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Note

Stimulation by Caffeic Acid, Coumalic Acid, and Corilagin of the Germination of Resting Spores of the Clubroot Pathogen *Plasmodiophora brassicae*

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Some chemicals were examined for their effects on the germination of resting spores of the clubroot pathogen *Plasmodiophora brassicae*, and on the control of clubroots in Chinese cabbage. Caffeic acid, coumalic acid, and corilagin stimulated the germination of *Plasmodiophora* spores and prevented the formation of clubroots in Chinese cabbage. Clubroot might be controlled by agents with germination-stimulating effects.

Key words: germination; *Plasmodiophora brassicae*; caffeic acid; coumalic acid; corilagin

Clubroot caused by *Plasmodiophora brassicae* Woronin is one of the most intractable diseases of cultivated cruciferous crops in the world.[1)] Recently, Wallenhammar attributed the decreasing yield of spring oilseed rape in central Sweden to infection with *P. brassicae*.[2)] Clubroot caused by *P. brassicae* is a serious disease of crucifers in Japan also, and an important factor of the death of cole crops.[1)] In Nagano Prefecture, a central area of successive cropping of Chinese cabbage (*Brassica campestris* L. pekinensis group), there are many problems related to clubroot.

Because of the spontaneous germination of *P. brassicae* resting spores and the short-lived character of the primary zoospores without hosts, rotation of susceptible crops with noncruciferous crops could control clubroots (S. Horiuchi, personal communication). However, the successive cropping of cruciferous crops has caused difficulties control of this disease.

Campbell was the first to report that pentachloronitrobenzene incorporated into the soil controls clubroot in transplanted cauliflower.[3)] The fungicides flusulfamide[1)] and fluazinam[4)] have been used for the control of clubroot and biological control agents also have been studied. Narisawa *et al.*[5)] found an clubroot-suppressing effect in an endophytic fungus, *Heteroconium chaetospira*, which inhabits the root cortex. Arie *et al.*[6)] reported that epoxydon isolated from *Phoma glomerata* (Corda) Wollenweber *et* Hochapfel, an anti-auxin, reduces clubroot symptoms in Chinese cabbage.

Suzuki *et al.* reported the existence of a factor that stimulates the germination of resting spores in the root exudate of turnip (*B. campestris* subsp. *rapa* rapifera group) and lettuce (*Lactuca sativa* L.).[7)] We have reported that a dry powder or an extract from the leaves of Posidonia (*Posidonia australis* Hook. F.) stimulate the germination of resting spores, and suppress the formation of clubroots in Chinese cabbage; for example, infected soil can be treated with 5% (w/w) dry Posidonia powder 7 days before planting.[8)] These results suggest that GSF exist not only in crucifers but also in other kinds of plants. In this study, we examined the effects of some chemicals commonly found in plants on the germination of *P. brassicae* resting spores.

The extract of *Posidonia oceanica* (L.) Delile contains phenolic compounds such as chicoric acid that may activate the growth of *Staphylococcus aureus* Rosenbach[9)] and inhibits HIV integrase.[10)] Cariello and Zanetti[11)] showed that the content of chicoric acid in *P. oceanica* is reduced in older and dead leaves, and suggested that caffeic acid may be the final product of the chicoric acid metabolism. Therefore, in this study, we examined the effects of caffeic acid on the germination of *P. brassicae* resting spores. Because the GSF may be adsorbed on to the artificial resins such as HP20 or XAD4,[7,8)] other chemicals having such characteristics also were examined.

If the germination of the resting spores could be stimulated synchronously by the application of these agents, we may be able to develop a new biological method for the control of clubroot. The purpose of this study was to find GSF and examine their clubroot-control effects.

Effects of caffeic acid, coumalic acid, and corilagin on germination of resting spores of Plasmodiophora brassicae. Clubroots on the cultivar Muso of Chinese cabbage (Takii Seeds) plants were obtained from a Nobeyama field in Nagano Prefecture, Japan, an

