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Occurrence of Non-infectious Phyllody Disease of Strawberry in Thailand

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ABSTRACT

Strawberries showing phyllody symptom on fruits has been observed in No.50 cultivar grown in northern region of Thailand since 1996. The symptomatic strawberries produce very poor fruit quality and unmarketable. The disease was epidemic in many strawberry growing areas. The abnormality symptom is similar to the green petal disease which caused by phytoplasma. Several methods including electron microscopy, serological methods, and PCR were used to detect phytoplasma in symptomatic strawberries. All methods gave negative results and phytoplasma could not be detected. In addition, the disease was proved not transmitted to healthy strawberry and periwinkle by grafting and dodder transmission. Evidently, this phyllody disease of strawberry is not caused by phytoplasma. It is possibly caused by physiological disorder and is called non-infectious phyllody disease that was previously found in strawberry in Italy.

Key words: non-infectious phyllody, strawberry

INTRODUCTION

Strawberry (*Fragaria* spp.) has been cultivated in the North of Thailand for many years, especially in Chiang Mai and Chiang Rai. Initially, it was used as the replacement crop for opium cultivation for the hill tribe people in the north. The project was carried out in 1974-1979 by Royal Project and Kasetsart University (Punsri *et al*., 1978). Now, strawberry has become an economic crop with the increasing demand for both domestic consumption and exporting. Japan is a major market for importing of processed strawberry from Thailand. However, current problem of strawberry production in Thailand is the unavailability of high quality planting-materials which are free of diseases and insects. Farmers have used the old stocks of uncertified runners every year and this leads to the accumulation of diseases and insects, particularly in predominant Tioga cultivar. Thus, yield obtained from these runners is very low. Farmers have to use more chemicals to control both diseases and insects. To get a better yield, new cultivars have been introduced and recommended to farmers, such as

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Dover, No.50, Toyonoka, and Selva. These new cultivars replacing Tioga, especially No.50 cultivar grown in Mae Rim and Doi AngKhang, showed symptoms similar to those caused by phytoplasma (Breakbane *et al*., 1971; Converse *et al*., 1988). The symptoms are foliaceous growth arising from the achenes and phylloid fruits. Symptomatic fruits were unmarketable. This disease was found increasingly in many areas of strawberry fields during the growing season. It may be a limiting factor that causes serious problem for strawberry cultivation and becomes epidemic throughout the growing areas.

According to the typically reported phyllody symptom, phytoplasma has been implicated as the etiological agent of this disease found in strawberry. In this study, we describe several methods including electron microscopy, serological detection, molecular detection by PCR and transmission experiment to detect phytoplasma in phyllody symptomatic strawberry from the Northern Thailand.

MATERIALS AND METHODS

Plant samples

Strawberry plants were surveyed and collected from different growing areas in the North of Thailand; Ban Boe Kaew, Huay Namrin, Doi Pui Research Station, Samaeng, and Doi Inthanon during 1997-1998. Both symptomless and symtomatic plants showing phyllody disease (Figure 1) were collected for detection of phytoplasma. Strawberry plants with phyllody symptoms which were found in Doi Pui Research Station were usually marked and prunned off to remove the infected parts. These plants were left in the field for further growth observation.

(a)

(b)

Figure 1 Typical symptoms of the noninfectious phyllody disease on strawberry No.50 cultivar, (a) phylloid fruits known locally as pineapple disease and (b) foliaceous growth of leaves arising from the tip of fruits.

Detection of phytoplasma by electron microscopy

The samples for electron microscopy were prepared by Ultrathinsection. Tissues were collected from leaves and fruits of strawberry. Midribs including petioles were excised into small pieces of about 1×2 mm. The preparation of samples was performed as described by Spurr (1969) and the samples were examined with electron microscopy.

