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Possible Association of Indole-3-Acetic Acid Production by *Xanthomonas axonopodis* pv. *glycines* with Development of Pustule Disease in Soybean

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This report concerns the role of indole-3-acetic acid (IAA) in bacterial pustule disease of soybean. Pustule production in soybean leaves caused by *Xanthomonas axonopodis* pv. *glycines* was accompanied by a drastic increase in IAA content of host tissues. The phytopathogenic bacterium synthesized IAA in a tryptophan concentration-dependent manner when grown in a defined minimal medium. In complex media, however, the pathogen showed no response to tryptophan feeding, implying that the bacterial biosynthetic machinery of IAA is strictly regulated by nutrient availability of its growth environments. The results may suggest that IAA of bacterial origin and tryptophan of plant origin be involved in the process of pustule symptom development in soybean.

Key words: Glycine max *L., indole-3-acetic acid, pustule disease,* Xanthomonas axonopodis *pv.* glycines.

As a pathogen invades plant tissues, it may release biologically active compounds that account for production of symptoms characteristic of a particular disease. One such compound is IAA. The involvement of IAA in plant pathogenesis has long been known, particularly in gall-forming bacteria, such as *Pseudomonas savastanoi*, *Agrobacterium tumefaciens*, and *Erwinia herbicola*.[1,2]

Xanthomonas axonopodis pv. *glycines* causes pustule disease of soybean, which is characterized by small raised pustules surrounded by chlorotic halos on the surface of affected leaves. The pustule formation is a result of hypertrophic growth of host cells responding to the bacterial infection.[3,4] Since cell enlargement is closely associated with auxin effects in plants, IAA synthesis by the pathogen may be related to hypertrophy of host tissues. However, this remains still unclear. In fact, doubts have been cast on the importance of bacterial IAA production for the pathogenicity of *X. axonopodis* pv. *glycines*.[5]

In this study, we found that soybean leaf tissues infected by *X. axonopodis* pv. *glycines* had a markedly increased IAA content compared with noninfected tissues. Further, it was demonstrated that the pathogen in a minimal medium, which mimics plant fluids of the intercellular space where the pathogen resides, was capable of induced production of IAA when Trp was supplied.

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Abbreviations: CFU, colony forming unit; FW, fresh weight; IAA, indole-3-acetic acid; Trp, tryptophan.

Materials and Methods

Bacterial culture. Eight strains of *X. axonopodis* pv. *glycines* strains were used for examining bacterial IAA production *in vitro*, as compiled in Table 1. These strains caused mild to severe disease in soybean cultivars except for strain SL2098 (unpublished results). IAA production *in planta* induced by bacterial infection was studied with strain 8ra, because this strain is the best characterized among those examined in terms of a secretion system that may be involved in secreting some virulence factors hitherto unidentified.[6] Cultures grown to saturation in Luria-Bertani (LB) broth were diluted 1 : 100 in a minimal medium modified from XVM2 medium of Wengelnik *et al.*,[7] incubated at 28°C for 24 h with shaking for adaptation. Harvested cells were resuspended in defined medium XVM2 to give an OD_{600} of approximately 1.0, diluted 1 : 1000 in the same medium additionally containing filter-sterilized Trp at various concentrations (1 μM-2.5 mM), and then grown to an OD_{600} of 0.4-0.6 at 28°C with shaking. To analyze IAA production in a rich medium, cultures saturated in LB medium were diluted 1 : 1000 and incubated in the same medium to grow to an OD_{600} of 1.2-1.5. Rifampicin was incorporated into selective media at a concentration of 60 $\mu g \cdot ml^{-1}$. The medium composition of the modified XVM2 is: casamino Acid (0.03%), glucose (0.2%), $CaCl_2$ (1 mM), $FeSO_4$ (0.01 mM), KH_2PO_4 (3.2 mM), K_2HPO_4 (6.4 mM), $MgSO_4$ (5 mM), $(NH_4)_2SO_4$ (10 mM), and NaCl (20 mM), adjusted to pH 6.7. The inoculum for IAA assay *in vivo* was prepared by growing *X. axonopodis* pv. *glycines* strain 8ra on LB agar supplemented with rifampicin for 24 h at 28°C. Bacterial cells were removed from the agar surface with a swab and suspended in 10 mM $MgCl_2$. The cell concentrations were determined turbidimetrically and adjusted by dilution in 10

mM $MgCl_2$.

Plant materials. Soybean plants (*Glycine max.* L., cv Pella) were cultivated in a greenhouse maintained at ca. 25°C under natural illumination. At the stage of fourth trifoliate, they were spray-inoculated with bacteria suspended in 10 mM $MgCl_2$ at 10^8 CFU·ml^{-1} and kept growing for 15 more days in the greenhouse. Then, leaves were harvested, immediately frozen in liquid nitrogen, and stored at −70°C until used for IAA analysis.

