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Title: Increase in soil pH due to Ca-rich organic matter application causes suppression of the clubroot disease of crucifers

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Abstract

Clubroot disease of cruciferous plants caused by the soil-borne pathogen *Plasmodiophora brassicae* is difficult to control because the pathogen survives for a long time in soil as resting spores. Disease-suppressive and conducive soils were found during the long-term experiment on the impact of organic matter application to arable fields and have been studied to clarify the biotic and abiotic factors involved in the disease suppression. The fact that a large amount of organic matter, 400 t ha^{-1} y^{-1} farmyard manure (FYM) or 100 t ha^{-1} y^{-1} food factory sludge compost (FSC), had been incorporated for more than 15 y in the suppressive soils and these soils showed higher pH and Ca concentration than the disease conducive soil led us to hypothesize that an increase in soil pH due to the long-term incorporation of Ca-rich organic matter might be the primary cause of the disease suppression. We have designed a highly reproducible bioassay system to examine this hypothesis. The suppressive and conducive soils were mixed with the resting spores of *P. brassicae* at a rate of 10^6 spore g^{-1} soil, and *Brassica campestris* was grown in a growth chamber for 8 d. The number of root hair infections was assessed on a microscope. It was found that the incorporation of FYM and FSC at 2.5% (w/w) to the conducive soil suppressed the

infection and that the finer particles (≤5 mm) of FSC inhibited the infection and

increased soil pH more effectively. Neutralization of the conducive soil by $Ca(OH)_2$,

$CaCO_3$ and KOH suppressed the infection, but the effectiveness of KOH was less than

those of $Ca(OH)_2$ and $CaCO_3$. Acidification of the suppressive soils by H_2SO_4,

promoted the infection. The involvement of soil biota in the disease suppression was

investigated using the sterilized (γ-ray irradiation) suppressive soils with respect to soil

pH. The γ-ray irradiation promoted the infection at pH 5.5, but no infection was

observed at pH 7.4 irrespective of the sterilization status. All these observations

suggest that soil pH is a major factor in disease suppression by organic matter

application and that Ca and soil biota play certain roles in the suppression under the

influence of soil pH.

Keywords

Calcium; Clubroot disease; Organic matter; *Plasmodiophora brassicae*; Soil-borne

pathogen; Soil pH; Suppressive soil

1. Introduction

Clubroot of cruciferous plants is a major disease that is widespread throughout the world. It is caused by the soil-borne obligate parasite, *Plasmodiophora brassicae* classified amongst the Protist (Castlebury and Domier, 1998; Ward and Adams, 1998). The life cycle of *P. brassicae* consists of two phases: the primary phase is characterized by germination of the resting spore in the rhizosphere and subsequent infection of the root hair, and the secondary phase is characterized by colonization and proliferation in the root cortex (Ingram and Tommerup, 1972). It is difficult to control the disease because the pathogen survives in soil for a long time as resting spores. The breeding of resistant plant cultivars is one of the strategies to control clubroot, and several genes (Piao et al., 2004) and loci (Suwabe et al., 2003; Hirai et al., 2004; Rocherieux et al., 2004) involved in resistance to the disease have been identified from *Brassica* sp. Biological control using a fungal endophyte has also been proposed (Narisawa et al., 1998). Another approach is controlling the soil environment to suppress the disease, and analysis of disease-suppressive soils has been conducted for this purpose (Young et al., 1991; Murakami et al., 2000a). The involvement of soil pH in the occurrence of clubroot has been suggested: liming is a conventional technique to control the disease (Dobson et al., 1983; Campbell et al., 1985; Webster and Dixon, 1991; Murakami et al., 2002a; Tremblay et al., 2005). Young et al. (1991) suggested that gentisic acid, a

phenolic compound, found in disease-suppressive soils was involved in disease suppression. In addition to these abiotic factors, it has been suggested that the microbial community plays an important role in the suppression of clubroot (Murakami et al., 2000a).

Recently, a large amount of organic waste from sewage sludge, farming practices and the food industry has caused environmental pressures such as eutrophication of fresh water due to nitrogen-leaching and greenhouse gas emissions. It is becoming necessary for industries producing organic waste to organize recycling systems. The incorporation of organic waste into arable fields is one option for dealing with the problem. In particular, organic waste from farming and food factories is usually safe (i.e. free from toxic elements such as heavy metals) and thus suitable for incorporation into arable fields. Enrichment of soil organic matter improves soil aeration, physical structure, drainage, water holding capacity, nutrient availability and microbial activity. There are many examples showing that organic matter application can effect soil-borne diseases. Chicken litter decreased the population densities of the root-knot nematode *Meloidogyne incognita* (Riegel et al., 1996; Riegel and Noe, 2000) due to an increase in soil microbial activity (Riegel and Noe, 2000). The incidence of verticillium wilt of potato caused by the fungal pathogen, *Verticillium dahliae*, was reduced by animal

manure application (Conn and Lazarovits, 1999). Incorporation of composted sewage sludge also significantly reduced the lettuce drop caused by the fungus *Sclerotinia minor* (Lumsden et al., 1986). An excess application of organics to the field, however, can cause eutrophication of terrestrial freshwater systems. The influence of long-term application of organic matter to soil on crop productivity and nitrogen-leaching has been investigated in an experimental field of the Nagoya University since 1987 to define an appropriate level of organic matter application. The clubroot disease was first observed on cabbage in 1997 in the field, but little or no symptom was found in the plots in which a large amount of organic matter had been incorporated. It has been confirmed through this field experiment that the suppressive effect in the plots is not temporary and that the soils of the plots can be designated as 'suppressive soils'.