Serological detection

The detection method was F $(ab')_2$ indirect ELISA (Sarindu and Clark, 1993) using antisera against phytoplasmas causing sugarcane white leaf, sesame and sunn hemp phyllody and sunn hemp witches' broom. F $(ab')_2$ was first added to the wells of the microplate, followed by diluted antigen (1:20; 1:40; 1:80 w/v). The IgG used as detecting antibody was diluted in a 1:40 (w/v) buffer extract of healthy plant tissue and then added to the wells. Bound antibody was detected with horseradish peroxidase-labelled protein A (PA-HRP). The substrate was 3,3´,5,5´-tetramethylbenzidine. The antigens detecting antibodies and PA-HRP were diluted in PBS-TPO (PBS + 0.05% Tween 20, 2 g/L ovalbumin and 20 g/L polyvinylpyrrolidase)

DNA extractions and PCR conditions

DNA was extracted from leaf tissues by the use of modified Dellaporta's method (Dellaporta *et al*., 1983). Plant samples (200 mg) were ground in extraction buffer (0.1 M Tris, pH 8.0, 50 mM EDTA, 0.5 M NaCl) added with 5 µl of β-mercaptoethanol. The samples were centrifuged at 10,000 rpm for 5 min and the supernatant was collected and extracted with 1 vol. of PCI (phenol :chloroform : isoamyl alcohol - 25:24:1). The mixture were mixed vigorously by vortex. The tube was spinned at 10,000 rpm for 5 min and the supernatant was collected and added with 33 µl of 20% sodium dodecyl sulphate (SDS). The sample was incubated at 65°C for 10 min and then added with 160 µl of 5M potassium acetate (KoAC). The tube was centrifuged at 10,000 rpm for 5 min and the supernatant was collected and added with 0.5 vol. of isopropanol to precipitate DNA by centrifugation at 10,000 rpm for 5 min. The DNA pellet were kept and then washed with 500 µl of 70% ethanol. After extraction, DNA was resuspended in sterile water and used as the template for PCR.

The universal primer pairs R16F2/R2, (R16F2 : 5´ ACG ACT GCT GCT AAG ACT GG 3´ and R16R2 : 5´ TGA CGG GCG GTG TGT ACA AAC CCC G 3´) (Lee *et al*., 1993) were used to amplify phytoplasma 16S rDNA. The PCR mixture contained 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 2.5 mM $MgCl_2$, 200 µM of dNTP mixture, 0.4 pmole of each primer, 0.2 units of Taq Polymerase (Promega) and 2 µl of DNA templates in a final volume of 25 µl of reaction mixture. The PCR amplification was performed by denaturing DNA at 94 °C for 2 min followed by 30 cycles of denaturing at 94 °C for 1 min., annealing at 50 °C for 2 min and extending at 72 °C for 2 min. Then, it was followed by a final extension at 72 °C for 7 min. PCR products were analyzed with electrophoresis through 0.8% agarose gel stained with ethidium bromide and DNA bands were visualized using a UV transluminator.

Transmission experiment

Efforts to transmit disease from the symptomatic strawberry plants to periwinkle and healthy strawberry were carried out by grafting and dodder (*Cucusta campestris*) transmission.

The petiole-insert leaflet technique was done to transmit phytoplasma from phyllody-showing strawberry to healthy and periwinkle strawberry plants. All grafted plants were kept in the insect-proof greenhouse at Doi Pui Research Station.

In the case of dodder transmission, dodders was grown from seeds and placed on the recipient host, periwinkle. The symptomatic strawberries were used as donor plants and dodders were used as the bridge between the recipient host and donor plants. All plants were kept at 25-27°C in the insect-proof greenhouse at Plant Virology Section, Department of Agriculture, Bangkok. The dodder bridges were left for 3-4 weeks for transmission before discarded. The symptom development on

periwinkle and healthy strawberry was recorded.

RESULTS AND DISCUSSION

Attempts to detect the phytoplasma in strawberry showing phyllody symptom were performed by the use of electron microscopy, serological method and PCR detection. There were no phytoplasma detected in symptomatic strawberry. Phytoplasma could not be found in the phloem cells of strawberries showing the phyllody symptom by the use of electron microscopy. Also, indirect ELISA method gave the same results with no phytoplasma detected in symptomatic strawberries. In addition, no PCR product was detected with DNA template extracted from both strawberry showing phyllody symptom and the healthy strawberry, whereas the 1.2 kb PCR fragment can be detected in DNA extracted from all other phytoplasma infected plants (Figure 2). All methods gave the negative results. The transmission experiments by grafting and dodder transmission were negative. The grafting experiments showed that the survival of both periwinkle and healthy strawberry scions was 70%(21/30) and 60%(18/30), respectively, and none of them developed symptom within two months after grafting. The similar results were found in doder transmission. The periwinkle recipient plants showed no sign of phyllody disease two months after transmission. Thus, the phyllody disease of strawberry could not be transmitted to periwinkle and healthy strawberry.