Measurement of IAA in bacterial cultures. Liquid cultures were centrifuged (8000 *g*, 10 min), and the supernatant was filtered through a 0.45 m filter. Aliquots of the filtrates (1-5 m*l*) were acidified with 0.1 N HCl to pH 3.0 and applied to a C18 cartridge (1 g sorbent, Bond Elut Junior, Varian, Walnutcreek, CA) that had been pretreated with methanol followed by 2% acetic acid. After washing with 3m*l* of 2% acetic acid, 4 m*l* of methanol containing 2% acetic acid was passed through the cartridge. The pass-through fraction was dried by vacuum evaporation at 40°C, and the residue containing IAA was redissolved in 2 m*l* of 0.2 M imidazole buffer (pH 7.0) and diluted four times with double distilled water. Further purification of IAA was performed on an amino anion exchange column (Bond Elut LRC NH_2, Varian, Walnutcreek, CA) according to the procedures of Chen *et al.*[8] The final purification and concurrent quantification were done by HPLC on a 30 cm × 3.9 mm Nova-Pak C18 column (5 μm, Waters, Milford, MA). The running solvent was 30% methanol plus 1% acetic acid at a flow rate of 0.7 m*l*/min. IAA elution was monitored by fluorescence (280 nm excitation, 360 nm emission) using a Hitachi F-4500 fluorescence spectrometer equipped with a 18 μ*l* micro-flow cell unit.

Measurement of IAA in soybean leaves. Free IAA from soybean leaf tissues was isolated essentially following the procedures of Chen *et al.*,[8] which consisted of three steps, such as extraction with addition of [$^{13}C_6$]IAA (Cambridge Isotope Laboratory, Andover, MA) as an internal standard, separation in a conditioned amino anion exchange column, and purification by HPLC in a C18 column. However, as a heavy pigmentation of the extract was found to severely interfere with the recovery of IAA when its levels were low, we adopted one more step in order to remove pigments before going into the ion exchange chromatographic step. The extract (about 2-4 m*l* from 0.3-0.6 g of sample) in 65% isopropanol with 0.2 M imidazole buffer (pH 7.0) was diluted two times with double distilled water and passed through a Waters C18 cartridge (Sep-Pak Plus C18). This allowed the extract to get rid of most pigments, retained in the cartridge, without influencing IAA. The flow-through fraction and an additional fraction recovered in 1 m*l* of the diluted extraction medium were combined, diluted with double distilled water to reduce the isopropanol concentration to about 10%, and then applied to an amino anion exchange column. The final step of IAA purification by HPLC was the same as described above. GC-MS in the selected ion monitoring (SIM) mode was used to quantify IAA, as described by Cohen *et al.*[9] The fluorescent fractions from HPLC were pooled, reduced to dryness under N_2, redissolved in 100 μ*l* methanol, and methylated using etherial diazomethane.[10] The sample was then taken up in 50 μ*l* n-heptane and subjected to IAA analysis in a HP GC-MS (6890 plus GC/5973 MS, Hewlett-Packard, San Fernando, CA) equipped with a 30 m × 0.25 mm fused silica capillary column (Hewlett-Packard 5MS). Injections were made in the splitless mode with an injection volume of 2 μ*l*. The injector was maintained at 250°C, and the oven was at 80°C for 2 min followed by a ramp at 20°C/min to 240°C held for 5 min. The interface temperature was 250°C. The carrier gas was He at 1 m*l*/min. SIM was done using a 4-ion program with a dwell time of 50 msec for each ion. The monitored ions were of mass-to-charge ratio (*m/z*) 130, 136, 189 and 195.

Results

In such complex matrix as plant extracts, it is challenging to quantify IAA usually at very low concentrations. However, some modifications of the procedure described by Chen *et al.*[8] enabled us to measure IAA down to the levels of 2 *n*g · g^{-1} FW from 0.2 to 1.0 g of frozen leaf materials. The representative example of the selected ion chromatograms used for analysis of IAA in the [$^{13}C_6$]IAA-fortified extracts is given in Fig. 1,

