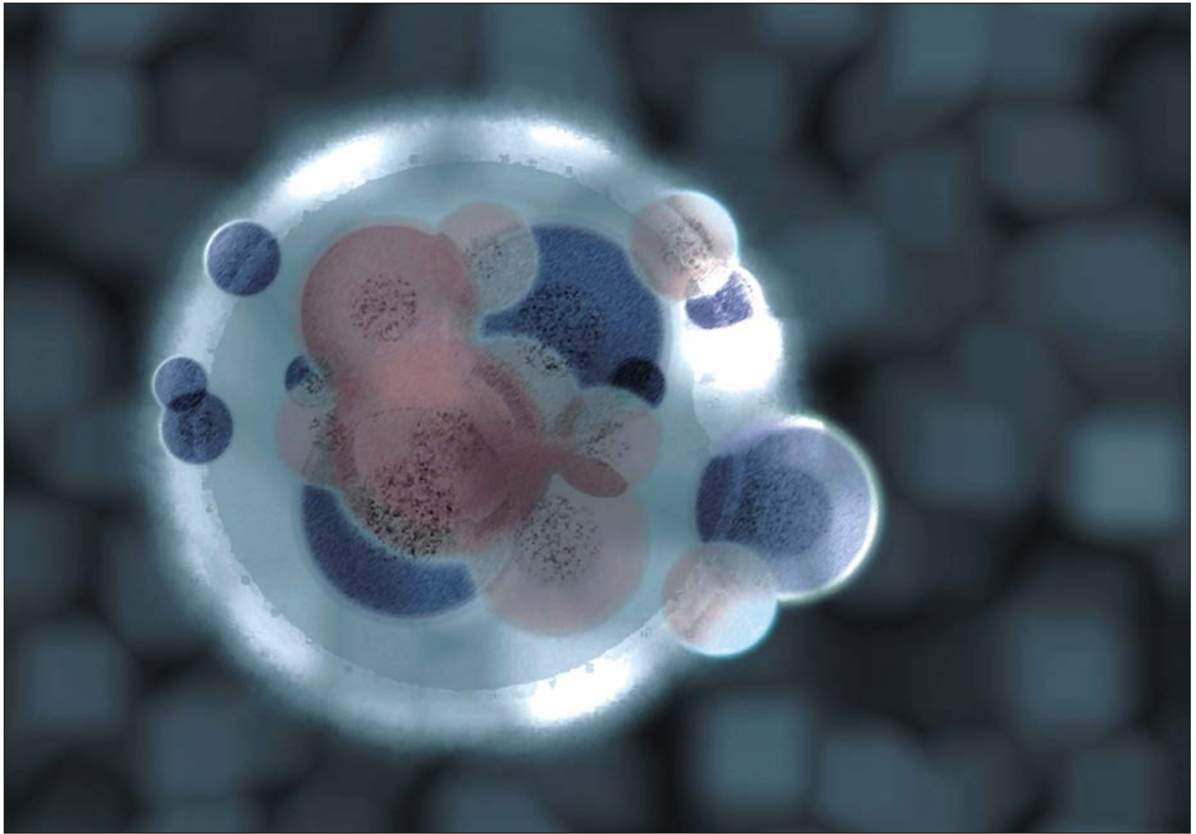

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Biosynthesis of Indole-3-Acetic Acid by the Gall-inducing Fungus *Ustilago esculenta*

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Abstract: *Ustilago esculenta* incites the formation of an edible gall and prevents inflorescence and seed production in the aquatic perennial grass, *Zizania latifolia*. As compared to the healthy tissues, the edible galls had higher amounts of indole-3-acetic acid (IAA), which could be synthesized from the host plant and/or the fungal pathogen. In this study we investigated the ability for IAA production by *U. esculenta*. The production of IAA in culture by *U. esculenta* was solely dependent on the presence of tryptophan. The addition of thiamine (vitamin B_1) to medium greatly enhanced fungal growth, whereas IAA production was completely inhibited. Maximum amount of IAA (*ca.*1.0 μg mL^{-1}) was obtained after 8-day incubation. The production of IAA was highly correlated with the amount of tryptophan. The optimum temperature for IAA production ranged from 20 to 25°C. However, IAA production was significantly reduced when fungus was grown in the fluctuated temperatures, indicating that a constant temperature has a profound effect on IAA production. The corn smut pathogen *U. maydis* also synthesized IAA using tryptophan and accumulated a maximum amount of IAA (*ca.*1.2 μg mL^{-1}) at day 3, then production was declined steadily thereafter. In contrast, the sugarcane smut pathogen *U. scitaminea* produced less amount of IAA (*ca.*0.53 μg mL^{-1}) compared to other two species. In addition to tryptophan, *U. esculenta* apparently could convert indole-3-acetamide (IAAld), indole-pyruate (IPA) and indole-lactic acid (ILA) into IAA. However, indole-3-acetamide (IAM) was evidently not a suitable precursor for IAA production. The results suggest that biosynthesis of IAA in *U. esculenta* from tryptophan proceeds through IPA and IAAld.

