TEM OBSERVATION OF SOYBEAN BACTERIAL PUSTULE

อาการของโรคใบจุดนูนของถั่วเหลือง จากการสังเกตด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบแสงผ่าน

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ABSTRACT

Histopathological symptom of soybean bacterial pustule caused by Xanthomonas campestris *pv.* glycines *was examined by transmission electron microscope (TEM). Leaves of soybean cultivar SJ4 were inoculated with strain Xcg 039. After leaf tissue occurring symptom, it was prepared for TEM observation. Pustules were composed of enlarged parenchyma cells with a thin shell of cytoplasm and a few sedimentary chloroplast. Vascular parenchyma in infected tissue contained more cytoplasm than healthy tissue with small vacuole. Nucleus of diseased tissue was irregular or pleiomorphic shape. The increased number of cells indicated that cell division as well as cell enlargement, was a mechanism of pustule development.*

บทคัดย่อ

จากการตรวจสอบอาการของโรคใบจุดนูนในระดับเนื้อเยื่อของใบถั่วเหลืองที่ได้รับการปลูกเชื้อด้วย *Xanthomonas campestris* pv. *glycines* สายพันธุ์ Xcg 039 โดยใช้กล้องจุลทรรศน์อิเล็กตรอนแบบแสงผ่าน

(TEM) พบว่าเนื้อเยื่อพาเรงคิมา (parenchyma) แต่ละเซลล์ตามบริเวณจุดแผลจะมีขนาดขยายใหญ่ขึ้น ผนังหุ้มไซโทพลาซึม (cytoplasm) ค่อนข้างบางและมีคลอโรพลาสต์ (chloroplast) อยู่น้อยกว่าปกติ ปริมาณไซโทพลาซึมใน vascular parenchyma ของเนื้อเยื่อพืชที่เป็นโรคจะมีมากกว่าในเนื้อเยื่อพืชปกติ ในขณะที่จำนวนแวคิวโอล (vacuole) จะน้อยลง พร้อมกันนั้นนิวเคลียสก็จะมีรูปร่างเปลี่ยนแปลงไปจากเดิม การที่เซลล์พืชที่เป็นโรคมีขนาดใหญ่และมีจำนวนมากผิดปกติชี้ให้เห็นกลไกของโรคใบจุดนูนที่ทำให้ถั่วเหลืองมีการแบ่งเซลล์และเซลล์ขยายใหญ่กว่าเดิม

INTRODUCTION

The bacterial pustule caused by *Xanthomonas campestris* pv. *glycines* (Nakano) Dye 1978 damages some parts of soybean included leaf, stem and pod which most symptom severely occurs at leaves.[10] The seriously damaged leaves defoliate causing decreased photosynthesis of the plant which in turn resulted in low yield and low quality.[7,8] In Thailand, the losses due to this disease is considerably high in rainy season and particularly great in SJ4 varieties which are commonly grown in Thailand.[9]

Xanthomonas campestris pv. *glycines* can infect soybean leaf through natural opening and wounds. [4,6] Wergin and Mace [14] reported that after one day of infection through stomata, small colony was formed at the wall of spongy parenchyma cell on lower substomatal chamber. Cell wall surrounded by the pathogen became gradually by hydrolyzed in the next day, a part of microfibrill wall was split from mesophyll cell and progressively loosen. After 3 days of inoculation there was a big difference between plant varieties. In susceptible soybean varieties, cells enlarged, thus pushed the lower epidermis out as pustules, while in resistant varieties the enlargement of cells did not occur. However, cisternae of endoplasmic reticulum, the increased number of ribosomes and abundant vesicles were observed in cytoplasm. These organells have the relationship with protein synthesis and extracellular secretion. A virulent strain of bacterium was limited only in mesophyll cell wall of susceptible leaves and after that, it was surrounded by fibrillar and granular material at mesophyll cell wall. On the contrary, the virulent strain was not area limited or surrounded, but remained free and intracellularly increased in number.[2] Groth and Braun[3] reported that both resistant and susceptible varieties, the bacterium was able to increase in number within the first 10 days

of inoculation on the leaves. In susceptible varieties, the ratio of number of pathogen to pustules was 1:1 while 10:1 in resistant varieties.