The occurrence of clubroot disease in the field, however, is not constant year by year. Therefore, it is required to establish a reproducible experimental system under controlled environmental conditions to improve our knowledge of this disease caused by an unculturable pathogen. The objectives of this study were to identify the biotic and abiotic factors involved in disease suppression by organic matter application in a model bioassay system.

2. Materials and methods

2.1 Experimental field, assessment of disease incidence and soil sampling

Seven treatments have been designed for the field experiment on the influence of long-term application of organic matter on crop productivity, soil chemical and physical properties and nitrogen-leaching in Nagoya University, Aichi, Japan since 1987. The following three treatments (plots) in which clear difference in the disease incidence were observed were chosen from seven original treatments. The conventional treatment plot has been amended with chemical fertilizer (270-520 kg N, 200-520 kg P_2O_5, 240-520 kg K_2O ha^{-1} y^{-1}) and 40 t ha^{-1} y^{-1} farmyard manure (FYM) since 1987. The FYM treatment plot has been amended with 400 t ha^{-1} y^{-1} FYM since 1987. The food factory sludge compost (FSC) treatment plot has been amended with 100 t ha^{-1} y^{-1} of the FSC since 1993. The FYM was compost of cattle feces and rice straw. The FSC was compost of dehydrated activated sludge and corn gluten feed discharged from a cornstarch factory. The properties of the FYM and the FSC are shown in Table 1. The pH of FYM and FSC were 9.8 and 7.3, respectively, and both of the composts showed high levels of base content. The size of each plot was 3×17 m

(*n*=1), and each plot consisted of two rows. Melon (*Cucumis melo* L. cv. Prince Melon)

and cabbage (*Brassica oleracea* L. var. *capitata* cv. Hukamidori) were cultivated in

1997, and sweet corn (*Zea mays* L. cv. Peter Corn) and chinese cabbage (*B. rapa* L. var.

pekinensis cvs. Satokaze and Tomikaze) have been cultivated since 1998. Crop residue

was incorporated by a rotary cultivator after each harvest.

The incidence of clubroot disease has been assessed annually after the crucifer crop

cultivation since 1997. Twenty cabbages were chosen randomly in each plot, and the

presence or absence of clubroot galls was assessed visually. Disease incidence was

expressed as percentages of clubbed plants.

About 3 kg of soil samples were taken after the harvest of cabbage in 2003 from 5

cm below the surface (20 cm in depth) from three randomly chosen spots in each row

(6×3 kg soil from each plot) of the conventional, FYM and FSC plots, combined,

mixed thoroughly, air-dried, passed through a 5 mm sieve and stored at room

temperature for chemical analyses and bioassay.

2.2. Soil chemical properties and resting spore density

A subsample was taken from each of the stored soils for the following chemical

analyses (*n*=1). Soil pH and electric conductivity were measured in H_2O (1:5, w/v).

Total C and N were measured by a CN coder. Total K, Ca and Mg were analyzed by an

atomic absorption spectrophotometer after digestion with conc. HCl. Total P was

determined by the ammonium vanado-molybdate method after the Kjeldahl digestion.

Available phosphate was determined by the method of Olsen and Sommers (1982).

Cation exchange capacity was determined by the method of Schollenberger and Simon

(1945). Exchangeable bases were extracted with 1 M ammonium acetate and

determined by an atomic absorption spectrophotometer.

The resting spore density of the soils was determined by a modified method of

Murakami et al. (2000b). Three subsamples (4 g soil) were taken from the stored soils

(*n*=3), suspended in 80 mL deionized water (DIW) and shaken vigorously for 1 min.

The pH of the suspension was adjusted to 10 with 1 M NaOH and the solution was

sonicated for 5 min at 50 kHz with the Ultrasonic cleaner VS-100 (Iuchi Corp., Osaka).

Then the pH of the suspension was readjusted to 9 by NaOH and the solution was

shaken vigorously for 1 min, and passed through a 38 μm sieve and centrifuged at 900

$\times g$ for 10 min at room temperature. The residue was washed with DIW and

centrifuged three times and suspended in DIW in a final volume of 50 mL. The

suspension was mixed thoroughly, and a 100 μL aliquot was mixed with an equal

volume of 200 µg mL^{-1} Fluorescent brightener 28 (Sigma-Aldrich Japan, Tokyo) solution (Takahashi and Yamaguchi, 1987). The numbers of the resting spore were counted on a Thoma blood corpuscle chamber using a fluorescence microscope under UV-excitation.

