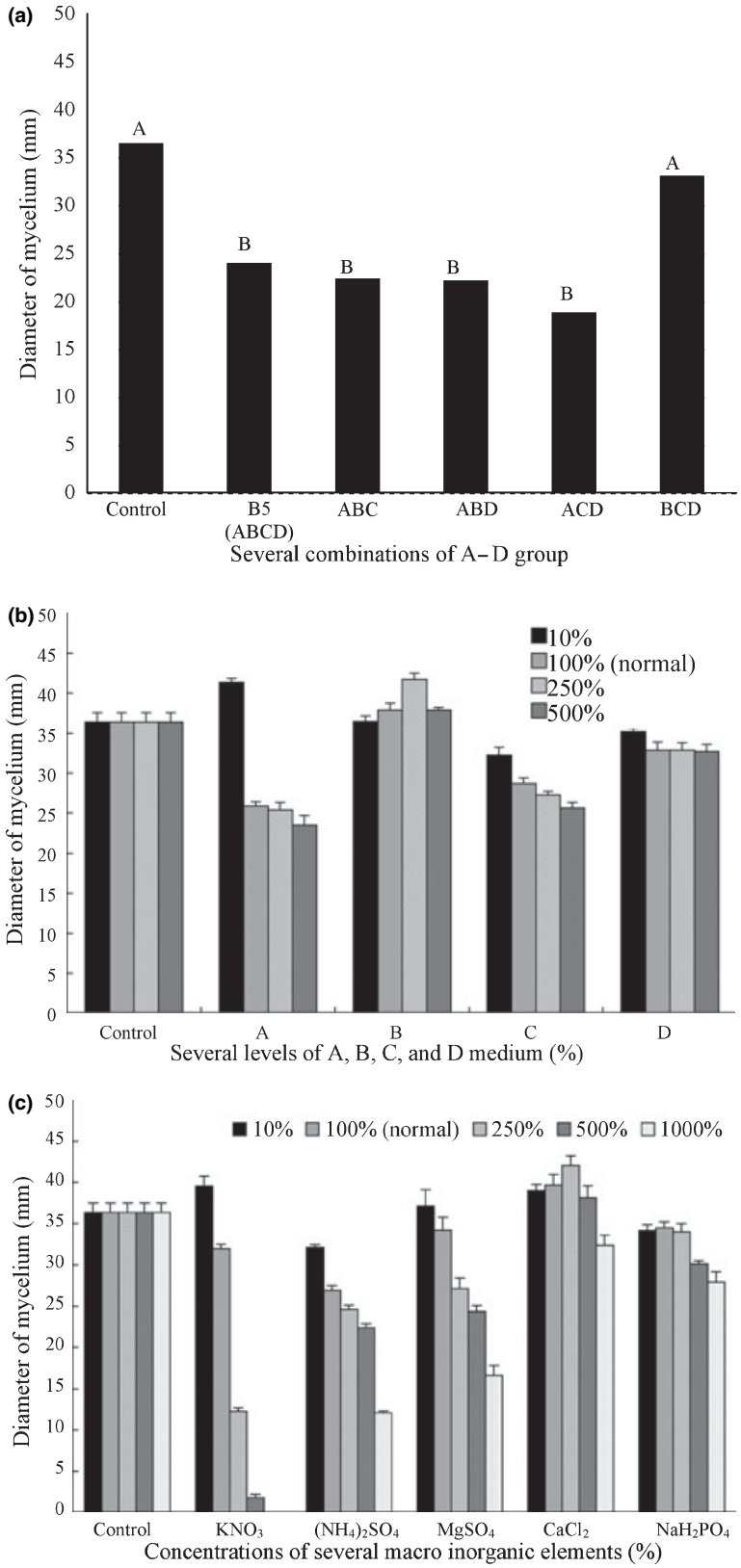

Fig. 5 Effect of B5 solution and four media (ABC, ABD, ACD and BCD; a), several concentrations (0%, 10%, 100%, 250% and 500%) of A, B, C and D medium (b), and various levels (0%, 10%, 100%, 250%, 500% and 1000%) of KNO_3, $(NH_4)_2SO_4$, $MgSO_4$, $CaCl_2$ and NaH_2PO_4 (c) on growth rate of PJ-H30 after 7 days incubation at 23°C. The medium ABC, ABD, ACD and BCD consists of 100% of (A) macro inorganic nutrients + (B) micro inorganic nutrients + (C) Fe-EDTA solution, (D) A + B + organic elements, A + C + D, and B + C + D, respectively. Numbers followed by the same letter are not significantly different according to an analysis of variance and least significant difference ($P < 0.05$). Bars indicate standard error of the mean