The thorough investigation on disease causing bacteria including the infected plant structure by examination of microscopic structure enabled to understand the attribute of the pathogen on the infected area of plant, as well as the changing of plant structure after infection. This would be used as a guide in other aspects of diagnosis in order to elucidate the possible mode and extent of spread of the pathogen including the outstanding of morphological aspects of pathogenesis of *X. campestris* pv. *glycines.*

MATERIALS AND METHODS

Seeds of the soybean cultivar SJ4 were planted in clay pots in a greenhouse. Inoculation was made with culture of *X. campestris* pv. *glycines* strain Xcg 039 after 35 days. Bacterial suspension containing 1 x 10^8 cfu/ml was sprayed with force by a chromatographic bottle onto the leaves until water-soaking appeared. Inoculated plants were left in the green-house under the condition of high humidity and temperature around 32-35°C for 10 days. When the pustule was appeared, infected leaves were brought for microscopic structure observation.

Tissue for transmission electron microscopy (TEM) was prepared by cutting infected portion into slices of 1 mm thick and 5 mm long. These small segments were vacuum-infiltrated with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and the solution was changed in 12 h later. The samples were rinsed, 2 h after vacuum-infiltration with 0.1 M phosphate buffer, pH 7.2 (3 changes for 30 min each). Following the rinse, the samples were placed in 2% osmium tetraoxide solution for 2 h at 4°C, and then rinsed in distilled water with 3 changes for 30 min each. The samples were treated for 1 h at 4°C with a saturated solution of 3% uranyl acetate solution and rinsed in distilled water. Then dehydrated in an acetone series of 30, 50, 70, 90 and 100% for 30 min each at room temperature (25-28°C). Once the dehydration was completed, the leaf segments were infiltrated, under vacuum, with increasing concentration of plastic media (Spurr's epoxy resin) : epoxy and 100% acetone (1:2) for 1 h, epoxy : acetone (2:1) for 1 h, and 100% epoxy overnight. The epoxy-infiltrated samples were poured into plastic containers (also with epoxy) and placed in a vacuum oven at 10°C for 8 h.

The embedded tissue was sectioned with a glass knife on a LKB 8800 ultramicrotome. The ultrathin sections were stained with Reynold's lead citrate and examined with a Hitachi HU 12 A at 75 KV.

RESULTS

The TEM examination of soybean bacterial pustule symptom revealed that whether pustules are visible or invisible by naked eyes, the cell enlargement was observed in the cross sections. In natural infection of *X. campestris* pv. *glycines* under the favorable environmental conditions, the symptoms were typical pustules that can be seen by naked eyes, but under unfavorable conditions it develops only as flat lesions.[10]

The days after inoculation, leaves became yellow-green chlorosis and widespread of pustules. The TEM of pustule revealed the appearance of very large, rounded parenchyma cells that produced swellings in the leaf and eventually resulted in collapse of epidermal cells and rupture of the cuticle.

A section from a pustule area, the leaf thickness increased compared with a symptomless area because of cell enlargement in the palisade cell layer and the contiguous paraveinal mesophyll. The paraveinal mesophyll is however, a characteristic cell layer between the palisade and spongy parenchyma cell layers.[15] Most pustules that had developed by 10 days after inoculation protruded from the abaxial side of the leaflet. In some pustules, palisade parenchyma cells are involved as well and protruded from both sides of the leaflet. In large, bilateral pustules, and increase in cell number was evident. Affected palisade cells were elongated, confluents, and some exhibited cross walls indicating cell division.