Three subsamples (20 g soil) were taken from the stored soils (n=3), and phenolic acids were extracted with 100 mL 0.5 M NaOH on a reciprocal shaker (150 rpm) under a nitrogen atmosphere at 25˚C for 1h. The suspension was centrifuged at 9,000 × g for 10 min at 4˚C, and the supernatant was filtrated on an Advantec No.3 filter paper (Advantec, Tokyo). The pH of the filtrate was adjusted to 1.0 with 2 M HCl to precipitate humic acids, and the solution was centrifuged at 12,000 × g for 10 min at 4˚C. The supernatant was extracted three times with 100 mL ethyl acetate, and the solvent was combined and evaporated by a rotary evaporator. The residue was dissolved in 20 mL DIW and subjected to HPLC analysis with the Hitachi LaChrom system equipped with the 4.6 × 250 mm ZORBAX-ODS column (DuPont, Tokyo) and the Hitachi L-7455 diode array detector. The mobile phase was water/acetic acid/1-butylalchol (650:1:10) at a flow rate of 1.0 mL min^{-1}. Phenolic acids were identified by corresponding retention time and an absorption spectrum in a range of 200-300 nm wavelength of the standard reagents.

2.3. Plug pot bioassay system

The *P. brassicae* inoculum culture was established using the clubroot galls of chinese cabbage from the field as an initial inoculum and maintained by the following procedure. *B. campestris* L. var. *rapifera* group, cv. Komatsuna Rakuten was inoculated with the suspension of the resting spores and grown in a growth chamber (25˚C, 16/8 h light/dark) for 40 days, and the galls were harvested and stored at −30˚C. To prepare the inoculum for the bioassay, the frozen galls were thawed in tap water, washed thoroughly, surface-sterilized with 70% ethanol for 30 sec, homogenized in DIW and filtered through eight layers of cheesecloth. The filtrate was centrifuged at $600 \times g$ for 5 min, and the pellet was washed four times with DIW then suspended in DIW. The number of spores in the suspension was determined according to the method of Takahashi and Yamaguchi (1987). The spore suspension was mixed with soil at a rate of 10^6 spore g^{-1} soil. Ten seeds of *B. campestris* were sown on the soil in the 4.5 cm square plug pots (60 mL in vol) (*n*=3) and grown in the growth chamber. The seedlings were thinned to four plants pot^{-1} 4 days after sowing and irrigated from the bottoms of the pot through a capillary sheet. Eight days after sowing, the roots were

harvested, washed and stained with 50 mg L^{-1} aniline blue in 5 g L^{-1} phenol solution

for 24 h at room temperature. The number of the zoosporangial clusters of *P. brassicae*

formed in the root hairs of the top 2 cm tap root were counted under a microscope.

2.4. Experimental designs for plug pot bioassay

To assess the effect of organic matter application on root hair infection in the

conventional plot (conducive soil), the FYM and FSC were mixed into the soil from

the conventional plot (conventional soil) at a rate of 2.5% (w/w) (corresponded to 3.3 t

ha^{-1}). Soils from the FYM and FSC plots (suppressive soils, designated as FYM-soil

and FSC-soil, respectively) were used as positive controls. The effect of the particle

size of FSC was investigated using the conventional soil mixed with 2.5% (w/w) FSC

in the sizes of >5 mm, 1-5 mm or <1 mm which were fractionated by corresponding

sieves. The effect of soil pH on the disease suppression was investigated using the

neutralized conventional soil and the acidified FYM- and FSC-soils. A preliminary

experiment was conducted to determine the appropriate levels of chemicals for pH

adjustment. One kg of the conventional soil (pH 5.6) was mixed with 2.5 g $Ca(OH)_2$,

$CaCO_3$ and KOH for neutralization. $CaSO_4$ (2.5 g kg^{-1} soil) was employed as a

negative control which increased the Ca levels but not the soil pH. To acidify the

suppressive soils, 150 mL 0.5 M H_2SO_4 was added to 1 kg of the FYM- and FSC-soils.

Subsamples of these soils were taken ($n=1$), and exchangeable Ca^{2+} levels were

measured as described above. The involvement of soil biota in the disease suppression

was investigated with respect to soil pH using sterilized soils. The FYM- and FSC-

soils were irradiated with 50 kGyγ-ray, and the pH was adjusted with 1 M H_2SO_4 to

5.5.

2.5. Statistics

The StatView software (SAS Institute Inc., Cary, NC) was used for all statistical

analyses. Analysis of variance (ANOVA) and the Turkey-Kramer's multiple range tests

as a post hoc test of ANOVA were applied for the analyses of the results obtained from

the phenolic acid analysis and bioassay.

3. Results

3.1. Incidence of clubroot disease in the field

Clubroot disease was first observed in the conventional plot in 1997, and the percentages of the disease incidence have been more than 75% since 1998 (Table 2). In contrast, the disease in the FYM plot was found only in 2001 (10 %) and 2003 (5%). The disease has never been observed in the FSC plot.