Key words: Hormone, IAA, thiamine, tryptophan, smut

INTRODUCTION

Association of a basidiomycetous fungus *Ustilago esculenta* P. Henn. with the perennial aquatic grass, *Zizania latifolia* (Griseb.) Turcz., results in an edible smut gall. The gall is derived from a hypertrophy of host tissues due to colonization of pathogen and usually located in the stem region of 3-4 nodes[1,2]. The gall is edible with unique flavor and tendency. Therefore, the crop is cultivated as a vegetable in southern Asia. It is commonly called "gau-soon" or "kal-peh-soon" in Taiwan. The gall resembles unhusked ears of corn, but with an attenuated and curved apex. Based on the color of the outer sheath, three cultivars (green, white and red) are commonly infected with *U. esculenta* in Taiwan[2].

U. esculenta colonizes intra- and intercellularly in the most of vegetative tissues except leaves and roots[2,3]. The crop in the field is propagated using asexual rhizomes and new grass sprouts are repeatedly infected with fungus. The fungus completely sterilizes the inflorescence production and seed production. The crop is grown in flooded paddies and the temperature of the water is critical for gall development. Inappropriate temperature limits gall development and induces early production of dark-colored and sandy teliospores. Gall development is also closely related to the host genotype (cultivars) interactions. The edible galls are harvested twice a year for 'green' and 'white' cultivars, whereas only once for 'red' cultivar.

Gall formation includes an increase of the size and number of host cells[4], suggestive of the involvement of a high IAA content within the swelling tissues. The hypertrophy of tissues also implicates cytokinin involvement in the gall formation[4]. The imbalance of IAA and cytokinin due to phytopathogen infections has been suggested to be important for overgrowth, hypertrophy and tumor formation in many plant-microbe interactions[5-8]. Although higher levels of IAA and

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cytokinins are often found in the gall tissues[4], the role of these two plant regulators in gall development has not been yet experimentally demonstrated. Furthermore, the increased level of plant growth regulators in affected plants can result from direct production by pathogens, stimulated synthesis of the host plant, or suppression of degradation by pathogens[9]. To define the role of plant growth regulators in the development of the unique galls, we first investigated the production of IAA by *U. esculenta* in culture, the effects influenced its production and its possible biosynthetic pathway. The abilities for IAA production among *Ustilago* species were also compared.

MATERIALS AND METHODS

Biological materials and media: Fungal isolates of *Ustilago esculenta* P. Henn. was collected from the gall tissues in *Zizania latifolia* (Griseb.) Turcz. Isolate of *U. scitaminea* was generously provided by Dr. HW Hsieh (Department of Plant Pathology, NCHU). Isolate of *U. maydis* was obtained from an innately infected corn gall from the experimental field of Taiwan Agricultural Research Institute (Wufeng, Taiwan). All fungal cultures were maintained on potato sucrose agar (PSA) plates and were subcultured once every month. The Czapek's solution used as a basal medium for IAA production, the source of inoculum and growth conditions were as described previously[3]. The gall tissues of *Z. latifolia* were collected from commercial field located in Puli, Taiwan. The healthy plant producing inflorescences was collected from the same field and was propagated in a pot outside the greenhouse (Taichung, Taiwan).