Inspection of host cell wall surfaces in pustules disclosed colonies of bacteria associated with enlarged cells, often at cell junctions. Bacteria made imprints on cell walls, suggesting wall degradation or secretion of surface materials by host or bacterial cells, and also indicating that bacteria formed a real association with host walls and were not simply deposited there during critical point drying, which must be considered as a possibility in other location.

Strands of material, possibly bacterial extracellular polysaccharide (EPS), were often present near bacteria.

The TEM observation of cross-section infected tissue also revealed the presence of enlarged and division of cells around the lesion site. Such morphological alteration was observed earlier on the lower epidermis rather than the upper epidermis. Many bacteria were observed on the cells surrounding lesions. Few bacteria were found in the center of the lesion or collapsed tissue. Although the pathogens were located at the tissue adjacent to the lesion, they were still closed to the drying cells.

The TEM study disclosed that the pathogen induced the severe effects on host cells. A typical enlarged parenchyma cells in a pustule is shown in Figure 1. The smaller spongy parenchyma cells in the pustule at higher magnification displayed ultrastructural features characteristics of the affected cells in cultivar SJ4, including thickening and apparent loosening of the wall matrix.

Pustules often abutted or surrounded minor leaf veins. A comparison of symptomless tissue versus pustular tissue indicated that bundle sheath cells in affected tissue contained more cytoplasm than usual with many small vacuoles, small chloroplasts (Figure 2B) and large pleiomorphic nuclei (Figure 2A).

DISCUSSION

The investigation revealed the presence of *X. campestris* pv. *glycines* within cells around the pustules. In natural conditions or in the field, the raised lesion of pustules might not be seen and showed only flat-simple lesion with naked eyes. Under electron microscopes, however the pustules showed more distinct morphological feature. The tissue of the pustule showed enlargement as well as division of cells which were similar to the observation of Hedge[4]. The pathogen was hardly found at the drying area since such area stopped function and became collapsed. Therefore, it was necessary for this bacterium to use adjacent cells for its continuous living. Moreover, some chemical substances which are needed for bacterial growth might be secreted when host cells die.

The tissue appeared normal and bacteria were not observed in the visibly unaffected areas of spray-inoculated leaves. In the unaffected tissues that were adjacent to a pustule, a large amount of bacteria embedded in an amorphous substance were occasionally observed in the substomatal of intercellular spaces of the spongy mesophyll. When bacteria were abundant, disruption of individual mesophyll cells and layers was more evident. Masses of bacteria sometimes either filled an area normally occupied by palisade cells or were surrounded by matrix that entirely filled a large area of the mesophyll. The nearby palisade and spongy parenchyma cells were moved to epidermis with subsequent thickness and disruption of the epidermal cells. When lesion became apparent, the vascular bundles of veins and epidermal cells were distinguishable, although the epidermal cells were compressed and distorted. The space previously occupied by the mesophyll contained with masses of bacteria. The necrotic areas eventually became dehydrated, matted together, and completely indistinguishable and were compressed, without bacteria. Bacteria did not occur in the vascular bundles of veins or rib vein of spray-inoculated leaves. The rib tissue of vein were usually devoid of and unaffected by bacteria, including leaf areas where the rib vein served as a boundary that separated unaffected from infected tissue.

Bacterial cells were consistently found on the surfaces of enlarge host cells when critical point dried material was examined. Bacteria were ofter partially embedded in host cell walls, so that artifacts of drying could be discounted. These bacteria were neither free in the intercellular space nor immobilized. The results indicated that the causal bacteria were more closely associated with stimulated cells.