level as that of the control, when medium BCD was used. Various concentrations of A, C and D groups could reduce the growth rate, except for 10% A and D groups. However, B group slightly increased the growth of the isolate (Fig. 5b). Therefore, the macro inorganic nutrients group was the most effective for the reduction of fungal growth.

A concentration of 10% (2.47 mM) KNO_3 slightly increased the fungal growth, whereas 100–1000% (24.7–247 mM) KNO_3 led to a marked suppression of

Table 2
Effect of potassium or calcium concentration on shoot and root dry weight and potassium or calcium concentration in non-inoculated soybean plants of *Glycine max* cv. Chusei-Hikarikuro

	Dry weight (mg)				Concentration (% of dry weight)			
	Shoot		Root		Shoot		Root	
Concentration[a](%)	KNO_3[b]	$CaCl_2$	KNO_3[b]	$CaCl_2$	KNO_3[b]	$CaCl_2$	KNO_3[b]	$CaCl_2$
0	199 a	215	59 a	59 a	1.363 a	0.117 a	1.438 a	0.105 a
10	199 a	219	60 a	88 b	1.838 b	0.169 b	2.075 b	0.184 b
100	229 b	223	73 b	79 b	3.538 c	0.199 c	8.700 c	0.233 c
250	235 b	225	46 c	75 b	4.088 d	0.233 d	9.538 d	0.335 d
500	–	232	–	60 a	–	0.266 e	–	0.502 e
1000	–	233	–	58 a	–	0.389 f	–	0.698 f

[a]Eight non-inoculated seedlings (cv. Chusei-Hikarikuro) in each trial were subjected to different potassium or calcium combinations (0%, 10%, 100%, 250%, 500% and 1000% of KNO_3 and $CaCl_2$). Several concentrations of 10%, 100%, 250%, 500% and 1000% of KNO_3 and $CaCl_2$ indicate 2.47, 24.7, 61.75, 123.5 and 247 mM of KNO_3, and 0.1, 1.0, 2.5, 5.1 and 10.2 mM of $CaCl_2$.
[b]A concentration of 500–1000% $KNO_3$was phytotoxic to soybean plant growth.
Columns numbered without letters and followed by the same letter are not significantly different according to an analysis of variance and least significant difference ($P < 0.05$).

mycelium growth ($P < 0.01$; Fig. 5c). The diameter of PJ-H30 increased slightly in the presence of 10–500% (0.1–5.1 mM) of $CaCl_2$ in comparison with the control ($P < 0.05$). However, 1000% (10.2 mM) $CaCl_2$ led to a slight suppression of mycelium growth for the isolate ($P < 0.05$). All levels of $(NH_4)_2SO_4$, NaH_2PO_4, and 100–1000% $MgSO_4$ could suppress growth rate of PJ-H30 isolate (Fig. 5c).

Relationship between potassium or calcium concentrations in soybean plants and disease incidence

There was a difference between the dry weight of shoot of cv. Chusei-Hikarikuro at the time of inoculation using varied concentrations of KNO_3 (Table 2). Application of 100–250% (24.7–61.75 mM) KNO_3 increased the growth of shoots, compared with the control plants. A concentration of 500–1000% (123.5–247 mM) KNO_3 was toxic to soybean plants; plant growth was highly inhibited. However, there was no significant difference between the dry weight of shoot of the cultivar with several different calcium concentrations of $CaCl_2$. The potassium and calcium concentration (percentage of dry weight) in shoots increased significantly, compared with the control. The potassium and calcium concentration in shoots were correlated with the concentration in the agar medium: $r = 0.914$ (KNO_3) and $r = 0.972$ ($CaCl_2$) on cv. Chusei-Hikarikuro.