Purification and detection of IAA: For all experiments, 10^6 sporidial mL^{-1} suspension of *Ustilago* isolates was inoculated in 300 mL Erlenmeyer flasks containing 99 mL Czapek's solutions with 1 mM tryptophan. For substrate feeding experiments, the sporidial suspension was added to 10 mM phosphate buffer (pH 7.2) containing 100 μM each of indole-pyruvic acid (IPA), indole-3-acetamide (IAM), indole-3-acetadehyde (IAAld), or indole-3-lactic acid (ILA). All cultures were incubated at 25°C on a Model 711 shaker (Hotech, Taipei, Taiwan) set at 100 rpm. Each of 10 mL cultural solutions at the end of the incubation period was extracted three times with equal volume of dichloromethane, then the organic phase was combined and evaporated by nitrogen gas or with a Model R110 of Rotavapor (Brinkmann, Büchi, Switzerland). Indole compound was dissolved in 1 mL of 50% methanol containing 1 M acetic acid, then passed through a 0.2 μm pore size of PTFE membrane. The resulting solution was analyzed by a Model 1330 High Performance Liquid Chromatography (HPLC) (Bio-Rad, Richmond, CA, USA). Components in the solution were separated on a Lichrocart RP-18 reversed phase column (125x4 mm, 5 μm) (Merck, Germany) at room temperature, using 40% methanol containing 0.2 N acetic acid (pH 2.8) as a mobile phase. Presence of indole compounds and tryptophan were detected by a Model 1305A of UV monitor (Bio-Rad) at 280 nm wavelength. IAA and other indole compounds were verified by analysis of the authentically commercial product at the same conditions. To purify IAA from plant tissues, 20 g of each sample was ground in liquid nitrogen and extracted twice with 80% methanol solution containing 0.01 N acetic acid and 100 mg L^{-1} BHT [2,6-bis-(1,1-dimethylethyl)-4methyl phenol] for 12 h at 4°C. After removing methanol, the pH of aqueous solution was adjusted to 3.2 using 1 N HCl and extracted three times using dichloromethane. Indole compounds were dissolved in methanol/acetic acid solution and analyzed by HPLC as described above. In this study the recovery rate for tryptophan and IAA from cultural solution was approximately 90% in any experiments. Quantification of indoles was performed using regression line obtained for authentic indole standards. Each treatment was run with duplicates and all experiments were repeated twice.

Oxygen consumption during IAA production: Oxygen consumption was conducted in buffer containing 16.6 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) (pH 8.0), 66.6 mM KCl and 1 mM tryptophan as described by Comai and Kosuge[10,11]. Control was buffer without adding tryptophan. The reactions were conducted at 25°C for 2 days, then the oxygen consumption was measured with a Model SD-70 of oxygen detector (Suntex, Taipei, Taiwan).

RESULTS

IAA and tryptophan could be easily detected as distinct peaks by HPLC (Fig. 1A and B). The retention time for tryptophan and IAA was 2.7 and 3.3 min, respectively. The amounts of IAA in healthy and in various stages of gall tissues were first determined. The gall tissues were divided to seven stages based on the size and the color of the outer sheath (Fig. 2A). As compared to that of healthy plant, all stages of gall tissues had higher amounts of IAA despite considerable variations of IAA among different stages (Fig. 2B). The highest levels of IAA were detected at the first two stages (I and II). Higher levels of IAA in gall tissues could be resulted from *de novo* biosynthesis by the host and/or pathogen.

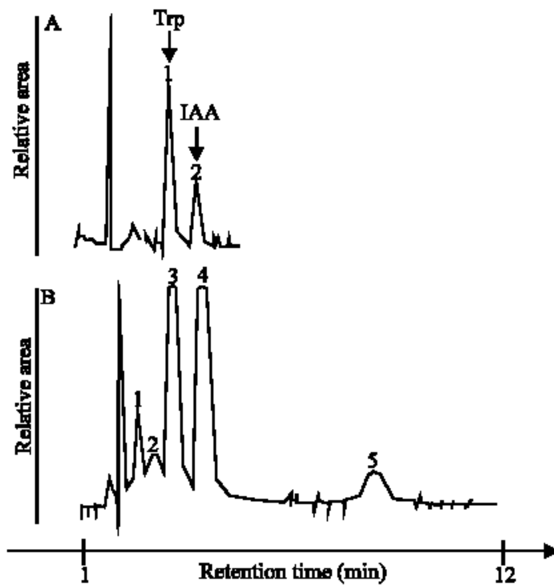

Fig. 1: HPLC chromatogram of standard 100 nM L-tryptophan (Trp, peak 1) and 10 ng mL^{-1} IAA (peak 2) (Sigma, St. Louis, MO, USA). (A). HPLC chromatogram of dichloromethane extract of *Ustilago esculenta* culture filtrate (B). Peak 3 and 4 appeared to be tryptophan and IAA, respectively. Peaks 1, 2 and 5 remain to be identified

The ability of *U. esculenta* to produce IAA in culture was then determined. To test the importance of tryptophan as a precursor for IAA biosynthesis, the fungus was grown in Czapek's solution supplemented with 0.1, 1, 10, 20, 40 mM tryptophan and indole compounds were extracted from the medium. No IAA or any other indole was detected in the absence of tryptophan, whereas in the presence of tryptophan IAA accumulated to high levels (Fig. 3). The amounts of IAA increased with increasing tryptophan concentrations, providing further support that IAA biosynthesis is tryptophan dependent.