The effect of bacteria on the leaf cells was suggested that the successful infection may involve numerous factors, including attachment of the pathogen or its extracellular polysaccharide to the host cell wall[13], the ability of extracellular polysaccharide to maintain a lesion spot in the intercellular spaces, the presence of appropriate bacterial enzymes or immobilizing materials or toxins such as auxin-induced cell stimulation [5,12] and the ability to overcome host antibacterial substances. Indoleactic acid (IAA) and cytokinins of host and/or bacterial origin are known to be involved in pathogenicity by *Agrobacterium tumefaciens, Pseudomonas syringae* pv. *savastanoi* and *Rhodococcus faciens* and are

considered to be necessary but not sufficient to produce the tumors, galls, knots or other tissue distortions caused by these organisms. A primary role of IAA in crown gall is believed to be nutrient accumulation in affected areas by induction of a high electrochemical proton gradient across the cell membrane [11]. IAA caused cell elongation and multiple other effects on cellular metabolism. It may be involved in the cell enlargement in bacterial pustule. *X. campestris* pv. *glycines* produces IAA *in vitro*. The absence of pustule formation at high concentration of bacteria may be resulted from hyperauxinic condition in which IAA-medicated cell elongation is inhibited and lateral expansion increases[1].

The technique of the sample preparation for TEM, was observed that bacteria are not anchored to cell wall by EPS or by immobilizing materials may be moved about in the intercellular space or washed out together by the multiple fluid exchanges in fixation and dehydration process. Infiltration with plastic for TEM may trap some bacteria in the middle of the intercellular space. Neither technique gives straight forward evidence about the location of free bacteria. Moreover, TEM does not see a single layer of bacteria on a cell wall as a colony unless there is another distinguishing feature, such as a colony border of EPS. However, if unsharped razor blade was used to cut plant leaves, the sampling can be destroyed by the cutting. This might lead to misinterpretation. Therefore, if the study of diseased tissue was conducted along with other procedures such as, the color staining of plant sample with microscopic examination, a more realistic result would be obtained.

CONCLUSION

The primary effect of *X. campestris* pv. *glycines* on soybean leaf ultrastructure was cell enlargement ; whether a mitotic effect was also present was less certain. However, the clear increase in cell number in some pustules and the presence of cross wall indicated that at least instances, cell division occurred. If cytokinins were involved, their role appeared to be secondary. Overgrowths develop at the wound site because of stimulation of mitotic activity in cells around the wound indicating a primary role for cytokinins in symptom development.

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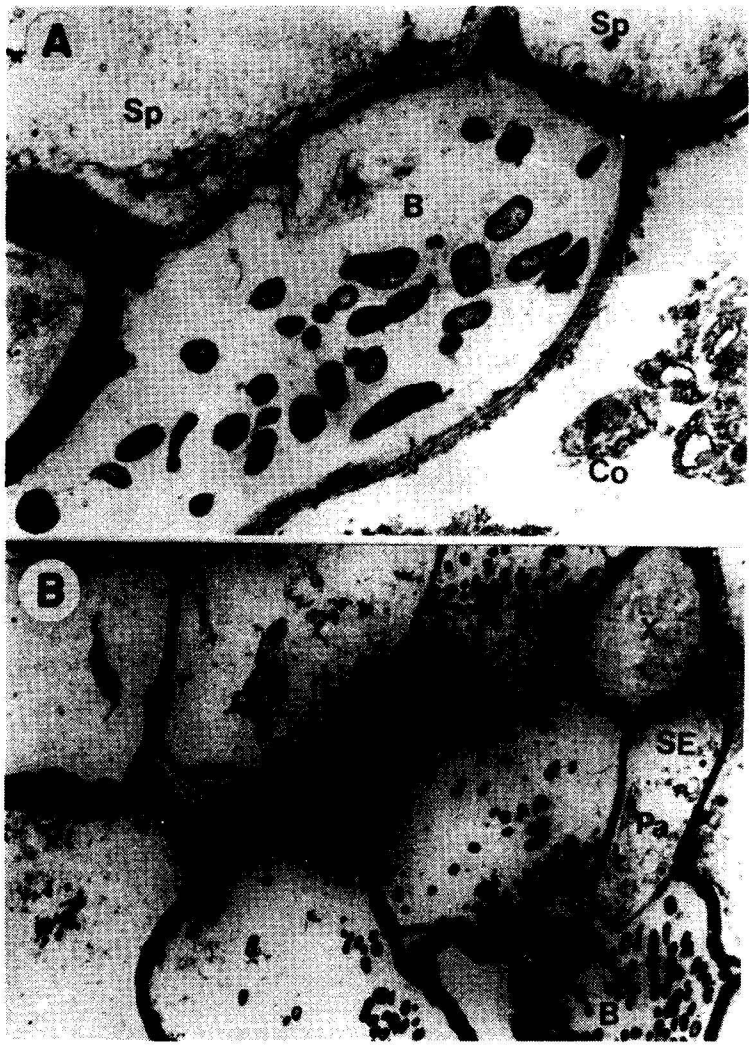