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Abbreviation: GSF, germination-stimulating factor

area severely infected with the clubroot pathogen *P. brassicae*. The clubroots were washed in tap water and stored at −20°C until use. Resting spores of *P. brassicae* were obtained from the clubroots of Chinese cabbage by the method of Williams.[12] The suspension of spores was adjusted to 1×10^7/ml, 1 mM caffeic acid, coumalic acid, or corilagin was added to the suspension, and spores were incubated at 25°C for 1 day or 7 days in the dark. Caffeic acid and coumalic acid were dissolved in 0.1 M ammonia and the dilutions were used in all experiments. A drop of the suspension was placed on a glass slide and stained with Fluorescent Brightner 28 (Sigma). One hundred spores were counted three times under UV excitation with a fluorescent microscope. The stage of germination was evaluated as described by Hata *et al*.[8] Stages were 0 for the presence of a developed cell wall, 1 for the presence of both a cell wall and germination cave, 2 for the disappearance of the cell wall and clear development of a germination cave, and 3 for the disappearance of both the cell wall and germination cave, and the appearance of dark red pigment. The experiment was replicated five times.

The stained cell-wall layer of the ungerminated resting spores showed intense light-blue fluorescence (germination stage 0). The germination stage of resting spores without any treatment remained 0 without exception (Table 1). In the treatment with 1 mM caffeic acid, 25% of the spores proceeded to stage 1 or 2 after the treatment for 1 day, and after treatment for 7 days, most of the spores proceeded to stage 1 or 2. Not only caffeic acid but also 1 mM coumalic acid and 1 mM corilagin stimulated the germination of resting spores. In the treatment with coumalic acid, 63% of the spores proceeded to more than the stage 1, with corilagin, all of the spores reached stage 2.

Table 1. Effects of Some Chemicals on the Germination of Resting Spores of *Plasmodiophora brassicae*

Chemical	Days of incubation	Frequency distribution (%) of spores with different stages of germination			
		0*	1	2	3
1 mM Caffeic acid	7	11	65	24	1
	1	75	24	1	0
1 mM Coumalic acid	7	37	18	9	36
1 mM Corilagin	7	0	0	100	0
Not added	7	100	0	0	0

* The germinated and ungerminated spores were directly counted under UV excitation with a fluorescence microscope. The stages of germination were as follows: 0, presence of developed cell walls; 1, presence of both cell wall and germination cave; 2, disappearance of cell wall and clear development of germination cave; 3, disappearance of both cell wall and germination cave, and dark red inside.

Effects of caffeic acid, coumalic acid, and corilagin on the clubroot formation in Chinese cabbage seedlings. A soil mixture of sterilized loam soil and sand (1:1, v/v; pH 5.8) was corrected to pH 6.5 with calcium hydroxide, mixed with the above-mentioned suspension of the spores (1×10^4/g soil), and incubated for 7 days with 1 mM caffeic acid, 1 mM coumalic acid, 1 mM corilagin, or no addition. Fifteen seeds of the Muso cultivar of Chinese cabbage were sown in infested soil in 10-cm Jiffy pots. The germinated seedlings were thinned to 10 plants per pot after 10 days. The plants were grown at 25°C under a 16-h photoperiod of 3,000 lx. The plants were fertilized weekly with 15-15-18 ($N:P_2O_5:K_2O$) soluble fertilizer (4 g/l, 40 ml/pot) beginning 2 weeks after sowing. Sodium molybdate (0.25 g/l, 40 ml/pot) was applied only at the time of the first fertilizing. On day 35 after sowing, the plants were harvested and the number, length, and fresh weight of leaves were recorded. Then the soil was washed out from the roots and the weight and length of the roots and the severity of the clubroot were examined. The degree of clubroot formation was recorded on a scale of 0–3 as described by Buczacki and Moxham:[13] 0 for no clubroots; 1 for slight swelling on taproot, lateral root, or both; 2 for moderate swelling on taproot, lateral root, or both; and 3 for severe swelling on taproot, lateral root, or both. This experiment was replicated four times.

The degree of formation of clubroots in the soil infested with the spore suspension treated with caffeic acid, coumalic acid, or corilagin was 0, but that in the soil infested with the spore suspension without any treatment was 2.8 (Table 2). No undesired effects of these agents on the number of leaves and their

Table 2. Effects of Caffeic Acid, Coumalic Acid, and Corilagin on the Growth of Chinese Cabbage and on Clubroot Disease When Grown in Soil Infested with Resting Spores of *Plasmodiophora brassicae*

Chemical	Number of leaves	Length of leaves (cm)	Weight of leaves (g)	Length of roots (cm)	Weight of roots (g)	Degree of formation of clubroot
1 mM caffeic acid	8.00±1.00*	10.50±0.58	2.90±0.82	16.28±3.87	0.20±0.08	0.0
1 mM coumalic acid	8.00±0.71	9.92±1.01	2.94±1.77	13.52±3.39	0.20±0.06	0.0
1 mM corilagin	8.20±1.64	10.7±2.39	3.03±0.82	11.52±3.91	0.26±0.05	0.0
Not added	8.20±0.84	9.56±1.27	2.10±0.95	10.86±3.68	0.36±0.12	2.8

The Muso cultivar was used.