3.2. Abiotic and biotic factors involved in disease suppression

Soil chemical properties of the conventional, FYM- and FSC-soils are shown in Table 3. The soil pH of the conventional soil was acidic, pH 5.7, whereas those of the FYM- and FSC-soils were neutral, pH 7.4 and 7.2, respectively. The values of electric conductivity, total C, N, P, cation exchange capacity and exchangeable Ca^{2+} in the FYM- and FSC-soils were more than twice as much as those of the conventional soil. Particularly, exchangeable Ca^{2+} content in the FYM- and FSC-soils was about 8- and 6-fold greater, respectively, than that in the conventional soil. The density of *P. brassicae* resting spores in the field soils were within a range of 10^5-10^6 spore g^{-1} soil: the conventional, FYM- and FSC-soils contained 10.4 ± 1.7, 6.5 ± 0.4 and 6.6 ± 0.9 $\times 10^5$ spore g^{-1} dry soil ($\pm$ SE), respectively. The most abundant phenolic acid in the

three soils was p-coumaric acid, and the concentration in the conventional soil (24.0 ±

3.0 mg kg^{-1}) was significantly lower than that in the FYM-soil (47.0 ± 2.6 mg kg^{-1}) but

higher than that in the FSC-soils (8.4 ± 1.4 mg kg^{-1}) (Table 4). The levels of p-hydroxy

benzoic acid, syringic acid and vanillic acid were not significantly different among

these soils. Gentisic acid was undetectable in all of the soils.

The plug pot bioassay system was designed to investigate the influence of various

abiotic and biotic factors on the infection, and first we tried to reproduce the disease

occurrence/suppression in the field using the conducive (conventional) and suppressive

(FYM- and FSC-) soils. The bioassay system well reflected the field situation: the high

level of root hair infection of *P. brassicae* on *B. campestris* was observed only in the

conventional soil, and no infection in the FYM- and FSC-soils was observed (Fig. 1).

No difference in the development of tap root and root hairs of *B. campestris* among the

treatments was observed at the time of assessment. Based on the results, we considered

that the bioassay was a reliable model system to investigate the suppression

mechanism of the clubroot disease.

Application of the FYM and FSC to the conventional soil significantly reduced the

number of root hair infections (Fig. 2). The particle size of the FSC greatly affected the

suppressive effect. A particle size for the FSC ≤5 mm suppressed the root hair infection

significantly and increased the soil pH effectively (Fig. 3). Based on these observations

in addition to the fact that the suppressive soils, the FYM- and FSC-soils, showed

higher pH than that of the conventional soil (Table 3), it was hypothesized that an

increase in soil pH due to the organic matter application might be the primary cause of

the disease suppression. To examine this hypothesis, the following experiment was

conducted. Application of $Ca(OH)_2$ or $CaCO_3$ to the conventional soil increased the pH

from 5.6 to 7.5 or 7.0, respectively, and decreased the number of the root hair infection

significantly (Fig. 4). Application of $CaSO_4$ doubled the exchangeable Ca^{2+} levels and

reduced soil pH slightly, but did not suppress the infection. Application of KOH

increased soil pH to 7.3 and reduced the number of root hair infections significantly,

although the levels of the suppression by KOH were lower than those by $Ca(OH)_2$ and

$CaCO_3$. For further confirmation, the FYM- and FSC-soils, were acidified by H_2SO_4.

The root hair infection was completely suppressed in the original FYM- and FSC-soils

but was promoted by the addition of H_2SO_4 which decreased the pH to less than 6.

The involvement of soil biota in the disease suppression in the FYM- and FSC-soils

was investigated with respect to soil pH. The root hair infection occurred both in the

control and sterilized treatments of the FYM- and FSC-soils at pH 5.5 (Fig. 5). The

levels of the infection, however, were significantly higher in the sterilized treatments

than in the controls. Zero or negligible infection was observed at pH 7.4 in these soils

irrespective of sterilization status.

4. Discussion

4.1. Application of Ca-rich organic matter causes the disease suppression

In the present study, it was clearly demonstrated that clubroot disease caused by

Plasmodiophora brassicae was suppressed by the incorporation of farmyard manure

(FYM) or food factory sludge compost (FSC) and that an increase in soil pH was the

primary cause for the suppression by the application of these organic matters. Although

there are more than sufficient data to demonstrate that organic matter applications

suppress disease, not all types of organic matter are effective in the suppression of

soil-borne diseases. Manure application increased the severity of stalk rot of maize

caused by *Macrophomina phaseolina* (Osunlaja, 1990) and the number of galls caused

by root-knot nematode *Meloidogyne hapla* (Szczech et al., 1993). Hoitink and Fahy

(1986) suggested that chemical, physical and biological characteristics are involved in

disease suppression in soil amended with organic matter, but the mechanisms

underlying disease suppression/promotion by organic matter application are of a complex nature and not always well understood. In the present study, however, the mechanism of the clubroot suppression by organic matter was clarified experimentally. The types of organic matter used in the study were rich in Ca, and the successive application of the material to the soils might result in the accumulation of Ca that suppressed the disease through neutralizing the soil in our field.