Fig. 1 **Transmission electron micrographs showing enlarged cells in a pustule caused by *Xanthomonas campestris* pv. *glycines* (x 6000)**

A : in a pustule the enlarged cell had a thin shell of cytoplasm and a few sedimentary chloroplast (Co). Bacteria were within the intercellular space (B). Spongy parenchyma (Sp) cells exhibited the typical cytoplasmic feature of the tissue infected by the pathogen.

B : vascular parenchyma in diseased tissue contained more cytoplasm than healthy tissue. B = bacteria, Pa = vascular parenchyma, SE = seive element, X = xylem.

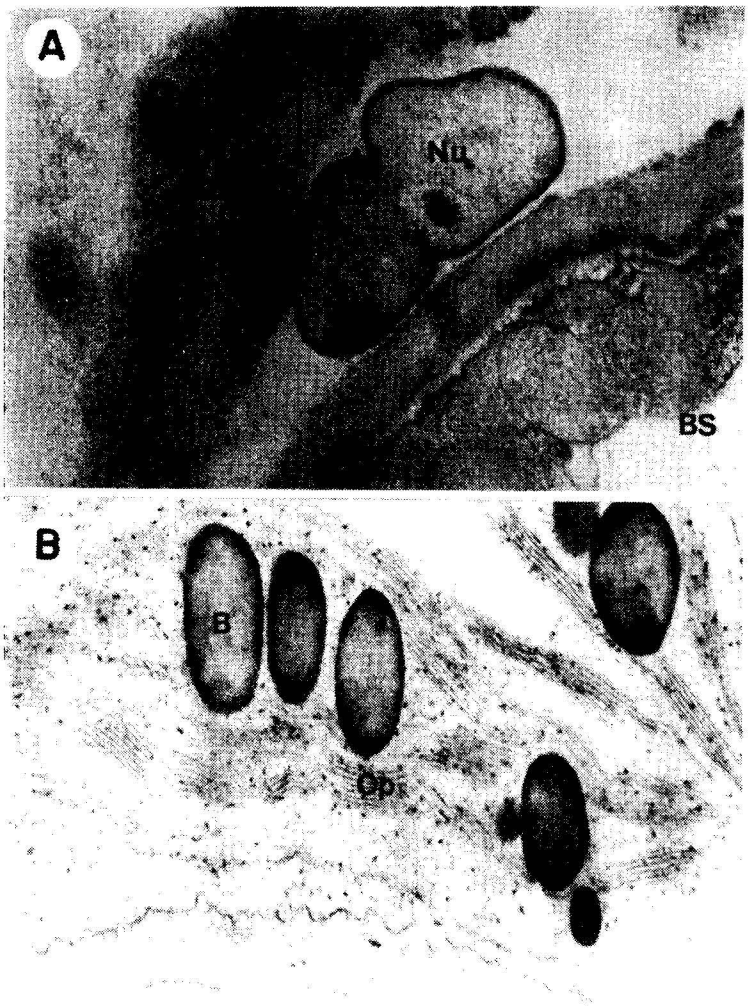

Fig. 2 **Ultrastructure of infected soybean leaves in a pustule induced by *Xanthomonas campestris* pv. *glycines***

A : a large irregularly shaped nuclei (Nu),

BS = bundle sheath cells.

B : the cytoplasmic volume was occupied by small chloroplasts (Cp),

B = starch granules.