The conditions required for optimum production of IAA *in vitro* were further investigated. A time-course study revealed that the amounts of IAA increased exponentially after 2 d in the Czapek's solution amended with tryptophan (Fig. 4). *U. esculenta* barely grew in the Czapek basal medium and tryptophan had no effect for growth stimulation (data not shown). Thiamine (vitamin B_1) has been proved to be important for growth enhancement in *U. esculenta*[3]. However, in the presence of thiamine, IAA production was completely inhibited in the medium containing tryptophan precursor (Fig. 4). IAA production was also inhibited when *U. esculenta* was grown in the medium containing yeast extract or host plant extract decoctions (data not shown).

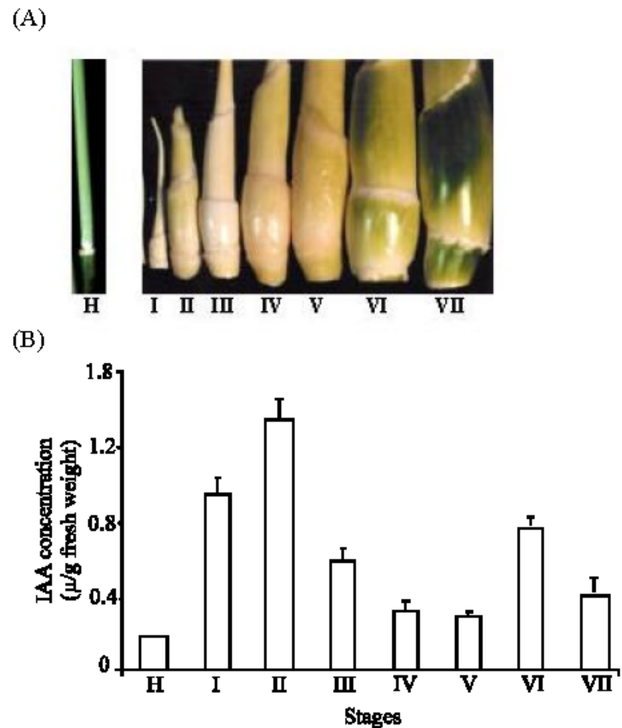

Fig. 2: The healthy, uninfected perennial aquatic grass, *Zizania latifolia* (H) and various stages of edible galls incited by a phytopathogen *Ustilago esculenta* (A). Identification and quantification of free indole-3-acetic acid (IAA) from the perennial aquatic grass and gall tissues using HPLC (B)

The effect of temperature on the IAA production was also determined. The result indicated that the optimum temperature for IAA production was around 25°C (Fig. 5). The amounts of IAA decreased as temperatures were dropped below 20°C. Only a trace amount of IAA was detected when the fungus was incubated at 30°C. Interestingly, the optimum temperatures for IAA production were also correlated with those for optimum growth. Temperature fluctuations (15/20°C and 15/25°C, day/night) significantly decreased the production of IAA (Fig. 5). When temperature was fluctuated between 15°C (12 h) and 20°C (12 h), the rate and amounts of IAA production were markedly reduced. The reduction of IAA production was much significant when temperature was fluctuated between 15°C and 25°C (12 h each), suggesting that a constant temperature is one of the critical factors for IAA biosynthesis in *U. esculenta*.

