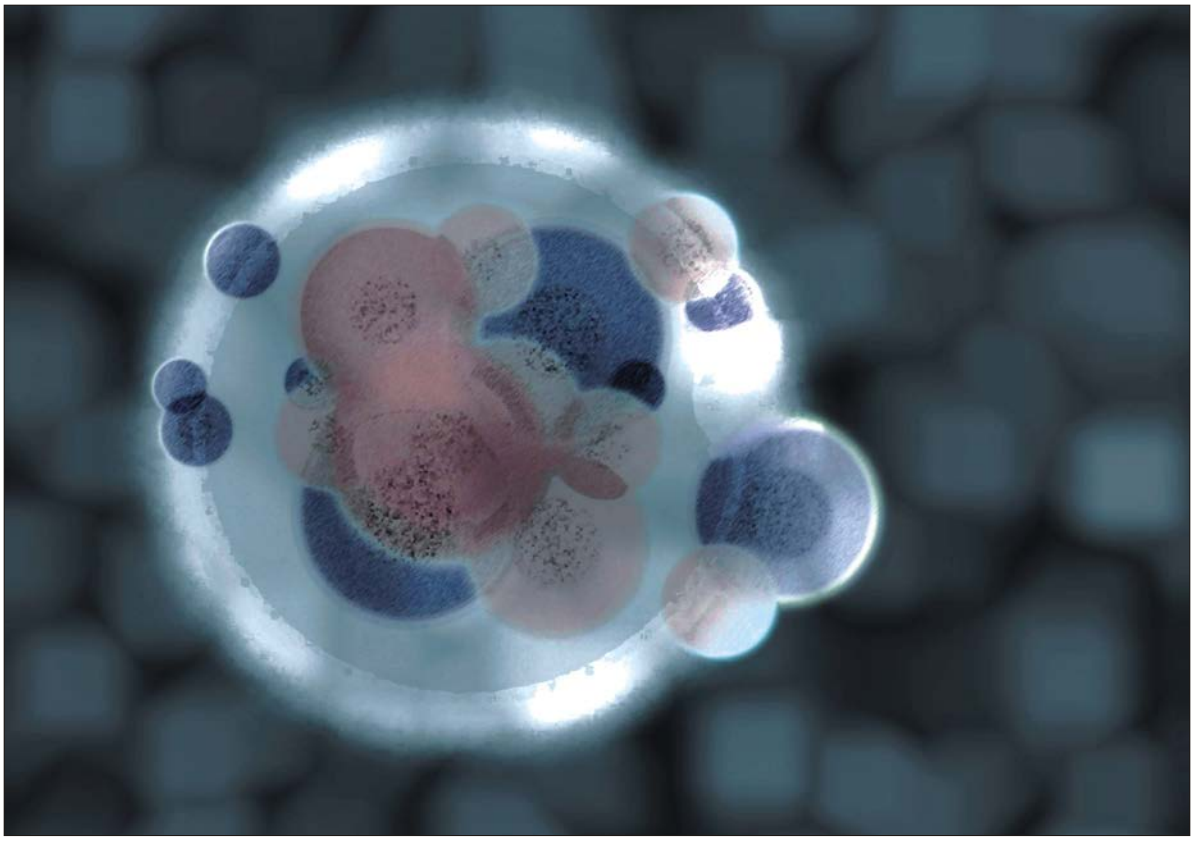

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Physiological Regulation of Biosynthesis of Phytohormone Indole-3-acetic Acid and Other Indole Derivatives by the Citrus Fungal Pathogen *Colletotrichum acutatum*