The density of the resting spores of *P. brassicae* in soil is one of the most significant factors for the disease incidence and severity (Murakami et al., 2002b). Naiki et al. (1978) demonstrated that the disease occurred at very low spore density e.g. in a range from 10 to 10^3 spore g^{-1} soil. In our field, the spore density in the FYM- and FSC-soils (suppressive soils) was lower than that of the conventional (conducive) soil but still much higher than the range reported by Naiki et al. (1978). In addition, the infection did not occur in these suppressive soils even though the soils were enriched to 10^6 spore g^{-1} soil in the plug pot bioassay (Fig. 1). It is apparent that the suppression of the disease in the FYM and FSC plots in the field was not due to the lower density of the resting spores in comparison to the conventional soil.

Young et al. (1991) conducted comparative analysis of phenolic compounds in clubroot-suppressive and conducive soils and found that the concentration of gentisic

acid in suppressive soils was higher than that in conducive soils, although the total

content of phenolics was higher in the conducive soils. In addition, the disease was

significantly suppressed by the addition of gentisic acid to the conducive soil. In our

experimental field, the concentration of gentisic acid in the soils was under the

detection limit. Phenolic acid might have originated from plant residues in the FYM

which had been applied to the conventional and FYM plots for more than 15 years, and

this could be a reason why *p*-coumaric acid was greater in the conventional and

FYM-soils than that in the FSC soil. It is likely that the involvement of phenolic acid

in the suppression of clubroot disease may be circumstantial, and at least in our study

there was no evidence that phenolic acid was involved in the disease suppression.

4.2. Soil pH as a major factor for the disease suppression

In the present study, the effect of soil pH on the disease suppression was clearly

discriminated from that of Ca by the highly reproducible bioassay. The infection in the

conventional soil was suppressed not only by $CaOH/CaCO_3$ but also by KOH. In

contrast, the infection in the FYM- and FSC-soils was promoted by H_2SO_4 which did

not reduce the Ca concentration. It is controversial whether Ca is a major factor of the

disease suppression. Fletcher et al. (1982) applied several Ca compounds at different pH values and demonstrated that an increase in Ca levels suppressed the disease irrespective of pH. On the other hand, Myers and Campbell (1985) found a decrease in the infection with increasing pH levels and a limited effect of Ca on the suppression of the disease development under low pH conditions. In our experiment, KOH increased soil pH to the same extent as $CaOH/CaCO_3$ but was less effective in terms of the disease suppression. In addition, the levels of the infection in the acidified FYM- and FSC-soils were significantly lower than that in the conventional soil in which Ca content was much less than the suppressive soils. These observations suggest that soil pH plays a major role in the suppression of the disease and that the effect of Ca is additive or limited under neutral pH.

Dobson et al. (1983) found that the particle size and the degree of mixing of lime affected the effectiveness of liming: thorough mixing and finer particles of lime achieved a more uniform pH distribution in soil, resulted in less infection. In the present study, it was demonstrated that the finer particle compost increased pH and suppressed the disease more effectively.

Most soils inhibit fungal germination and growth to a certain extent, a phenomenon known as soil fungistasis (Dobbs and Hinson, 1953). Many factors affect the intensity

of fungistasis including soil physical and chemical traits, environmental changes, fungal characteristics, the community composition, and the metabolic activities of other soil microbes. Murakami et al. (2000a) compared the disease occurrence in suppressive and conducive soils with or without sterilization and found the suppressive effect of the soil biota. It was demonstrated, in the present study, that soil biota was also involved in the disease suppression in the soils to which a large amount of organic matter had been incorporated. However, the suppressive effect was observed only under low pH, and it is suggested that soil biota may play a certain role in the disease suppression, but the effect is under the influence of soil pH.

4.3. Reproducibility of plug pot bioassay

Various environmental factors such as soil moisture (Dobson et al., 1982) and temperature (Gabrielson and Robak, 1988) affect the occurrence of clubroot disease, and it is very difficult to conduct different sets of experiment under equal conditions even in greenhouse pot experiments. The establishment of a reproducible experimental system under controlled environmental conditions, therefore, was essential to clarify the mechanism of the disease suppression. The reproducibility of the plug pot bioassay

is high enough to obtain the same results in different sets of experiments. However, it does not reproduce absolutely 'the same number of root hair infections' in each set of experiments e.g. the number of root hair infections in the conventional soil shown in Fig. 2 are different between (A) and (B). We consider that this is due to difference in the infection potential among the different inocula (the resting spores prepared from the different clubroot galls), which is most difficult to control. The potential (viability) of the resting spores may be under the influence of gall maturity, bacterial and fungal contamination in the gall, the length of storage of the gall in a freezer and other unknown factors. One might suggest a possibility to control the potential by the vital staining techniques (e.g. the assessment of esterase activity with fluorescent diacetate). However, we believe that the most reliable method to assess the potential is a bioassay such as the plug pot system, and it will be very important to equalize the potential by a preliminary bioassay if different lots of inoculum are to be used in the same series of experiment.

