



## Infection of *Plasmodiophora brassicae* in Chinese cabbage

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**ABSTRACT.** *Brassica* crops infected by *Plasmodiophora brassicae* can produce root galls (clubroots) and be prevented from growing normally. To understand the series of changes that occur in the host root during root gall production, the resistance character of 21 Chinese cabbage lines were identified and then resistant and susceptible lines were used for infection observation. Hydroponic technology system was used for plants growing, and the infection process of *P. brassicae* in the roots of resistant and susceptible Chinese cabbage was examined based on morphology and microscopic characteristics using microscope. In susceptible Chinese cabbage, the root hair infection stage occurred over approximately 7 days after inoculation, the cortical infection happened over approximately 14 days after inoculation, and clubroots formed in approximately 30 days after inoculation. However, in resistant Chinese cabbage, the pathogen could be prevented and maintained in the root hair infection stage. This research provides a foundation for the subsequent studies of cabbage resistance of *P. brassicae*.

**Key words:** Chinese cabbage; Hydroponic system; Infection progress; *Plasmodiophora brassicae*; Resistant lines; Susceptible lines

## INTRODUCTION

Clubroot of crucifers, caused by the obligate parasite *Plasmodiophora brassicae*, is characterized by the formation of conspicuous root galls. It was first discovered in Russia in 1878 and named *P. brassicae* (Voronin, 1878). Clubroot is a fungal disease present on cultivated and wild cruciferous plants. This disease was identified in the Mediterranean and southern Europe and is now widely distributed worldwide; the problem is particularly serious in temperate zones. Clubroot recently became common in most provinces and autonomous regions in China (Yang, 1990).

The life cycle of *P. brassicae* consists of 3 phases: survival in the soil, root hair infection, and cortical infection (Kageyama and Asano, 2009), but the effects in Chinese cabbage infected with *P. brassicae* are unclear, including the types of reactions and the lengths of different infection phases. In this study, we examined the morphological and internal changes of Chinese cabbage root during infection by *P. brassicae*. Changes in the root regarding gall development at different stages were examined using light microscopy and histological analysis. Our results may be useful for studies aimed at overcoming clubroot resistance in Chinese cabbage.

## MATERIAL AND METHODS

### Plant materials

The seeds of 21 lines of Chinese cabbage were kindly provided by the Liaoning Provincial Key Laboratory of Genetics and Breeding of Cruciferous Vegetable Crops, Shenyang. These lines are used to carry out resistant studies in the horticulture greenhouse of Shenyang Agriculture University.

### Preparation of spore suspension

Clubroots were collected from the test fields of Shenyang Agriculture University, cleaned in running tap water, and stored at -20°C. Resting spores were extracted as described by Castlebury et al. (1994) with some modifications. Mature root galls of Chinese cabbage were homogenized using a juice extractor (Joyoung, Ji'Nan, China). The homogenate was filtered through 8 layers of gauze and the filtrate was clarified by centrifugation 2 times (Biofuge Stratos, Thermo Scientific, Waltham, MA, USA) at 3000 rpm for 9 min and 4000 rpm for 12 min. The resting spores precipitate was resuspended in sterile water, adjusted to a concentration of  $1 \times 10^7$  spores/mL, and stored in 4°C.

### Resistance of different Chinese cabbage lines

For inoculation with soil bacteria, the clubroots collected from severely disease-affected areas in Shenyang were prepared as spore suspension. The spore suspension was mixed with turf to a final concentration of  $1.0 \times 10^7$  spores/g turf. The mixture was decayed over 2 days and then used as inoculated soils. Seeds of Chinese cabbage were surface-sterilized with 70% ethanol for 1 min and 10% hydrogen peroxide for 1 h, followed by rinsing 3 times in sterile distilled water (Asano et al., 1999). Surface-sterilized seeds were incubated for 1 day in the dark at 25°C. The seedlings were planted in the decayed inoculated soil and grown in

the greenhouse at 20°-25°C. The roots were collected approximately 4 weeks after inoculation and washed thoroughly under running tap water to remove soil particles. Next, the incidence of disease was examined.