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Abstract: Present study investigated the regulation and production of phytohormone indole-3-acetic acid (IAA) and other indole derivatives by the fungus *Colletotrichum acutatum*. Using HPLC and chromogenic stains after fluorescence thin-layer chromatography, biosynthesis of IAA and other indoles, including indole-acetaldehyde (IAAld), indole-acetamide (IAM), indole-lactic acid (ILA), indole-pyruvic acid (IPA) and tryptophol (TOL) were solely dependent on the presence of tryptophan (Trp) and were highly regulated by carbon and nitrogen sources by *C. acutatum*. As compared to other sources, the production of IAA increased drastically (up to 12-fold) using mannitol or galactose as the sole carbon source, or using ammonium nitrate as the sole nitrogen source, whereas IAA was completely suppressed in the presence of ammonium chloride. The putative pathways for IAA biosynthesis by *C. acutatum* likely proceeded via Trp/IPA/IAAld and Trp/IAM intermediates. This study provides an opportunity to identify genes involved in IAA biosynthesis by using suppression subtractive hybridization approach and provides a nutrient base for screening IAA non-producing mutants of *C. acutatum*.

Key words: Hormones, phytopathogenic fungi, sweet orange, tryptophan, tryptophol

INTRODUCTION

Phytohormones such as indole-3-acetic acid (IAA, auxin), indole-related compounds, gibberellins, cytokinins, ethylene, abscisic acid and many other growth regulators interact with each other and play profound roles in developmental and physiological processes of plants. The imbalance of these growth regulators caused by pathogenic attack is the major cause of many disease symptoms, including hyperplasia, stunting, leaf epinasty, excessive root branching, tissue malformation and premature leaf drop[1]. Among various growth regulators, IAA has been demonstrated as an important pathogenicity factor in various bacteria-plant interactions[2]. Many fungal diseases such as smut gall as well as witches' bloom and leaf curl caused by *Ustilago maydis* and *Taphrina* species, respectively, are also likely due to the disturbance of endogenous IAA[3,4]. However, the roles of IAA and other indoles in the fungal-plant interactions have not yet been fully determined.

Colletotrichum acutatum causes Postbloom Fruit Drop (PFD) of sweet oranges and Key Lime Anthracnose (KLA). Isolates of this species causing both diseases infect flower petals resulting in blossom blight and inducing young fruit drop and the formation of persistent calyces in the affected trees[5]. The healthy flowers and young fruit next to affected flowers also tend to fall and form persistent calyces. In addition, the leaves around the affected flowers often become deformed with swollen veins. Molecular analysis using gene expression profiles indicated the genes encoding jasmonic acid and ethylene biosynthesis and auxin responsive genes were highly up-regulated in the citrus flowers after fungal infection[6]. The levels of ethylene and IAA also greatly increased after fungal colonization. Furthermore, using IAA transport inhibitors or other anti-auxin compounds could cause fruit in trees to be retained after fungal infection (Chung *et al.*, unpublished data). We therefore hypothesize that the imbalance of phytohormones, especially IAA, is likely involved in the formation of persistent calyces and in young fruit drop[7].

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Increased hormone levels in the affected flowers can result from *de novo* biosynthesis by the host plant and/or pathogen. Previous studies revealed that *C. acutatum* isolates were able to utilize tryptophan (Trp) as a precursor to synthesize low levels of IAA and other indole derivatives in culture, despite the fact that the production of those compounds was highly variable among isolates[8]. The production of secondary compounds in an organism can be regulated by genetic and physiological factors. The aim of this study was to understand the regulation and production of indole derivatives by the carbon and nitrogen sources in the phytopathogenic fungus *C. acutatum*. The results indicated that the production of IAA and other indoles were markedly regulated by carbon and nitrogen sources, suggesting differential expression of genes involved in IAA biosynthesis. The conditions for the production of IAA and other indoles obtained in this study shall facilitate the identification of genes up-regulated in *C. acutatum* using a suppression subtractive hybridization[9].