There was a difference between the dry weight of the root of cv. Chusei-Hikarikuro at the time of inoculation by the application of KNO_3 and $CaCl_2$ (Table 2). Concentrations of 100% (24.7 mM) KNO_3 and 100–250% (1.0–2.5 mM) $CaCl_2$ increased the growth of roots in cv. Chusei-Hikarikuro, whereas 250% (61.75 mM) KNO_3 and 500–1000% (5.1–10.2 mM) $CaCl_2$ reduced the growth, when compared with the control plants. These results show that there was a difference in the effect of potassium and calcium on shoot and root growth of cv. Chusei-Hikarikuro. The potassium and calcium concentrations in roots increased significantly when correlated with the concentration in the agar medium; $r = 0.888$ (KNO_3) and 0.982 ($CaCl_2$) on cv. Chusei-Hikarikuro.

The relationship between potassium or calcium concentration in plants and incidence of disease (16 days after inoculation) is illustrated in Fig. 6. Disease reduction was related to increased potassium and calcium uptake by the shoots and roots of cv. Chusei-Hikarikuro, except when the amount of potassium and calcium applied was more than 4.088% (potassium) and 0.389% (calcium) in shoots, 9.537% (potassium) and 0.697% (calcium) in roots of the dry weight in cv. Chusei-Hikarikuro.

Effect of KNO_3 and $CaCl_2$ on zoospore release of PJ-H30 isolate

Zoospore release from isolate of PJ-H30 cultured on PDA did not occur under several KNO_3 and $CaCl_2$ concentrations as well as SDW. However, zoospore release from the isolate on LBA was influenced greatly by several concentrations of KNO_3 and $CaCl_2$, as illustrated in Fig. 7. All levels (10–1000%; 2.47–247 mM) of KNO_3 significantly reduced the release of zoospore. Although 10–250% (0.1–2.5 mM) $CaCl_2$ increased zoospore release, a concentration of 500–1000% (5.1–10.2 mM) $CaCl_2$ significantly reduced the release of zoospore ($P < 0.01$).

Discussion

Information of host nutrition in relation to disease occurrence is considered important to control diseases and to change conventional agricultural practices. In the present study, B5 solution and macro inorganic elements in the B5 medium suppressed infection caused by *P. sojae* in cv. Chusei-Hikarikuro ($P < 0.01$; Figs 1–3); however, micro inorganic nutrients, Fe-EDTA solution and organic elements in the B5 solution were ineffective (Fig. 3). The application of KNO_3 and $CaCl_2$ contained in the macro inorganic nutrients group markedly affected resistance to pathogens (Fig. 4).