A survey of IAA production among *U. esculenta*, *U. maydis* and *U. scitaminea* revealed that all three species were able to utilize tryptophan as a precursor to

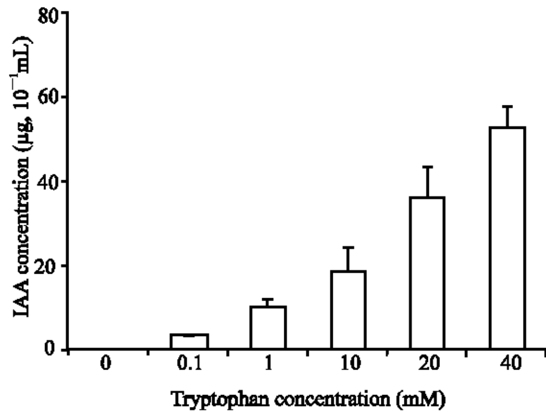

Fig. 3: Effect of different initial concentration of tryptophan on IAA production in *Ustilago esculenta*. Fungal isolate was inoculated into the Czapek's solutions containing various amount of tryptophan as indicated. The cultures were incubated under continuous shaking for 7 d. IAA and other indole compounds were extracted and determined by HPLC as described in the text

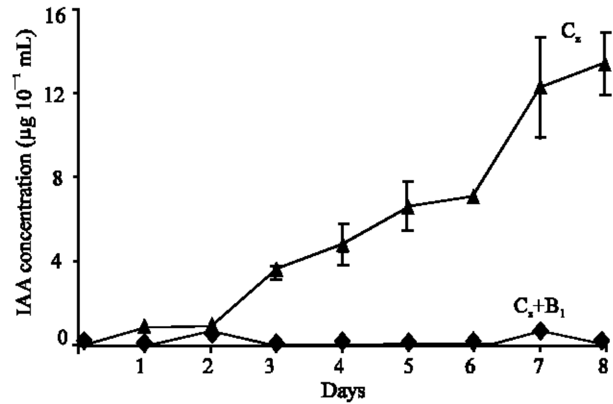

Fig. 4: Inhibition of IAA production by thiamine in *Ustilago esculenta*. Fungal isolate was inoculated in Czapek's solution containing 1 mM tryptophan and with or without 10 mM thiamine (vitamin B_1), then incubated under continuous shaking at 25°C. At the end of incubation periods, IAA was extracted with dichloromethane and analyzed by HPLC

synthesize IAA in cultures (Fig. 6). However, the amount and synthetic rate of IAA highly varied among *Ustilago* species. The IAA levels increased rapidly in *U. maydis* cultures during the early stages of incubation. It reached a maximum peak of IAA concentration (*ca.* 1.2 μg mL^{-1}) after 3 d, then IAA production was declined dramatically thereafter. The cultural medium became dark brown after 3 d although *U. maydis* continued to grow (data not shown). In contrast, *U. esculenta* produced IAA slowly but exponentially and reached a plateau (the maximum amount *ca.* 1.0 μg mL^{-1}) after 8 d. *U. scitaminea* produced IAA as much as *U. esculenta* did within the first 3 d, then IAA production no longer increased and reached a plateau (the maximum amount *ca.* 0.53 μg mL^{-1}). Both *U. esculenta* and *U. scitaminea* produced less pigmentation during incubation (data not shown).

The possible biosynthesis pathway of IAA in *U. esculenta* was investigated by feeding the putative precursors of IAA into the media. In the phosphate buffer, the test fungus barely grew. However, *U. esculenta* was able to convert indole-3-acetaldehyde (IAAld), indole-pyruvic acid (IPA) and indole-lactic acid (ILA), but not indole-3-acetamide (IAM) into IAA (Fig. 7). Feeding IAAld as a substrate resulted in accumulation of high levels of IAA. The amounts of IAA were lower while IPA or ILA was supplied, whereas IAA was undetectable while IAM was used as a primary source. To further exclude the possibility that IAM was a substrate for IAA biosynthesis in *U. esculenta*, the velocity of oxygen

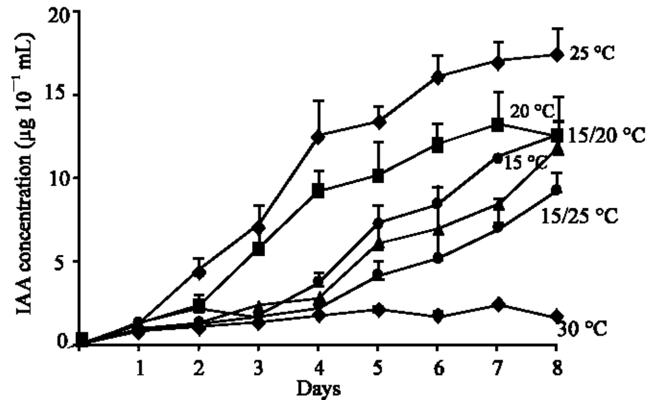

Fig. 5: Effect of temperature on IAA production by *Ustilago esculenta*. Sporidial suspension was inoculated into Czapek's solutions containing tryptophan at pH 6.5 and incubated under continuous shaking in the growth chambers with different temperatures as indicated. Temperature fluctuations were set for 12 h each of 15, 20°C and 12 h each of 15, 25°C, respectively. At the end of incubation periods, IAA was extracted and analyzed as described in the text

consumption was estimated during IAA production. If IAM involves in biosynthesis, significant amount of oxygen in the solution will be consumed due to the presence of tryptophane monooxygenase and IAM hydrolase in this pathway. The rates of oxygen