* Values are means and standard deviations (n = 10).

length and weight were observed. However, roots treated with these agents were longer and lighter than those without treatment. In untreated plants, clubroot formed.

Effects of incubation period of resting spores with caffeic acid on infection rate of root hairs and clubroot formation in Chinese cabbage seedlings. Resting spores (1×10^7/ml) with or without caffeic acid (0.1 or 1 mM) were incubated for 1 day or 10 days. Then a suspension of the spores was mixed with the above-mentioned soil mixture (1×10^4 spores/g soil, pH 6.5). Twenty seeds of the Muso cultivar of Chinese cabbage were sown and the germinated seedlings were thinned to 10 plants per ward after 14 days. The plants were grown as described above. The roots of 5 thinned plants were stained with 50 mg/ml Cotton Blue of 5% phenol solution for 24 h and the root hairs of 1 cm of taproot just under the hypocotyl stem were observed. By the counting of blue-stained primary plasmodia and zoospore systerna, the infection rate of root hairs was estimated. On day 35 after sowing, the plants were harvested and the clubroot severity was recorded as described above. This experiment was replicated twice.

The infection rate of root hairs in a one-day incubation of resting spores with 1 mM caffeic acid was higher than that in the control, but in 10 days of incubation with 0.1 or 1 mM caffeic acid, the infection rate was less than in the control (Table 3). The degrees of clubroot severity with 1 day of incubation with 1 mM caffeic acid and in the control were 3.0, but with 10 days of incubation with 0.1 or 1 mM caffeic acid, the degree of the clubroot severity was 0.0.

We previously showed that a Posidonia extract stimulated the germination of spores and the active component was adsorbed to resin HP-20.[8] Suzuki *et al.* also reported that the extract of crucifers contained a GSF, which is adsorbed by XAD-4.[7] Thus, the GSF seems to be ubiquitous in some plants. We found caffeic acid, coumalic acid, and corilagin, which significantly stimulate germination of spores of *P. brassicae* and prevent the formation of clubroots in Chinese cabbage.

We also previously showed that treating the infected soil with 5 w/w% dry Posidonia powder 7 days before planting reduces clubroot formation in Chinese cabbage, but that treatment on the day of planting results in greater clubroot formation than in the control.[8] Here, the infection rate of root hairs in 1 day of incubation of resting spores with 1 mM caffeic acid was higher than the control without treatment, but the rates with 10 days of incubation were lower than in the control. These results suggested that the timing of treatment of infected soil or incubation of the resting spores with GSFs is important for prevention of infection by the clubroot pathogen.

Table 3. Effects of Incubation Period of *Plasmodiophora brassicae* Resting Spores with Caffeic Acid on Infection Rate of Root Hairs and Formation of Clubroots in Chinese Cabbage

Concentration of caffeic acid, mM	Days of incubation	Infection rate (%) of root hairs	Degree of formation of clubroot
0.1	10	6.4c*	0.0
1	10	3.2c	0.0
1	1	70.1a	3.0
Not added	10	48.8b	3.0

* $P < 0.05$, Duncan's multiple range test ($n = 5$). No significant difference between groups with a same letter.

Throughout the life cycle of the pathogen, the resting spore and primary zoospore are the only stages that seem to be independent of host tissue. It is not clear whether secondary zoospores are released outside of the host tissue.[7] The resting spores of the clubroot pathogen are produced in the clubroots and can survive several years in the soil. Primary zoospores released after germination of the resting spores are the primary source of infection and apparently are short-lived without a host and are sensitive to fungicidal agents. Through stimulation of germination of the resting spores and because the primary zoospores are short-lived without host, Posidonia powder, caffeic acid, coumalic acid, and corilagin probably prevent the formation of clubroots in Chinese cabbage.

The practical application of the agents such as coumalic acid and caffeic acid in the field may be difficult. However, improvement in the application method such as combined application with other substances that control clubroot by other mechanisms may be effective.

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