### **Hydroponic growth of Chinese cabbage**

The seeds of resistant and susceptible lines were placed on Petri dish to accelerate germination for 3-4 days in a plant growth chamber (MLR-30H, Sanyo Biomedical, Osaka, Japan) at 25°C under a light-dark cycle of 16:8 h. The germinations were then transferred into the centrifuge tubes containing water and incubated for 4-5 days. When the first euphylla was available, the plants were transferred into Hoagland solution with a circulating system for which the solutions were changed once every 6-7 days to compensate for the nutrient uptake of the plants.

### **Cultivation and inoculation of plants**

Cultivation conditions and inoculation of plants were performed as follows. For cultivation, the concentration of Hoagland solution, pH 6.0, was used at 1X, 1/2X, 1/4X, and 1/8X concentrations. The optimal concentration was identified and used for cultivation. After cultivation for 3 days in a controlled environment (25°C, 16-h light, 70% humidity), plants were inoculated by adding 4 mL resting spore suspension in 1400 mL cultivate solution to a standard concentration of 10<sup>7</sup> spores/mL. Control plants were not treated with spores. Plants were harvested at designated times and used for the experiments.

### **Microscopic observation**

After inoculation, 5 seedlings from each line were randomly selected every 24 h and cleaned with tap water containing 0.05% Tween 80 for approximately 20 min. The taproot starting from hypocotyl was cut into 1-cm sections, used to prepare a squash slide, and the hair infection process was observed under a microscope (ECIPSE 80i, Nikon, Tokyo, Japan). Because of the difficulty in preparing the root on the 30th day, a frozen section was selected to prepare the squash slide. The root was cut into 1-cm sections and then embedded with Tissue Freezing Medium (CMI850UV-1-1, Leica, Wetzlar, Germany), cut into 10-µm thin sections, and observed under the microscope.

## **RESULTS**

### **Selection of clubroot-resistant and clubroot-susceptible lines of Chinese cabbage**

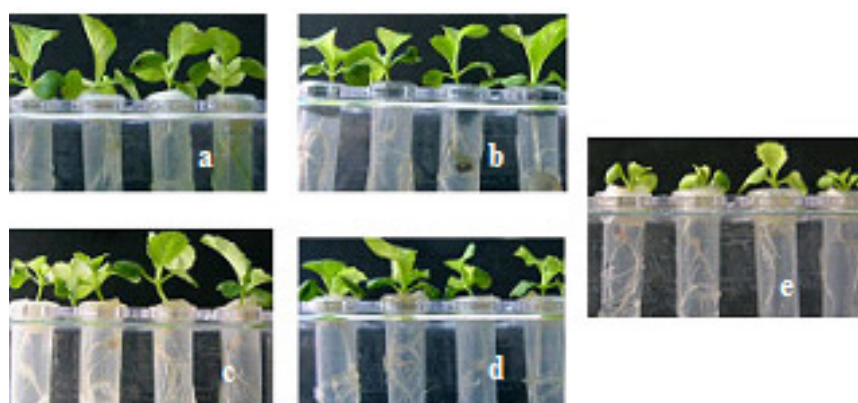
Resistant lines were identified using inoculated soil in the greenhouse in February 2013. The results showed that infection rates of lines Sy742, Sy179, and Sy210 were 0%, and thus were identified as clubroot-resistant lines; the infected rate of line Sy89, Sy112, Sy134, Sy143, Sy149, Sy166, Sy157, Sy191, and Sy174-2 were 100%, and thus these lines were considered to be clubroot-susceptible; other lines were considered to be disease-tolerant (Table 1).