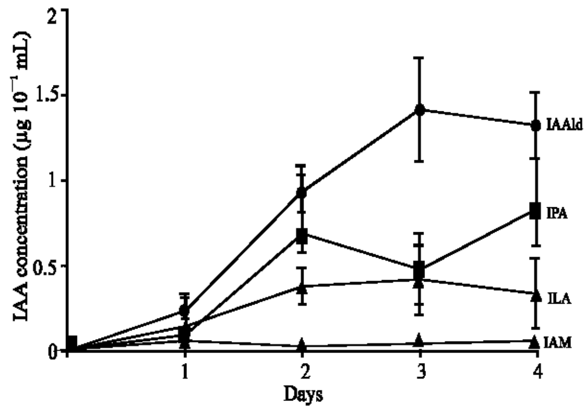

Fig. 6: Comparison of IAA production in cultures by *Ustilago esculenta*, *U. maydis* and *U. scitaminea*. Inoculation of sporidial suspension, conditions for incubation and IAA extraction and analysis were as previously described

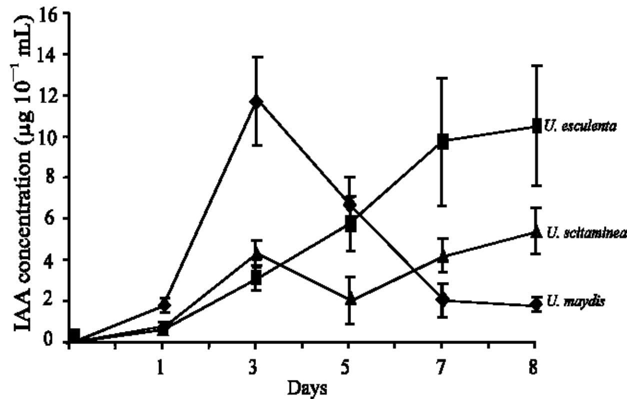

Fig. 7: Metabolism of indole-3-aldehyde (IAAld), indole-pyruvate (IPA), indole-lactic acid (ILA) and indole-3-acetamide (IAM) by *Ustilago esculenta*. Fungal isolate was grown in Czapek's solution containing tryptophan for 7 d, then harvested, washed with distilled water. The sporidial suspension was inoculated into 10 mM phosphate buffer (pH 7.2), then incubated at 25°C for additional period as indicated. Indole compounds were extracted daily and analyzed by HPLC

consumption were not significantly different when *U. esculenta* was incubated in the presence and absence of tryptophan (data not shown), suggestive of no role of monooxygenase involved in the IAA biosynthesis in *U. esculenta*.

DISCUSSION

The production of IAA by phytopathogens has been demonstrated as a pathogenicity factor and also a cause of gall formation in many plant-microbe interactions[5,8,12-19]. However, high levels of IAA in the infected tissues also can result from either the increased IAA biosynthesis by the host due to pathogen stimulation[20], or suppression of IAA degradation due to inhibition of IAA oxidase activity by pathogens[21]. To elucidate the mechanisms of gall development in Z. *latifolia* associated with *U. esculenta*, it is important to determine the ability of pathogen *in vitro* for IAA production. Since tryptophan is known to serve as a general precursor for IAA production in number of organisms, studies were undertaken on its metabolism in *U. esculenta* and other *Ustilago* species. For *U. esculenta*, relatively large quantities of IAA accumulated in cultural media when tryptophan was present. However, thiamine and nutrient media that support greatly for fungal growth completely aborted IAA production.