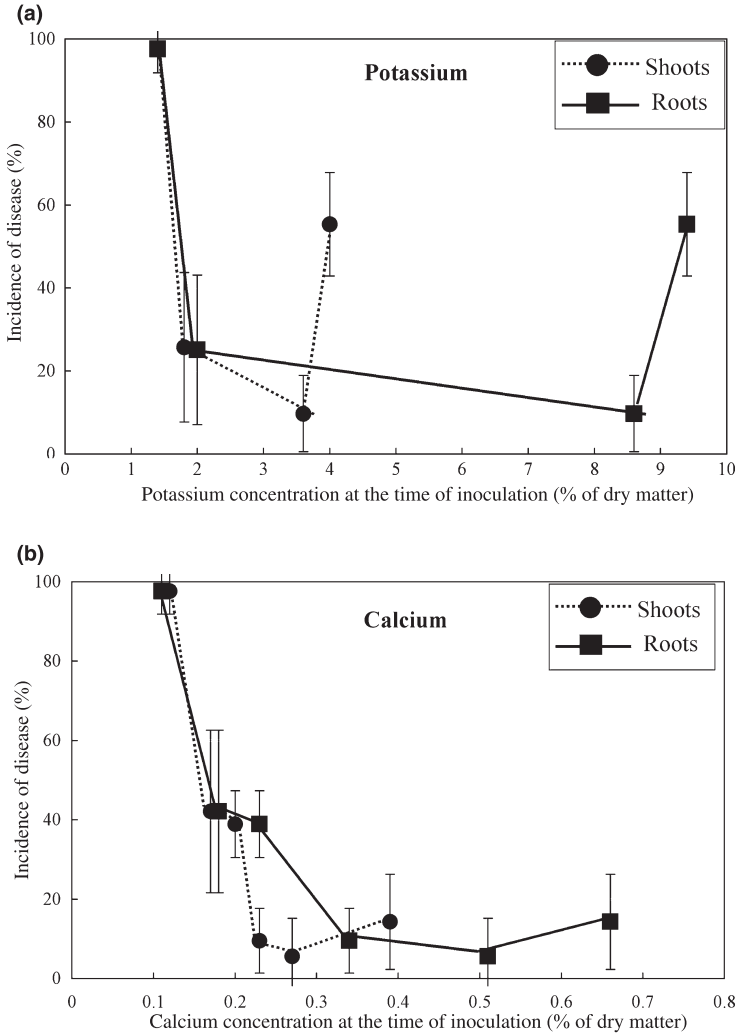

Fig. 6 Relationship between potassium (a) or calcium (b) content in shoots and roots and incidence of disease on *Glycine max* cv. Chusei-Hikarikuro 16 days after inoculation. Bars indicate standard error of the mean

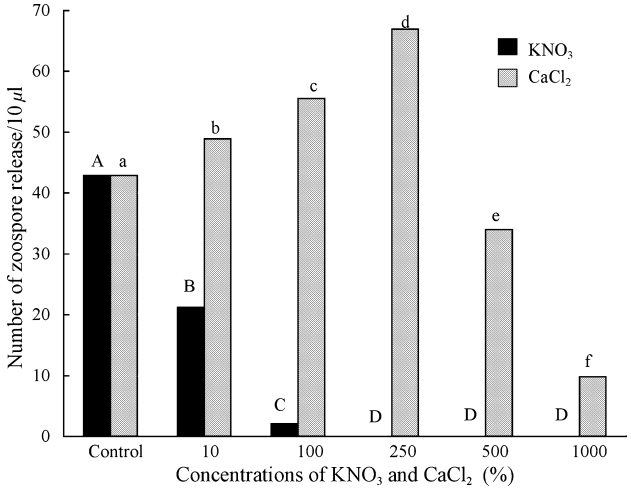

Fig. 7 Relationship between potassium or calcium concentration and zoospore release from isolate of PJ-H30 on Lima bean agar after 12 h incubation at 21°C. Black and shaded parts of the bar graph relate to KNO_3 and $CaCl_2$. Numbers followed by the same letter are not significantly different according to an analysis of variance and least significant difference ($P < 0.05$). Several concentrations of 10%, 100%, 250%, 500% and 1000% of KNO_3 and $CaCl_2$ indicate 2.47, 24.7, 61.75, 123.5 and 247 mM of KNO_3, and 0.1, 1.0, 2.5, 5.1 and 10.2 mM of $CaCl_2$

To evaluate the effect of inorganic elements on Phytophthora stem rot disease suppression, this study used B5 solution and the agar medium inoculation method (Sugimoto et al., 2003, 2005). The B5 medium established for soybean cell culture by Gamborg et al. (1968) includes several inorganic elements as well as organic elements. Therefore, B5 medium was considered a suitable solution for detection of effective nutrients of disease management. The agar medium inoculation method was used because of the possible effect of some inorganic elements and varying pH differences, which should be eliminated or minimized; some inorganic elements have suppressive effects on several pathogens (Huber, 1981; Marschner, 1995). Results in this laboratory study show that the applications of small amounts of potassium and calcium in the B5 solution, such as 10% (2.47 mM) KNO_3 or 10% (0.1 mM) $CaCl_2$ could significantly reduce infection by 25.7% and 42.1%, while the applications of 500% (5.1 mM) $CaCl_2$ and 100% (24.7 mM) KNO_3 was more effective in inhibiting infection in cv. Chusei-Hikarikuro (Fig. 4). It was previously reported that small levels of 0.4 mM $CaCl_2$ and $Ca(NO_3)_2$ treatments could greatly influence disease reduction of

Phytophthora stem rot on soybean (Sugimoto et al., 2005). The suppression of the incidence of disease suggests that the application of KNO_3 and $CaCl_2$ also affects the early stage of infection in cv. Chusei-Hikarikuro. Generally, the hypocotyl cell in the soybean cultivar, which is susceptible to pathogens, is infected 8–10 h after contact with *P. sojae* (Yoshikawa et al., 1978). According to this finding, it was thought that several levels of KNO_3 and $CaCl_2$ applications could affect the initial invasion of pathogens by direct suppression of fungal growth or by strengthening plant cell walls or by these multiple factors.