4.4. Conclusion

In the present study, it was revealed by the plug pot bioassay that an increase in soil

pH due to the enrichment of Ca was the primary cause for the suppression of clubroot

disease by organic matter application. We conclude that Ca-rich organic waste might

be useful for controlling the disease. The effectiveness will be improved by applying in

a particle size less than 5 mm. It is important that the amount of organic matter should

be enough to neutralize the soil but less than the levels which cause nitrogen-leaching

into underground water. So far, little is known about the mechanism of the suppression

of clubroot disease in high pH soil. Webster and Dixon (1991) suggested that lime

application did not eradicate the pathogen directly but might create unfavorable

conditions affecting processes such as invasion, colonization and symptom formation.

In this study, it was clearly demonstrated that the increase in soil pH suppressed the

root hair infection, and these results suggest that the neutralization of soil affects some

process in the primary phase of the life cycle i.e. the germination or the root hair

infection process. Experiments looking into the mechanisms of suppression for

primary infection are ongoing.

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Figure captions

Fig. 1. Bioassay of root hair infection of *P. brassicae* on *B. campestris* var. rapifera in the plug pot system. The conventional, farm yard manure (FYM)- and food factor sludge compost (FSC)-soils were mixed with the resting spores of *P. brassicae* at 10^6 spore g^{-1} soil, and *B. campestris* was grown in a growth chamber for 8 days. The number of zoosporangial clusters of *P. brassicae* formed in the root hairs was counted under a microscope after staining with aniline blue. The vertical bars indicate standard deviation (*n*=3).

Fig. 2. Effect of organic matter application on the number of root hair infections of *P. brassicae* on *B. campestris* var. rapifera in the conventional soil as revealed by the plug pot bioassay. **A**, the farm yard manure (FYM) was mixed with the conventional soil at 2.5% (w/w), and the FYM-soil was used as a positive control. **B**, the food factory sludge compost (FSC) was mixed with the conventional soil at 2.5% (w/w), and the FSC-soil was used as a positive control. N/A, not any. The vertical bars indicate standard deviation (*n*=3). The columns with the different letter are significantly different (Turkey-Kramer's multiple range test, *P*<0.05).

Fig. 3. Influence of the particle size of food factory sludge compost (FSC) on the number of root hair infections of *P. brassicae* on *B. campestris* var. rapifera in the conventional soil as revealed by the plug pot bioassay. The FSC were fractionated to the particles larger than 5 mm, 1-5 mm and smaller than 1 mm and mixed with the conventional soil at 2.5% (w/w). Soil pH before and after cultivation is shown. The vertical bars indicate standard deviation (*n*=3). The columns with the different letter are significantly different (Turkey-Kramer's multiple range test, *P*<0.05).

Fig. 4. Influence of soil pH and exchangeable Ca levels on the root hair infection of *P. brassicae* on *B. campestris* var. rapifera as revealed by the plug pot bioassay. Various chemicals were incorporated to control soil pH and Ca levels in the conventional, farmyard manure (FYM)- and food factory sludge compost (FSC)-soils. N/A, not any. Soil pH before and after cultivation is shown. The vertical bars indicate standard deviation (*n*=3). The columns with the different letter are significantly different (Turkey-Kramer's multiple range test, *P*<0.05).

Fig. 5. Effect of γ-ray sterilization and soil pH on the root hair infection of *P. brassicae*

on *B. campestris* var. rapifera as revealed by the plug pot bioassay. The control soil

received the filtrate of soil-water suspension of the non-sterilized soil after the γ-ray

irradiation. The soil pHs of the farm yard manure (FYM)- and food factory sludge

compost (FSC)-soils was adjusted with 1M H_2SO_4 or $Ca(OH)_2$. The columns with the

different letter are significantly different (Turkey-Kramer's multiple range test, *P*<0.05).

Table 1

Chemical properties of farmyard manure (FYM) and food factory sludge compost (FSC).

	FYM	FSC
Water content (g kg^{-1})	358	198
pH (1:10H$_2$O)	9.8	7.3
EC (1:10H$_2$O) (dS m^{-1})	8.85	5.68
Total C (g kg^{-1})	298	325
Total N (g kg^{-1})	25.0	46.0
C/N ratio	11.8	7.1
Total P (g kg^{-1})	58.1	54.1
Total K (g kg^{-1})	90.0	17.1
Total Ca (g kg^{-1})	58.3	91.1
Total Mg (g kg^{-1})	27.1	10.7

Table 2

Percentages of the disease incidence[a] in the experimental field of Nagoya University.

Plots	Year						
	1997	1998	1999	2000	2001	2002	2003
Conventional	40	85	80	75	90	80	90
FYM	0	0	0	0	10	0	5
FSC	0	0	0	0	0	0	0

[a]Twenty plants were randomly chosen from each plot. FYM, farmyard manure: FSC, food factory sludge compost.

Table 3

Chemical properties of the conventional, FYM- and FSC-soils collected from the experimental field of Nagoya University.