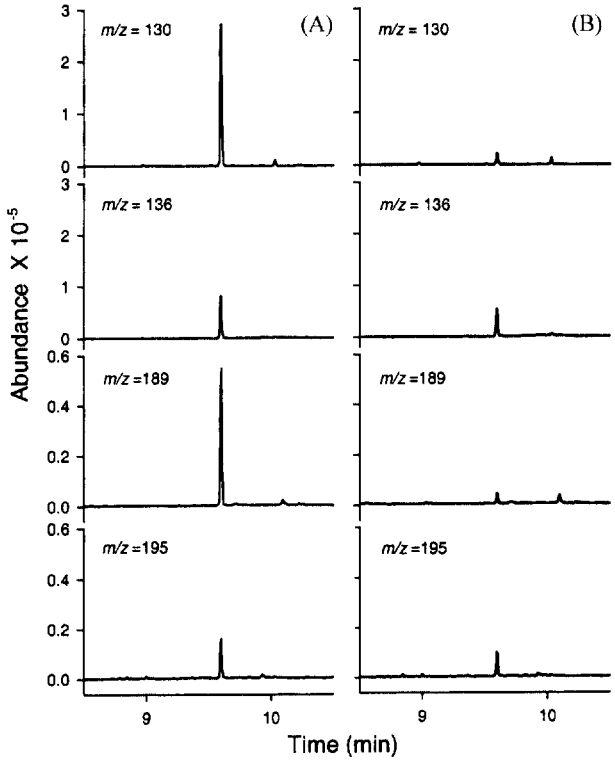

Fig. 1. SIM traces for GC-MS analysis of free IAA from [$^{13}C_6$]IAA-fortified extracts of soybean (cv. Pella) leaves. IAA samples, prepared from heavily pustuled leaves infected by *X. axonopodis* pv. *glycines* 8ra (A) and uninfected controls (B), were methylated and analyzed. IAA was quantified from the area ratio of quinolinium ion peak at *m/z* 130 to its [$^{13}C_6$]-labeled peak at *m/z* 136, which was confirmed by the area ratio of the respective molecular ion peaks at *m/z* 189 and 195.

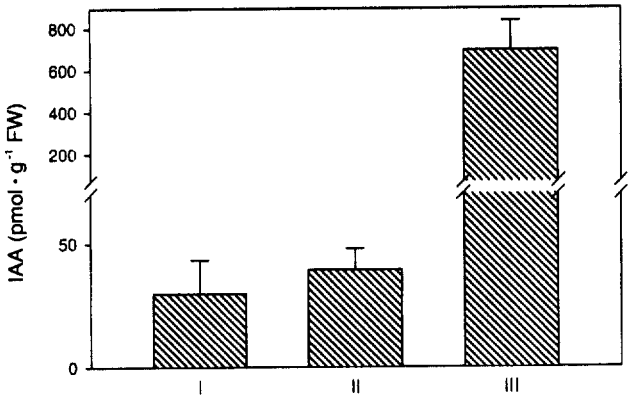

Fig. 2. IAA levels in leaf tissues of different segments taken from the same infected leaves. The segments of leaf area showing no pustule symptom (II) were separated from those with heavy pustule production (III), and both segments were assayed for IAA. Leaf IAA content of soybean that was not inoculated with the pathogen is also shown for comparative standpoint (I). The results presented are from the cultivar Pella. However, virtually the same results were obtained with other susceptible cultivars, such as Jangyeop and Taekwang. Data are given as mean ± SD (n = 5).

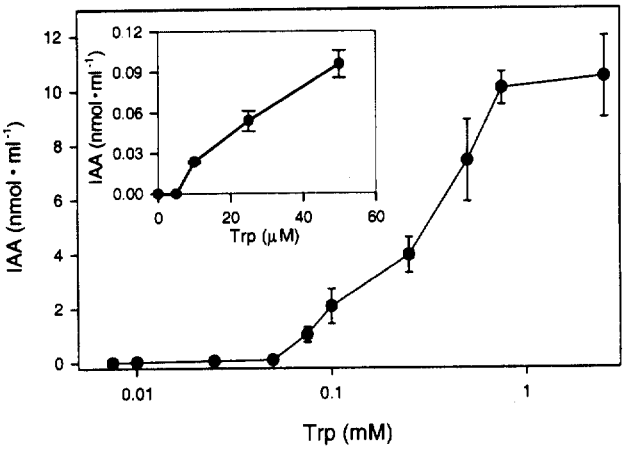

Fig. 3. IAA production *in vitro* by *X. axonopodis* pv. *glycines* 8ra at the late stationary-phase (62 h) of growth as a function of Trp supplied. Cell density was estimated with OD at 600 nm, and IAA levels of culture filtrates were normalized to values at $OD_{600} = 1$. Data are given as means of values from three separate sets of cultures, with error bars representing SD, and data below detection limit (0.01 nmol · ml^{-1}) were treated as zero. Inset shows data in an expanded scale for IAA production in low concentration range of Trp.

from which it can be noted that endogenous IAA level in leaves with heavy pustule development was strikingly higher than that of uninfected controls. The severity of infection seemed correlated with IAA content of the leaves (data not shown). Even in the same infected leaves, IAA was mostly found in heavily pustulated segments while only very low levels of IAA that were comparable to the controls were detected in symptomless segments (Fig. 2).