It was recently reported that some inorganic elements could reduce fungal infection by directly inhibiting fungal growth (Kao and Ko, 1986; Yamazaki and Hoshina, 1995; Sugimoto et al., 2005). To investigate this effect, the growth rates of PJ-H30 isolate on PDA containing several concentrations of nutrients were examined. As a result, B5 solution, macro inorganic elements (A group), three media (ABC, ABD and ACD) containing A group were effective for the reduction of mycelium growth, suggesting that macro inorganic treatments involved factors for suppressive effects of fungal growth. There was a significant relationship between mycelium growth and disease suppression (7 days after inoculation) when B5 solution, various levels of A group and the three media (ABC, ABD and ACD) were applied (Figs 1, 2a and 5). These results might be due to the multiple effects of direct suppression on mycelium growth in combination with the response of the host plant tissue to those nutrients.

Although mycelium growth of the isolate was influenced by the potassium and calcium concentration, no significant relationship was observed between inhibition of the fungal growth of the isolate and disease reduction (7 days after inoculation) at 10% (2.47 mM) KNO_3 and 10–500% (0.1–5.1 mM) $CaCl_2$ application, as illustrated in Figs 4a and 5c; the mycelium growth of PJ-H30 was enhanced slightly by the application of 10–500% (0.1–5.1 mM) $CaCl_2$ ($P < 0.05$); however, the incidence of disease (7 days after inoculation) was reduced significantly by the same concentration of calcium in comparison with controls ($P < 0.01$). This study supports previous findings (Sugimoto et al., 2005). On the other hand, the incidence of disease was reduced by the application of 10% (2.47 mM) KNO_3, although the mycelium growth slightly increased at the same concentration of potassium. The application of 100–250% (24.7–61.75 mM) KNO_3 led to a marked suppression of mycelium growth and disease incidence in comparison with controls ($P < 0.01$). This result shows that the application of calcium and potassium inhibits fungal growth at high concentration levels ($P < 0.05$). However, it did not relate directly to the calcium- or potassium-dependent resistance of soybean to pathogens. Moreover, the release of zoospore from the isolate on PDA never occurred during experiments because *P. sojae* mycelium cultured on PDA was used for the virulence test. The PDA did not interfere with the effect of calcium and potassium. Huber and Watson (1970) reported that some inorganic elements affect disease potential more than inoculum potential, and some nutrients may decrease disease even though the population of a pathogen is increased. Marschner (1995) suggests that good potassium and calcium fertility are associated with strong cell walls that enhance disease resistance and the ability of the crop to maintain firm. In view of these results, the disease reduction was predominately due to the response of the host tissue to potassium and calcium rather than a direct hyphal growth inhibition of pathogen by the application of potassium and calcium.

One theory suggests that disease suppression was due to the increased resistance to the disease by the increased levels of inorganic contents (Conway et al., 1992). It was therefore hypothesized that the concentration of potassium and calcium in plants could increase by the application of potassium and calcium 'by the time of inoculation', and that such applications may induce an improvement of the physical properties of cells in soybean seedlings.

The relationship between the potassium or calcium contents in plants and the incidence of disease was examined in an effort to test this theory. As shown in Table 2, the potassium and calcium concentrations in plants of cv. Chusei-Hikarikuro increased 'before inoculation' due to the application of potassium and calcium, which correlated with the concentration in the medium. This result indicates that increased potassium or calcium concentrations in plants were associated with disease reduction (Table 2 and Fig. 6). It was stated that the incidence of *Diaporthe sojae* in soybean disease decreased as the amount of potassium in the soil increased. Also the potassium concentration in leaves closely paralleled the potassium treatments and potassium content in the soil (Jeffers et al., 1982). Our results support the assertion that soybean seedlings acquired resistance to the pathogen, since the potassium and calcium content in plant increased before inoculation.