	Plots		
	Conventional	FYM	FSC
pH (H_2O)	5.7	7.4	7.2
Electric conductivity (dS m^{-1})	0.074	0.431	0.183
Total C (g kg^{-1})	23.4	127.7	56.7
Total N (g kg^{-1})	2.5	13.5	9.0
Total P (g P_2O_5 kg^{-1})	5.4	23.7	16.6
Available P (Olsen) (g P_2O_5 kg^{-1})	0.94	2.81	1.65
Total K (g K_2O kg^{-1})	1.7	2.8	1.5
Cation exchange capacity (cmol$_c$ kg^{-1})	13.0	44.5	34.6
Exchangeable Ca^{2+} (cmol$_c$ kg^{-1})	6.5	50.1	36.9
Exchangeable Mg^{2+} (cmol$_c$ kg^{-1})	1.5	15.5	2.5
Exchangeable K^+ (cmol$_c$ kg^{-1})	2.2	21.8	2.9

FYM, farmyard manure: FSC, food factory sludge compost.

Table 4

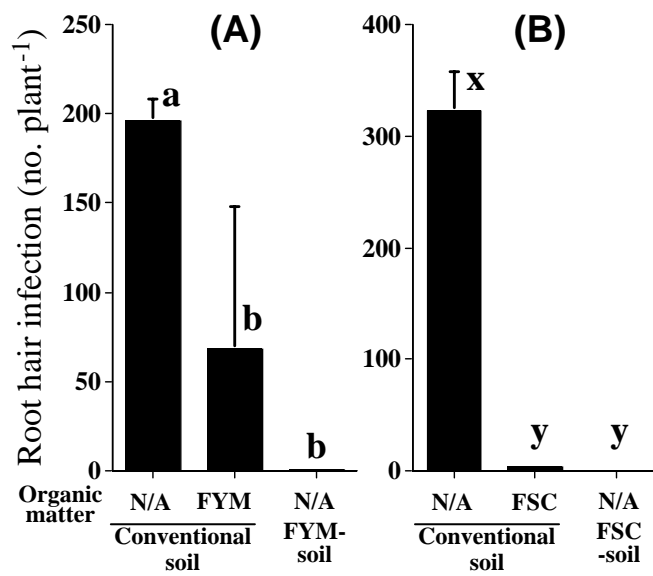Phenolic acids in the soils of the experimental field of Nagoya University.

	Plots		
	Conventional	FYM	FSC
		(mg kg^{-1} soil)	
Gentisic acid	n.d.[a]	n.d.	n.d.
p-Hydroxy benzoic acid	4.8 ± 3.0^{ns}	5.8 ± 2.6^{ns}	3.9 ± 2.5^{ns}
Syringic acid	3.3 ± 2.0^{ns}	4.6 ± 1.5^{ns}	2.0 ± 1.6^{ns}
Vanillic acid	4.4 ± 2.5^{ns}	5.8 ± 2.2^{ns}	2.9 ± 2.0^{ns}
p-Coumaric acid	24.0 ± 3.0 b[b]	47.0 ± 2.6 a	8.4 ± 1.4 c

[a] not detected.

[b]The different letter indicates significant difference among the treatments (Turkey-Kramer's multiple range test, $P<0.05$).