IAA production by *U. esculenta* has been suggested to correlate with the edible gall formation in *Z. latifolia*[4]. The conditions for IAA production and gall formation in the field are also closely correlated. Studies of the effect of temperature on the IAA production in cultures revealed that a constant temperature is crucial factor for IAA synthesis. Furthermore, the range of the optimum temperature for IAA production (20-25°C) is consistent with those of fungal growth[3] and gall development. Observations in the field revealed that some plants in the same field produced early galls around February-May if underground water was flooded into the field (the temperature of underground water was around 22°C in the region in the early spring). However, plants did not produce early galls after certain distance in the same field.

All of plants are propagated through asexual rhizomes and the genetic backgrounds between fungal isolates or host genotypes are identical. This argues that the early gall production is mainly due to a constant temperature generated by underground water. These results strongly suggested that the temperature of water surrounding the stem region of plants is one of the critical determinants for gall formation, probably by stimulating fungal growth and IAA production. This information shall be useful for gall production and management in the field by simply controlling water temperature.

U. maydis produced high quantities of IAA in culture within 3 d, then the production was steadily declined, indicating that *U. maydis* contains high activity of IAA oxidase or other IAA conjugating enzymes that further

degrade or modify IAA. It was also likely that a strong feedback inhibition of IAA synthetic enzymes by IAA itself or other intermediates are present in *U. maydis*. *U. scitaminea* produced much lower amount of IAA in culture compared to other *Ustilago* species, probably due to lower efficiency of IAA synthetic enzymes or a stronger feedback inhibition.

Several pathways for IAA biosynthesis from tryptophan have been identified in plants and microorganisms[22,23]. In general, tryptophan is the principal precursor for the production of IAA. In many plants, IAA biosynthetic pathway usually involves IAAld as an intermediate. For some other plants, indole-acetonitrile is an intermediate for IAA production[24]. Biosynthesis of IAA in the gall-inducing phytopathogenic bacteria, *Agrobacterium tumefaciens* and *Pseudomonas savastanoi*, proceeds from tryptophan to IAM, then IAA[10,11,25]. Another phytopathogenic bacterium, *Erwinia herbicola* can synthesize IAA either from IAM or IPA pathway, but with distinct patterns for expression and regulation[18]. Furthermore, IAA synthetic pathway in *P. solanacearum* (K-60) is completely different route, in which IAA is synthesized via kynurenine pathway[26]. Little is known about IAA synthesis in fungi. The biosynthesis of IAA by phytopathogenic fungi is assumed to proceed along the pathway similar to those of the host plant[9]. However, some divergent pathways leading to IAA production have been demonstrated. IAA synthesis in *Gymnosporangium* sp. involves tryptamine and IAAld as intermediates[14], whereas IPA and IAAld are two key intermediates for IAA synthesis in *M. lini* and *Taphrina* sp.[12,13,27]. More recently, both pathways involved in IAAld and IAM are identified in *Collectotrichum gloeosporioides* f. sp. *aeschynomene* and *C. acutatum*[28,29]. These exceptions demonstrate that IAA biosynthetic pathway in an organism should not be assumed without experimental documentation.

The possible synthetic pathway of IAA in *U. esculenta* was investigated by substrate feeding experiments. No IAA was accumulated by feeding IAM and no oxygen was significantly involved during IAA production, strongly suggesting that IAM is not a suitable precursor. However, feeding IAAld, IPA and ILA resulted in accumulation of IAA, implying that these compounds are possible intermediates for IAA production in *U. esculenta*. Feeding IAAld or IPA intermediate was inconclusive since either compound was unstable and tended to change spontaneously into IAA[29]. However, as proposed in *U. maydis*[8], IAA is also likely synthesized from tryptophan through IPA, then IAAld in *U. esculenta*. Recently, two NAD^+-dependent indole-3 acetaldehyde dehydrogenases that are responsible for conversion of IAAld to IAA are identified in *U. maydis*[30]. This further suggests the assumption that *U. esculenta* may synthesize IAA through IAAld pathway. Utilization of ILA as a substrate for IAA production likely represents a branch of pathway from IPA as suggested by Kaper and Veldstra[31], or another pathway through ILA exists in *U. esculenta*. However, no ILA was detected from cultures by HPLC chromatogram, suggesting that enzymes involved in IPA pathway also exhibit activities to convert ILA to IAA. ILA has been documented to be the major byproduct of IAA biosynthesis via the IPA/IAAld pathway[29,32], further confirming the presence of IPA/IAAld pathway. Further experiments using radioisotope-labeled substrates will be required to elucidate the whole biosynthetic pathway for IAA production.

ACKNOWLEDGMENTS

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