MATERIALS AND METHODS

Fungal strains and growth conditions. Fungal isolates (KLA 207 and Navel) of *Colletotrichum acutatum* J. H. Simmonds used in this study were isolated from surface-sterilized leaves and flower petals of infected plants. Methods for isolation, culture, maintenance and characterization were described elsewhere[10]. For indole production, fungal isolate (KLA207) was grown in Czapek's broth (pH 6.5) containing sucrose and sodium nitrate as a sole carbon and nitrogen source, respectively and 10 mmol L^{-1} tryptophan for 4 days. To investigate the effects of various carbon and nitrogen sources on the production of IAA and other indole compounds, 90 m mol L^{-1} of the carbon source (equivalent carbon content to that of sucrose) and 24 m mol L^{-1} of the nitrogen source (equivalent nitrogen content to that of sodium nitrate) were added to replace sucrose and sodium nitrate, respectively, in Czapek solution. Feeding experiments using various IAA intermediates were conducted in 1 m mol L^{-1} phosphate (pH 7.2) buffers with a fungal isolate (Navel). Fungal dry weight was estimated in a pre-weighted filter paper. At the end of the incubation period, the contents of each 25 mL Erlenmeyer flask were filtered through a pre-dried and pre-weighed Whatman No. 1 filter paper and washed with deionized water. The filters were dried at 90°C for 24-36 h.

Purification and quantification of indole compounds. Indole derivatives were purified using ethyl acetate and subsequently analyzed by HPLC using a Nucleosil 120-5 C18 reverse column (5 μm, 250x4 mm) (Richard Scientific, Novato, Calif.) as previously described[8]. Quantification of indoles was performed using regression line obtained for authentic indole standards. All experiments were conducted at least three times. Identification of indole-3-pyruvic acid (IPA) using HPLC was problematic. A chromatogenic reagent 2,4-dinitrophenyl hydrazine (2,4-DNPH) that specifically reacts with IPA by forming a distinct orange color was used to further verify IPA using thin-layer chromatography (TLC) plates with silica gel 60 F_{254} fluorescent plates (Fig. 1A and B). The conditions used for TLC separation and chromatogenic staining was conducted as previously described by Chung *et al.*[8].

RESULTS

Previous studies indicated that several major indole compounds including IAA, IAAld, IPA, IAM, ILA and TOL were purified from a cultural filter of *C. acutatum* and were readily separated and detected as a distinct peak by HPLC using a C18 reverse column, providing a reliable means for indole identification and quantification[8]. The production of IPA was further confirmed using fluorescence TLC plates followed by 2,4-DNPH staining due to its instability. By using fluorescent TLC plates, IPA with R_f 0.48 was visualized as a distinct band under short-wavelength UV light (Fig. 1A) and reacted with 2,4-DNPH to form a distinct orange color (Fig. 1B). The ethyl acetate extracts from fungal cultures grown in different carbon sources had similar bands at R_f 0.48 that also reacted with 2,4-DNPH to form orange colors, thus confirming the identity of IPA (Fig. 1).

A study was conducted to determine the effect of carbon sources on the production of IAA and indoles. In the presence of Trp, KLA207 isolate produced low level of IAA (5.3 μg mL^{-1} mg^{-1} fungal weight) in the original Czapek's broth containing sucrose and sodium nitrate as sole carbon and nitrogen sources, respectively. As shown in Fig. 2, the original Czapek's broth containing sucrose/sodium nitrate supported low levels of IAA, IAAld, IAM, TOL and IPA, whereas high ILA production was observed. The accumulation of IAA increased slightly when the fungus was grown in a medium containing fructose, glucose or maltose as the sole carbon. Highest levels of IAA (= 50 μg mL^{-1} mg^{-1} fungal weight) were detected when the fungus was grown in a medium containing galactose or mannitol as the sole carbon source. Compared to other carbon sources, mannitol also supported higher production of IAM, TOL and IPA. However, the levels of ILA were drastically reduced when the fungus was grown in fructose- and mannitol-containing media. No IPA was identified when the fungus was grown in the presence of fructose using 2,4-DNPH staining (Fig. 1B) or HPLC (Fig. 2).