This study confirms that potassium and calcium are effective nutrients in reducing Phytophthora stem rot disease, as illustrated in Fig. 6. However, the high levels of KNO_3 and $CaCl_2$ application caused a slight phytotoxicity on plant growth (predominantly on the roots) of cv. Chusei-Hikarikuro (Table 2). Gamborg et al. (1968) stated that the soybean cells appeared to tolerate high concentrations of potassium; however, the yields of dry weight decreased when the concentration of potassium nitrate exceeded 30 mM. Volpin and Elad (1991) reported that $CaCl_2$ induced the greatest disease suppression of Botrytis blight in rose, but there was a difference between cultivars in the effectiveness of calcium; this was due to a difference in chloride tolerance. Moreover, the incidence of disease increased on cv. Chusei-Hikarikuro when the amount of potassium applied as 100% (24.7 mM) KNO_3 was more than 3.53% in shoots or 8.70% in roots of the dry weight, as illustrated in Fig. 6. The incidence of disease

slightly increased when the amount of calcium applied as 500% (5.1 mM) $CaCl_2$ was more than 0.266% in shoots or 0.502% in roots of the dry weight. This trend was also found in a previous study (Sugimoto et al., 2005); the incidence of disease had already increased when 4 mM $CaCl_2$ was applied. The amount of calcium amended as 4 mM $CaCl_2$ was estimated 0.215% in shoots or 0.418% in roots of the dry weight. It is possible that a high level of NO_3^- and Cl^- concentration was a negative element for this disease management. Therefore, rates of potassium and calcium application need to be further evaluated to reduce the risk of phytotoxicity and disease enhancement on soybean when calcium and potassium are applied for disease suppression.

The effect of KNO_3 or $CaCl_2$ on zoospore release from *P. sojae* on LBA was investigated for an evaluation of the applicability of experimental methods and results to field situations. These results may strengthen the possibility of applying these inorganic elements to reduce the incidence of disease in practical agricultural situations. The application of 10–250% (0.1–2.5 mM) $CaCl_2$ enhanced zoospore release from PJ-H30 isolate (Fig. 7). A minimum concentration of calcium is necessary for production of zoosporangia or zoospore release by *Phytophthora* spp. (Halsall and Forrester, 1977; Sato, 1994; von Broembsen and Deacon, 1997). However, the microbial environment in the field differs from that of an experimental situation due to the presence of many different micro-organisms, as well as antagonistic or synergistic activities between potassium, calcium and other inorganic elements in the soil and nutrient solution. Huber (1981) reported that high-potassium and low-calcium conditions increased disease caused by *Phytophthora parasitica*. The level of potassium in plants depends on the availability of Mg and Ca. Potassium availability is enhanced by calcium (Marschner, 1995). It is necessary to examine effective levels of KNO_3 or $CaCl_2$ for disease suppression in the field.

The central role of the mechanisms involved in disease reduction needs further research. It was reported that several factors influence the effectiveness of potassium fertilizer in reducing incidence of disease (Marschner, 1995). The foliar application of potassium to the first true leaf of cucumber, before inoculation with powdery mildew, induced systemic protection from the disease organism (Reuveni et al., 2000). Resistance to *P. infestans* is associated with the K-induced accumulation of fungistatic levels of arginine in leaves (Alten and Orth, 1941). It has been noted that the Ca^{2+} ion signal is one of the earliest events in challenged cells, and the signal is essential for the activation of plant defence responses, such as phytoalexin biosynthesis, induction of defence-related genes and hypersensitive cell death (Knight et al., 1991). It is necessary to examine gene expression related to plant defence reaction and signalling in Phytophthora stem rot on soybean in response to the application of calcium and potassium.

In conclusion, this study showed that potassium and calcium amendments greatly influenced disease reduction of Phytophthora stem rot on soybean. The results suggest that this effect could be mediated by a response of the host tissue to increased potassium and calcium directly, and possibly by a direct inhibition of fungal growth (at the high levels of potassium and calcium). The application of a potassium and calcium solution (more than 2.47 mM KNO_3 and 5.1 mM $CaCl_2$, respectively) may be effective for the disease management of Phytophthora stem rot in the field through the inhibition the release of zoospore. We believe that the findings of this study can contribute to the construction of effective strategies for the management of Phytophthora stem rot on soybean.

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