The aim of this study was to elucidate the

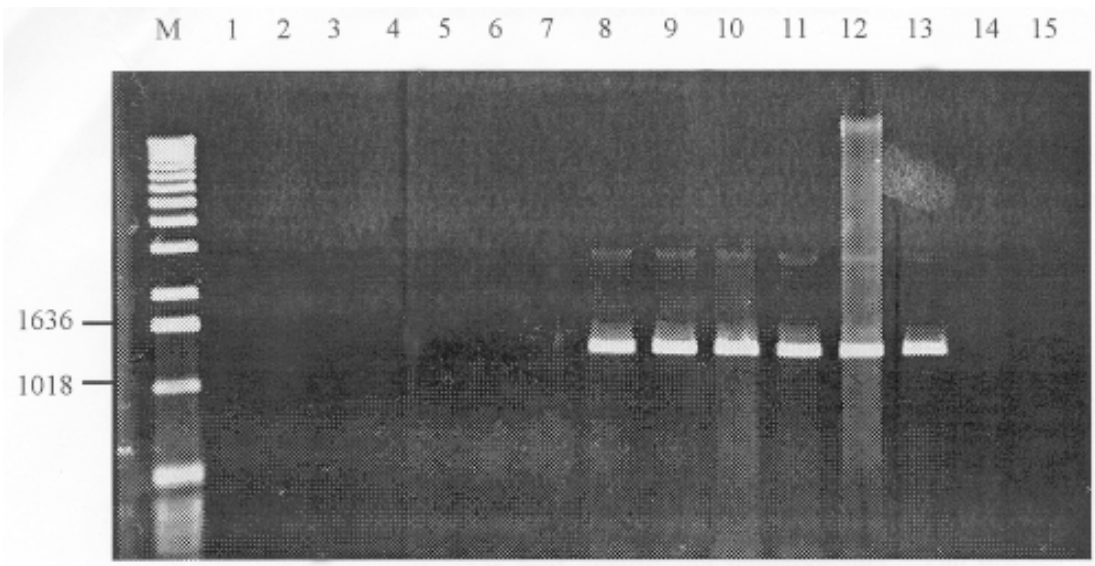

Figure 2 PCR of a 16S rDNA sequence from various phytoplasma using primer pair R16F and R16R2. PCR products were analyzed on 0.8% agarose gel electrophoresis. Lane M, 1-kb ladder marker (GIBCO, BRL), lane 1-3 and 4-7, strawberry showing phylloidy disease collected from Doi Inthanon and Ban Boe Kaew, respectively, lane 8-13, plant samples infected with phytoplasma as follows, crotalaria phyllody, crotalaria witches phyllody, eggplant little leaf, faba bean phyllody, gerbera phyllody, jute phyllody and mungbean phyllody, respectively, lane 14, healthy strawberry, lane 15, H_2O as negative control.

etiology of phyllody disease occuring in strawberry. The most striking symptoms were a pronounced foliaceous growth arising from the achenes and development of phylloid fruits which resemble the symptoms in the green petal disease caused by phytoplasma (Harrison *et al*., 1997). In order to find out whether phytoplasma is the causal agent of the phyllody disease in strawberry, electron microscopy, serological method, and PCR assay were used for phytoplasma detection. The results showed that no phytoplasma could be detected in symptomatic strawberries. It is likely that this disease was not caused by phytoplasma. From our observations, this disease might be caused by the physiological disorder which has been found frequently in some certain cultivars, especially No.50 . Marcone *et al.* (1996) reported that a similar phyllody disease has been found on strawberry grown in Northern Italy. It has been observed usually in the strawberry came from cold-stored runners. They concluded that it was non-infectious phyllody disease which might be a physiological disorder induced during storage (Marcone *et al*., 1996). In constrast, this disease had been reported in strawberry plants that were not kept in cold storage and was called Plymouth disease (McGrew and Posnette, 1970). Therefore, plant breeder should be aware of the physiological disorder before releasing or recommending the new cultivars developing with tissue culture techniques to the growers. In addition, to obtain new cultivars of strawberry with trueness to type and varietal purity, the breeding program of strawberry requires a very careful inspection until the new cultivars are stable and insensitive to variable environments. This will help to prevent the problem from the physiological disorder as non-infectious phyllody disease reported in this study.

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