Microbial IAA synthesis occurs through several different pathways, such as indole-3-acetamide pathway, indole-3-pyruvate pathway, and tryptophan side chain pathway.[2] Since Trp is the precursor for IAA *via* these pathways, diverse microbes with IAA genes produce increased amounts of IAA when growth media are supplemented with Trp. However, *X. axonopodis* pv. *glycines* has been reported to be unable to produce IAA *in vitro* unless Trp at a nonphysiologically high level, i.e. 0.05% (about 2.5 mM) is supplied to culture media.[5] Then, there might be a threshold of Trp concentration for inducing microbial IAA production *in vitro*. To ascertain this, *X. axonopodis* pv. *glycines* strain 8ra was cultured in a minimal medium (modified XVM2) supplemented with Trp at various concentrations (0-2.5 mM), and the culture filtrates were then analyzed for IAA. It turned out that there was no Trp concentration-threshold: the pathogenic strain synthesized IAA in a Trp concentration-dependent manner, showing its capability of producing IAA even at a Trp level as low as 10 μM (Fig. 3). The disagreement between the observation of Fett *et al.*[5] and ours regarding Trp requirement for the bacterial IAA synthesis *in vitro* is not thought to arise from the difference of bacterial strains tested in two laboratories. We rather assume that high concentrations of Trp should have been provided to the cultures so as to allow them to produce IAA to levels assessable by the then available methods with relatively low analytical sensitivity.

Table 1. IAA production *in vitro* by *X. axonopodis* pv. *glycines* in the presence of 2.5 mM Trp.

Strains	IAA production (nmol · ml^{-1})[a]	
	LB[b]	XVM2
8ra	0.17	12.22
LMG7403	0.12	8.71
LMG7404	0.19	19.89
SL1017	0.10	2.50
SL1018	0.15	10.51
SL1045	0.18	16.67
SL1157	0.13	5.96
SL2098	0.14	10.37

[a]Results are averages of two replications.
[b]Corrected for intrinsic IAA content (0.07 nmol · ml^{-1}) of LB broth from Difco.

IAA production was further studied with cultures of eight strains of *X. axonopodis* pv. *glycines*. The results indicated that all strains examined produced IAA to a significant extent when grown in a minimal medium supplemented with Trp and that the bacterial IAA synthesis was highly dependent on nutritional conditions of culture media. In rich media, such as LB and Kings B media, *X. axonopodis* pv. *glycines* produced only small amounts of IAA (0.1-0.2 nmol · ml^{-1}) even in the presence of 2.5 mM Trp, which was a clear contrast to the observation in the defined medium (Table 1).

Discussion

Plant cells normally regulate intracellular IAA pool in response to the developmental program as well as to environmental stimuli. When a high level of exogenous IAA is intro-

duced, plant cells may suffer loss of their transcending regulatory capacity, which can result in severe changes in their morphology. This is a hypothesis explaining the development of pustule disease of soybean in terms of cellular effects of excess IAA synthesized by *X. axonopodis* pv. *glycines*. Yet, no experimental data have conformed to the hypothesis although this pathogen has inherited ability to synthesize IAA.

To the best of our knowledge, this report is the first ever documented on increased production of IAA *in vivo* by *X. axonopodis* pv. *glycines* infection in association with development of pustule symptom in soybean. For the present, we do not know whether the IAA measured in infected tissues was mostly synthesized by the invading pathogen or by the host tissues responding in some manner to the bacterial invasion. Nevertheless, since minimal media for bacterial culture mimic plant fluids of the intercellular space where *X. axonopodis* pv. *glycines* resides,[11] our data for bacterial IAA production in modified XVM2 medium may lead to suggest that the pathogen can synthesize IAA in natural habitat if a certain level of Trp is available. Then, a relevant question is how intracellular Trp becomes available to the pathogen in the intercellular space. Changes in ion fluxes across the plasma membrane as one of the early-phase response of plant cells to bacterial invasion[12] might be linked with the process of Trp secretion from cells *via* alteration of the proton gradient that controls the plasmalemma transport of amino acids.[13]

Medium dependence of the bacterial response to Trp feeding for IAA production may be taken as an indication that IAA biosynthetic machinery in *X. axonopodis* pv. *glycines* is strictly regulated by some factor of the growth environments. A certain chemical substance contained in rich, complex media could be the factor, acting as either an inhibitor of the enzyme system involved in IAA synthesis pathway or a suppressor of IAA gene expression. However, this possibility may be ruled out because the same lack of response to Trp feeding was seen in the pathogen cultured in two different rich media, i.e. LB broth and Kings B medium. We therefore assume that overall nutrient availability *per se* may be a signal of the regulatory mechanism for IAA biosynthesis.

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