To determine the effect of nitrogen sources on the production of IAA and other indoles, five common nitrogen sources (calcium nitrate, potassium nitrate,

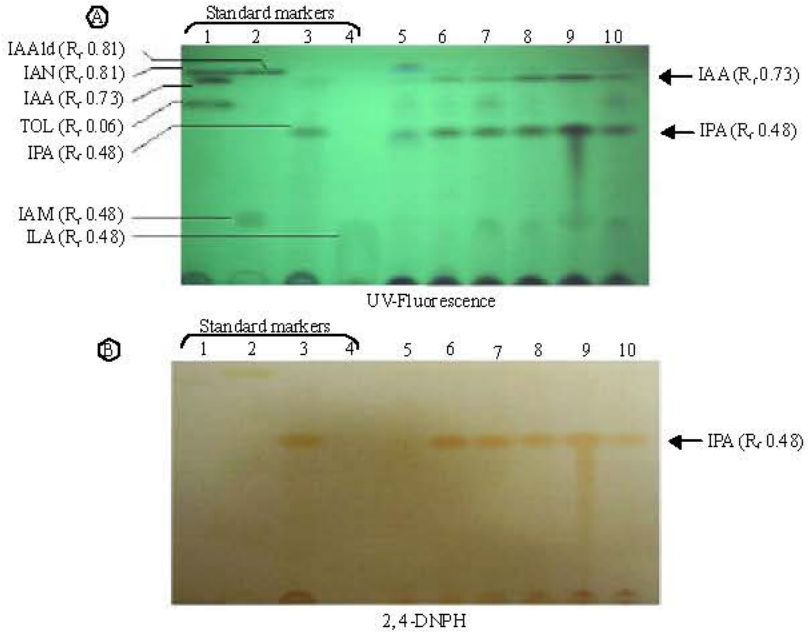

Fig. 1: Identification of indole pyruvic acid (IPA, indicated by arrow) produced by *Colletotrichum acutatum* using a fluorescence TLC plate (A) and the 2,4-DNPH chromogenic reagent (B). Ethyl acetate-extracted samples from fungal culture grown in a medium containing fructose (lane 5), galactose (lane 6), glucose (lane 7), maltose (lane 8), mannitol (lane 9), or sucrose (lane 10) as a sole sucrose source were loaded onto silica gel fluorescent plates and developed in a solution containing n-hexane, ethyl acetate, isopropanol and acetic acid. After development, the plate was visualized directly using a short wavelength UV light (A), or stained with 2,4-DNPH reagent (B). The authentic indole compounds with 5 m mol L^{-1} each (lanes 1-4) were loaded on the plates for reference. Their mobility (R_f) and related positions are indicated at the left