**Table 1.** Disease resistance investigation of different Chinese cabbage lines.

No.	Lines	Infected	Total	Infected rate (%)
1	Sy742	0	19	0
2	Sy179	0	19	0
3	Sy210	0	19	0
4	Sy100	6	18	33.3
5	Sy211	8	18	44.4
6	Sy129	13	33	56.5
7	Sy145	14	23	60.8
8	Sy124	16	23	69.6
9	Sy189	15	20	75.0
10	Sy092	9	11	81.8
11	Sy186	15	17	88.2
12	Sy209	16	18	88.9
13	Sy089	14	14	100
14	Sy112	16	16	100
15	Sy134	23	23	100
16	Sy143	19	19	100
17	Sy149	21	21	100
18	Sy157	20	20	100
19	Sy166	14	14	100
20	Sy191	20	20	100
21	Sy742-2	20	20	100

### Optimal concentration of Hoagland nutrient solution for Chinese cabbage

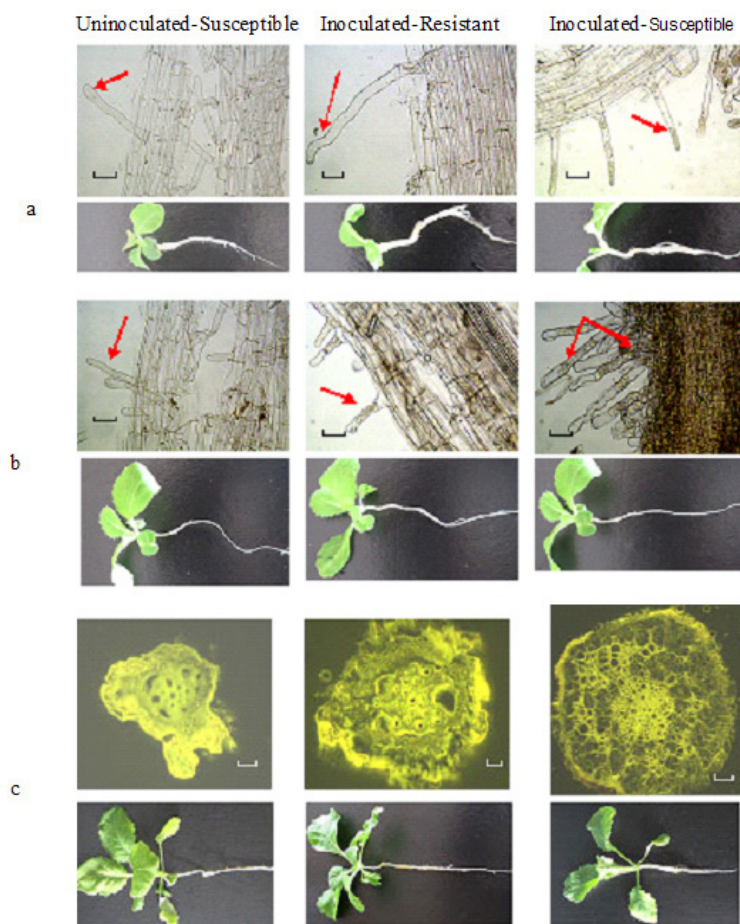
After determining germination and seedling survival in distilled water, Chinese cabbage seeds were cultivated in Hoagland nutrient solution at different concentrations of 1X, 1/2X, 1/4X, and 1/8X (Figure 1a-d, respectively), while water solution was used as the control (Figure 1e). Plants on the 10th day are shown in Figure 1. All plants cultivated in Hoagland nutrient solution at different concentrations grew better than those in water and showed greater blooming. Therefore, 1X and 1/2X Hoagland nutrient solution were used to cultivate Chinese cabbage. However, the roots cultivated in 1X solution turned green (Figure 1a). Thus, the 1/2x Hoagland nutrient solution was the most suitable for Chinese cabbage.



**Figure 1.** Chinese cabbage of the 10th day cultivated with different concentrations of Hoagland solution. a.-d. The concentrations of Hoagland solution are respectively 1X, 1/2X, 1/4X, and 1/8X. e. Water as control.