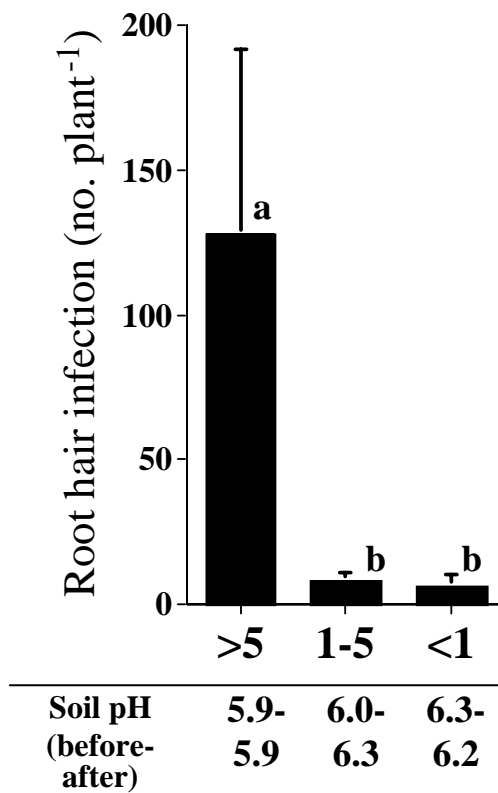FYM, farmyard manure: FSC, food factory sludge compost.
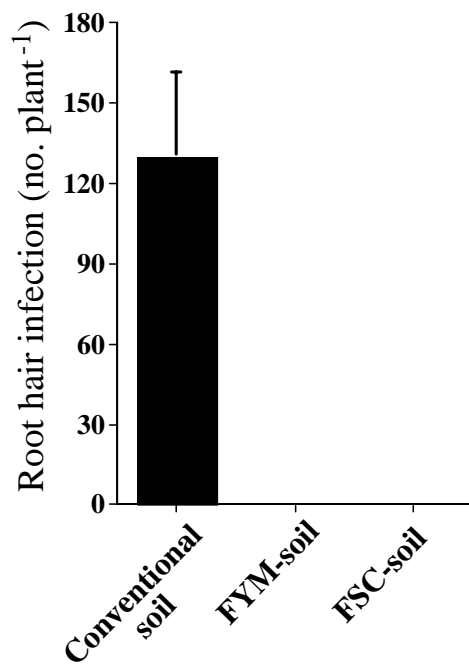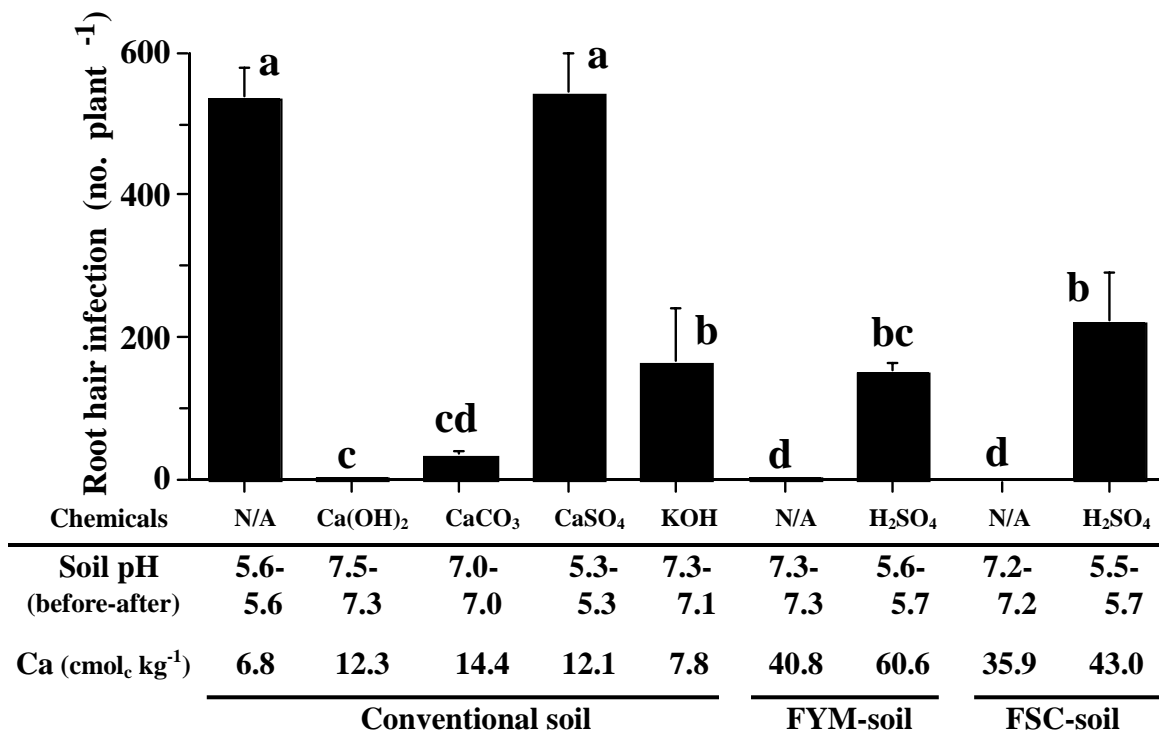
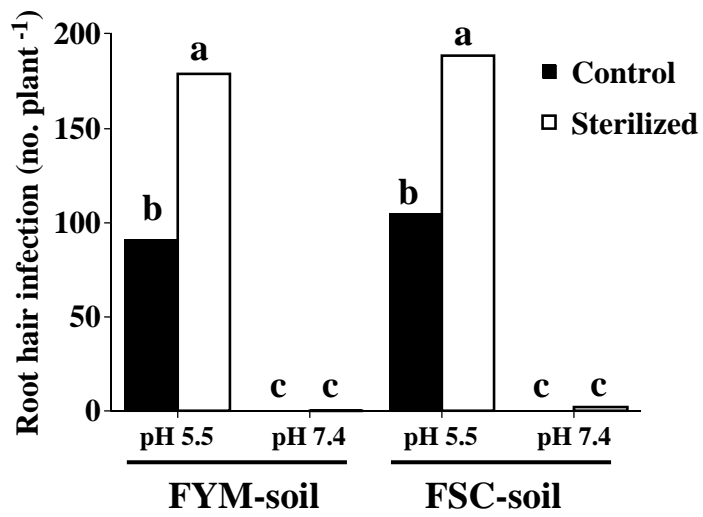Fig. 1.
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Fig. 2.
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Fig. 3.
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Chemicals	N/A	Ca(OH)$_2$	CaCO$_3$	CaSO$_4$	KOH	N/A	H$_2$SO$_4$	N/A	H$_2$SO$_4$
Soil pH (before-after)	5.6-5.6	7.5-7.3	7.0-7.0	5.3-5.3	7.3-7.1	7.3-7.3	5.6-5.7	7.2-7.2	5.5-5.7
Ca (cmol$_c$ kg^{-1})	6.8	12.3	14.4	12.1	7.8	40.8	60.6	35.9	43.0
			Conventional soil			FYM-soil		FSC-soil	

Fig. 4.

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Fig. 5.
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