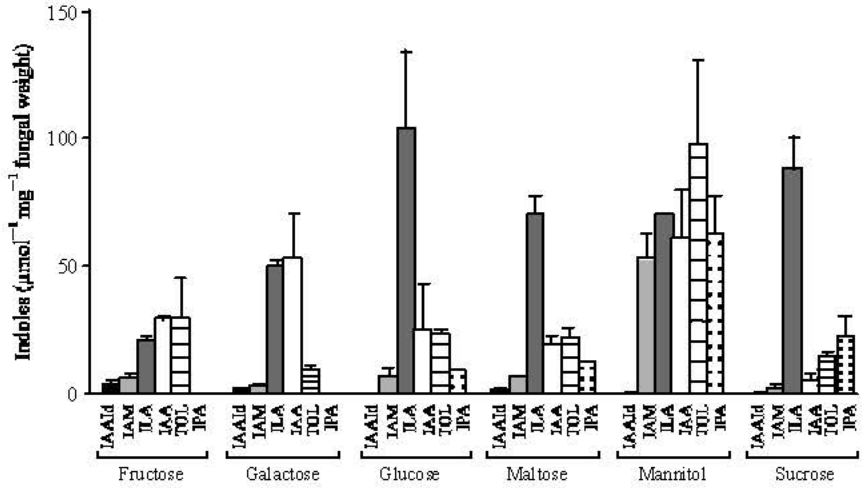

Fig. 2: Effect of carbon sources on the biosynthesis of indole-3-acetic acid (IAA), tryptophol (TOL), indolelacetic acid (ILA), indoleacetaldehyde (IAAld), indole-pyruvic acid (IPA) and indole-acetamide (IAM) by *Colletotrichum acutatum* grown in a medium supplemented with 10 m mol L^{-1} tryptophan. Fungal isolate KLA 207 was grown for 7 day and the filtrate was extracted with ethyl acetate. The resulting indole compounds were analyzed by HPLC using a C18 reverse column. The concentrations of indole compounds were calculated by reference to the peak area obtained for authentic standards using a linear regression curve. The data shown are the mean values and standard errors calculated from three independent experiments

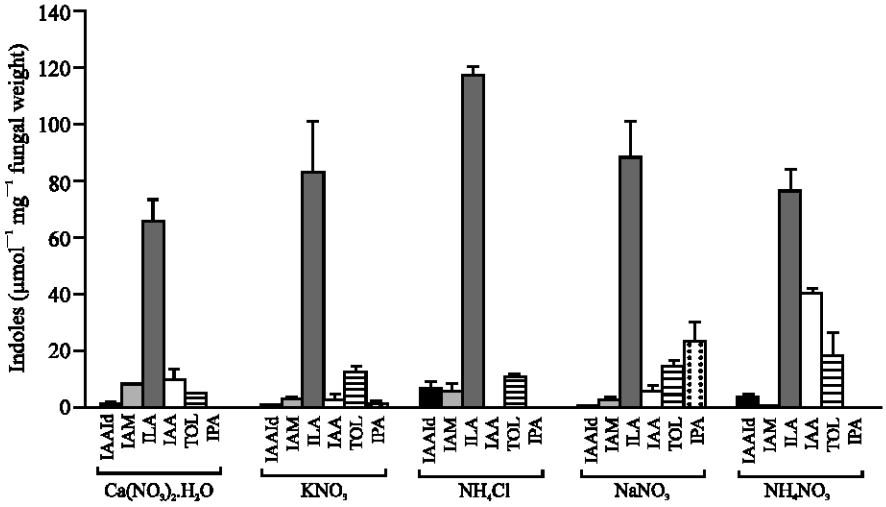

Fig. 3: Effect of nitrogen sources on the accumulation of indole-3-acetic acid (IAA), tryptophol (TOL), indoleacetic acid (ILA), indoleacetaldehyde (IAAld), indole-pyruvic acid (IPA) and indole-acetamide (IAM) by *Colletotrichum acutatum* grown in the medium supplemented with 10 mmol L^{-1} tryptophan. The data shown are the mean values and standard errors calculated from three independent experiments.