### Infection of Chinese cabbage with *P. brassicae*

The progress of root infection and clubroot formation was analyzed by hydroponic inoculation identification combined with microscopic observation. On the 7th day after inoculation, no difference was observed in external morphology among inoculated resistant and susceptible roots and non-inoculated susceptible roots, but microscopic observation revealed that the root hair of susceptible plants had begun to be infected (Figure 2a). On the 14th day after inoculation, no difference was observed in external morphology among the 3 type of roots; however, microscopic observation showed root hair of disease-resistant root in the protoplasm stage. Furthermore, the epidermis protoplasm of disease-susceptible roots had been infected (Figure 2b). On 25-30 days after inoculation, the gall was visible in roots of disease-susceptible lines, and the transverse sections of the roots were broken and were easily sliced, while the roots of non-inoculated and disease-resistant lines were hard and difficult to cut (Figure 2c).



**Figure 2.** External morphology and microscopic observation of Cabbage roots after inoculating with *Plasmodiophora brassicae*. **a.** The 7th day after inoculation. **b.** The 14th day after inoculation. **c.** The 30th day after inoculation. Scale bars = 50  $\mu$ m.



## DISCUSSION

Most previous studies of hydroponic nutrient solution used the Hoagland nutrient solution method, organic fertilizer, and the other specifically configured nutrient solutions. Hoagland nutrient solution has been widely applied, but the concentrations used in studies varied according to different plants and cultivation purposes (Li et al., 2007; Zhao et al., 2007). In this study, we found that all plants cultivated in Hoagland nutrient solution had bright, dark green leaves, which were superior to control plants (Figure 1a-d). Chinese cabbage cultured in Hoagland solution grew better than in water potentially because it contained relatively complete level of nutrients, allowing the plant growth normally, contributing to plant metabolism, and enhancing photosynthesis (Pan and Dong, 1999). The only study of Chinese cabbage hydroponic technology was reported for chromium stress, in which Hoagland nutrient solution was used (Wang et al., 2013); however, the concentration was not described in detail. By optimizing the nutrient solution concentration, we found that 1/2x Hoagland solution met the demands of Chinese cabbage growth (Figure 1b).

During the life cycle of the *P. brassicae*, pathogens can live through the winter and summer in soil or animal manure with non-rotted diseased plants. Under appropriate conditions, these resting spores release primary zoospores; when a zoospore swims to the surface of a root hair, it penetrates through the cell wall. This stage is termed the root hair infection stage, and inside root hairs, the pathogen forms primary plasmodia (Agrios, 2005). As reported previously, sporangia are produced 4 days after inoculation (Kageyama and Asano, 2009). In this study, we found that primary plasma inside the root hair of susceptible lines began to multiply, increasing gradually, and more than a quarter of the lines were occupied on the 7th day after inoculation (Figure 2a); Thus, this stage was referred to as the root hair infection stage, similarly to the results of Agrios (2005). Kageyama and Asano (2009) reported that at the root hair infection stage, there were no visible symptoms, which was confirmed in this study (Figure 2b). After secondary zoospores were released, the secondary plasmodia contained 2 nuclei in the early stages of growth, which developed into multinuclear plasmodia during the cortical infection or secondary infection stage (Kageyama and Asano, 2009). We found that 14 days after inoculation, the stage varied gradually from root hair infection to cortex infection, but no external differences were visible (Figure 2b). These plasmodia undergo a series of cell divisions and eventually form multinucleate secondary plasmodia that invade cells in the cortex and stele. Both infected and neighboring uninfected cells undergo extreme hyperplasia (cell division) and hypertrophy (cell expansion), leading to the formation of characteristic galls at 30 days (Figure 2c); these results agree with those of Malinowski et al. (2012) in normal cells, and greater rates of cell division were evident in infected plants on 16, 22, and 26 days after inoculation. Cell division had largely ceased by 32 days after inoculation, while cell division in the hypocotyls of infected plants appeared as a diffuse ring in the vascular cambium (VC) and neighboring progeny cells. Therefore, we clearly determined the life cycle of *P. brassicae*, the dynamic changes of Chinese cabbage roots, and specified the time of each life cycle stage of *P. brassicae* infection in Chinese cabbage. Our results may provide strategies for improving plant tolerance to pathogens. In addition, we found that the disease-resistance preventing pathogen damage mainly occurred during the second stage of cortical infection.

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