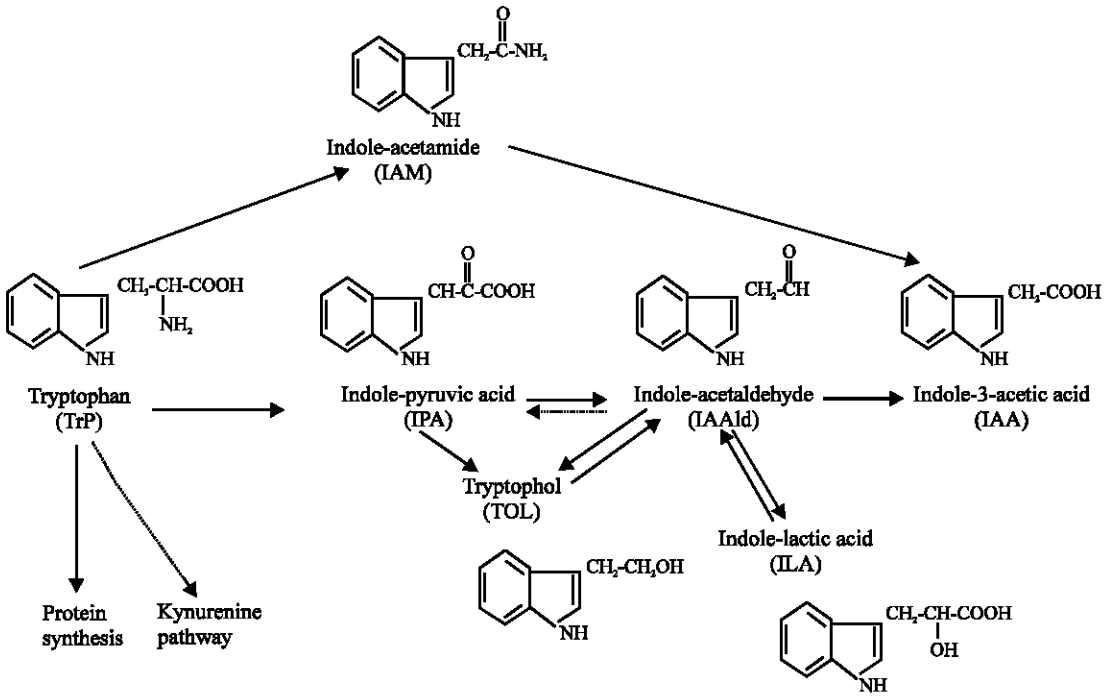

Fig. 4: Proposed routes of indole-3-acetic acid biosynthesis pathway from tryptophan detected in *Colletotrichum acutatum*. The results were compiled from feeding experiments using tryptophan (Trp) and other indole intermediates and from existing literatures[17,12,11,18].

ammonium chloride, sodium nitrate and ammonium nitrate) were tested and revealed that all nitrogen sources triggered high amounts of ILA in cultures (Fig. 3). In the presence of sucrose, the levels of IAA were suppressed in most of nitrogen sources tested except for ammonium nitrate. The medium containing ammonium nitrate/sucrose supported higher levels of IAA production, however, this medium markedly suppressed the production of IAM and IPA. The medium containing ammonium chloride/sucrose completely suppressed the production of IAA and IPA,

but resulted in increased IAAld accumulation compared to other nitrogen sources (Fig. 3).

The actual sequence of IAA biosynthetic pathways remains to be determined. However, repeated identification of IAAld, IPA, TOL, ILA and IAM from *C. acutatum* cultures supplemented with Trp suggests the existence of multiple pathways for IAA biosynthesis (Fig. 4). The ability of *C. acutatum* to metabolize indole intermediates into IAA could provide evidence for the presence of a metabolic pathway for IAA biosynthesis. Feeding experiments with different precursors were therefore conducted to determine if the fungus could synthesize IAA from metabolites of the IPA/IAAld and IAM pathways. Feeding IAAld or IPA intermediate was inconclusive since either compound was unstable and tended to change spontaneously into IAA and TOL (data not shown). However, the presence of IPA/IAAld pathway for IAA biosynthesis in *C. acutatum* was evident by the identification of IPA, IAAld, TOL and ILA.

IAM was consistently identified from a cultural extract of *C. acutatum*. The presence of the IAM pathway for IAA biosynthesis in *C. acutatum* was further supported with feeding experiment using the IAM intermediate. IAM remained unchanged in the absence of fungus, but was converted into IAA by *C. acutatum* after 24 h incubation (data not shown). Feeding of TOL or ILA, however, resulted in no detection of IAA. Although tryptamine (TNH_2) and indoleacetonitrile (IAN) were apparently not intermediates for IAA biosynthesis by *C. acutatum*, feeding these indoles led to the formation of low levels of IAA (data not shown). There was no indication that IAA was converted into a conjugated or oxidized form since it remained unchanged after 7 d.

DISCUSSION

We observed a significant increase of IAA in citrus flower petals after *C. acutatum* infection, leading us to hypothesize that IAA plays a pivotal role in the formation of persistent calyces and young fruit drop[6,7]. Since isolates of *C. acutatum* were capable of producing IAA and related indole compounds in culture, the higher levels of IAA in affected petals were likely, in part, due to the *de novo* biosynthesis by the causal fungus. To test how IAA affects the development of symptoms, we intend to create fungal mutants that are defective in IAA biosynthesis. Comparisons of the ability for the induction of persistent calyces and young fruit drop between wild type and IAA non-producing mutants will allow us to determine if the production of IAA by *C. acutatum* plays any role in increasing IAA and in the development of symptoms.

Previous studies revealed that *C. acutatum* isolates were able to utilize Trp as a precursor to synthesize IAA and other indoles in culture[8]. However, increasing the amount of Trp only slightly increased the levels of IAA, but significantly increased the levels of TOL and ILA. The Czapek's medium containing sucrose and sodium nitrate as sole carbon and nitrogen sources, respectively, supported low amounts of IAA production. In this study we investigated the effects of carbon and nitrogen sources on the production of IAA and other indole derivatives by *C. acutatum* isolates. In general, *C. acutatum* tended to accumulate higher levels of ILA and TOL in most nutrient conditions tested. Both TOL and ILA have been documented to be the major byproducts of IAA biosynthesis via the IPA/IAAld pathway[11,12]. TOL also has been suggested to act as a regulator in the levels of IAA and IAAld in plants[13].

In this study, the types of carbon or nitrogen had drastic effects on the accumulation of IAA and other indoles. The accumulation of IAA was increased almost 12 fold when the tested fungus was grown in a medium containing mannitol or galactose as the sole carbon source as compared to that of sucrose. The medium containing mannitol also markedly increased the accumulation of IAM, TOL and IPA, but decreased the production of ILA. Of the five nitrogen sources tested, all stimulated higher levels of ILA production. Ammonium chloride completely suppressed the production of IAA and IPA. In contrast, ammonium nitrate drastically increased the IAA production, but not IPA. It is unclear how different carbon or nitrogen sources regulate the indole production.

As with many microorganisms, the production of IAA and other indoles by *C. acutatum* was also solely dependent on the presence of tryptophan[8]. Tryptophan is a physiologically important substance and mainly used for protein synthesis. Tryptophan can also be metabolized by a variety of pathways such as kynurenine and indole pathways[11]. IAA can be synthesized from tryptophan through various intermediates, depending on organisms. The detailed pathways for IAA by *C. acutatum* remain elusive. However, judging from the identification of IPA, IAAld, TOL and ILA and the results of intermediate-feeding experiments in this study, *C. acutatum* also mainly utilized the Trp/IPA/IAAld pathway to synthesize IAA. Considerable evidence has also been accumulating in support of the fact that the IPA/IAAld pathway is the predominant IAA biosynthetic pathway operating in plants and fungi[14]. Interestingly, as with many bacteria and some fungi[15,16], isolates of *C. acutatum* also could utilize IAM as an intermediate to synthesize IAA. Since higher plants lack the IAM pathway, it seems that the existence of the IAM pathway

in a microorganism may override the regulatory mechanism for controlling the IAA level.

In conclusion, this study demonstrates that the production of IAA and other indoles by *C. acutatum* was affected by different carbon and nitrogen sources. It is tempting to speculate that genes involved in IAA and other indole biosynthesis might be highly inducible by different carbon or nitrogen sources. The specific knowledge gained about the regulation of IAA production in the isolates of *C. acutatum* will be very helpful in conducting genetic analysis of IAA biosynthesis. In particular, difference of IAA suppression and stimulation by ammonium chloride and ammonium nitrate may provide ideal conditions for gene identification for IAA biosynthesis using a suppression subtractive hybridization[9].

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