

## Identification of Auxin from *Pseudomonas* sp. P7014 for the Rapid Growth of *Pleurotus eryngii* Mycelium

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### 큰느타리버섯 균사체의 생육촉진을 위한 *Pseudomonas* sp. P7014으로부터 옥신 확인

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The promoting effect of *Pseudomonas* sp. P7014 on the mycelia growth of *Pleurotus eryngii* was investigated. An ethyl acetate fraction (F5) from the culture supernatant of the bacteria was confirmed to contain the growth promoting compound (GPC). The GPC was identified to be indole acetic acid (IAA) by TLC, HPLC, MS/MS, and NMR analyses. *P. eryngii* mycelia grew rapidly both on PDA and in PDB after the treatment of GPC. The promoting concentration of GPC was as low as 1.0 nM. Tryptophan, the aminated form of IAA, was confirmed to be the precursor of IAA. These results suggested that bacterial secreted compound was IAA and plays an important role in promoting growth of mushroom mycelia.

**Keywords:** *Pleurotus eryngii*, *Pseudomonas* sp. P7014, bioassay, growth promotion, indole acetic acid

King oyster mushroom (*Pleurotus eryngii*) is the third largest commercially produced mushroom (Bano and Rajarathnam, 1987; Obodai *et al.*, 2003) in the world. The increasing popularity of *Pleurotus* mushroom among consumers is not only due to its flavor, texture and shelf life but also to immunomodulating and oxidative enzyme production (Mark *et al.*, 2004; Silva *et al.*, 2005). In the cultivation of *Pleurotus* mushrooms, the biological properties of composts appear to be very important for inducing fruiting body formation. At least one mushroom species, *Agaricus bisporus*, growth and development are particularly affected by bacteria of the family *Pseudomonadaceae* (Grewal and Rainey, 1991). In addition, Cho *et al.* (2003) report that fluorescent pseudomonads promote the growth of *Pleurotus* sp. Representatives of the genus *Pseudomonas* are common in soil and on plants, and a few are associated with mushrooms. There

are numerous reports on the improvement of plant growth and crop yield upon inoculation with rhizosphere bacteria. The observed plant response to bacterial inoculation is attributed to the production of bacterial indole-3-acetic acid (IAA) (Barbieri and Galli, 1993; Okon and Vanderleyden, 1997). Unlike plants, mushrooms cannot synthesize auxins endogenously. However, the hormonal effects of auxins on mushrooms can be elucidated by studying exogenous effects. There is also ample evidence that numerous soil micro-organisms are actively involved in the synthesis of auxins in pure culture and in soil (Arshad and Frankenberger, 1998; Barazani and Friedman, 1999; Biswas *et al.*, 2000).

*Pleurotus sajor-caju* is cultured under submerged condition with plant growth hormones (indole-3-acetic acid, gibberellic acid, and kinetin) and twenty-eight percent more enhancements are observed with indole-3-acetic acid (Mukhopadhyay *et al.*, 2005). Wang *et al.* (2009) and Wang *et al.* (2010) report that the aposymbiotic growths of *Ramalina farinacea* and *R.*

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*fastigiata* are increased by plant hormones (indole-2-butyric acid and 2,3,5-tri-dobenzoic acid) on malt-yeast extract medium and the growths of lichen-forming fungi (*Nephromopsis ornata*, *Myelochroa irrugans*, and *Usnea longissima*) are promoted by plant hormones (auxin, abscisic acid, cytokine, and gibberellin), respectively. In contrast, there are a few reports on the improvement of mushroom growth upon inoculation with bacteria and very few of these are attributed to the production of IAA by bacteria.

IAA is the most important naturally occurring auxin and is implicated in many aspects of plant growth and development. Promotion of root growth is one of the beneficial effects of plant growth-promoting bacteria (Glick *et al.*, 1995). Most root-promoting bacteria synthesize IAA and their effects on plants mimic that of exogenously applied IAA. L-tryptophan (L-TRP), an amino acid, serves as a physiological precursor for the biosynthesis of auxins in plants and microbes (Frankenberger and Arshad, 1995).

Although many reports suggested that the addition of different pseudomonas species may have a beneficial effect on mushroom mycelium growth, the active factor or factors have not been conclusively identified. We examined bacterial extracts in order to identify compounds responsible for growth promotion. The work presented here is details an investigation of factors that are involved in enhancing *Pleurotus eryngii* mycelium growth secreted by bacteria.

## Materials and Methods

### Media, culture conditions, and growth promoting test of bacterium

A bacterium, *Pseudomonas* sp. P7014, used in this study was previously described by Kim *et al.* (2008) and identified by 16S rRNA analysis (GenBank accession no. EF154396). The effects of its culture supernatant on *P. eryngii* mycelium growth were examined as described previously (Kim *et al.*, 2008).

### Extraction and identification of growth promoting compound (GPC)

Filtered mycelia of *P. eryngii* were dried and weighed. The dry mass of mushroom mycelium, were grown in potato dextrose (PD, Becton Dickinson Co., USA) containing a range of GPC prepared concentration. Inoculated flasks were incubated at 25°C for 15 days. Bacteria in culture of *Pseudomonas* sp. P7014 were pelleted by centrifugation at (8,000 × g) for 30 min. The supernatant was then mixed with an equal volume of ethyl acetate and extracted by the procedure outlined in Tien *et al.* (1979). The extracts (ethyl acetate extraction) were filtered through a 0.45 µm membrane filter in

preparation for analytical thin layer chromatography (TLC), high-pressure liquid chromatography (HPLC). Additionally, the GPC was analyzed by LC-mass spectrometry (MS) and nuclear magnetic resonance (NMR).

TLC was done using aluminum silica gel 60 (Merck). The solvent system was ethyl acetate:isopropanol:ammonium hydroxide [45:35:20 (v/v)] to separate indole compounds. Specifically indole acetic acid (IAA, Sigma-Aldrich Co., USA) was detected on TLC plates by Salkowski's reagent (H<sub>2</sub>SO<sub>4</sub> 150 ml + H<sub>2</sub>O 250 ml + 0.5 M FeCl<sub>3</sub> · 6H<sub>2</sub>O 7.5 ml) staining. The TLC bands were extracted with methanol (Fisher Scientific Co., USA); further eluted quantified fractions were diluted with sterile water for getting appropriate concentration and tested for effectiveness by plate assay in PD agar medium. For each 100 ml of medium, 1 ml of an ethyl acetate fraction or 2 ml of eluted fraction, respectively, were added. Plates were then inoculated with a mycelium containing PDA block and incubated for 12 days at 25°C with 65±5% humidity.

For preparation of the compound fraction, the corresponding spots were scratched out of the thin-layer chromatograms, and the silica gel material was extracted with methanol. For further purification, 1 mg of extract was subjected to reversed phase high performance liquid chromatography (RP-HPLC; LC-908, JAIGEL-1H column, Japan Analytical Industry, Japan). The mobile phase for elution was acetonitrile/water (1:1, v/v) (Fisher Scientific Co.) at a flow rate of 2.5 ml/min and the absorbance of the separated IAA containing fractions were measured at 280 nm.

The GPC was analyzed by LC-mass spectrometry (MS) and nuclear magnetic resonance (NMR). Fractions containing tentative IAA (as determined by comparison with the retention time of authentic IAA) were pooled, concentrated, dissolved in 100 µl CDCl<sub>3</sub> and transferred for NMR at 300 MHz (Bruker DRX-600 equipped with a 2.5-mm SEI microprobe, Bruker Co., Germany). NMR data from <sup>13</sup>C NMR experiments was used to identify IAA by comparison with NMR data recorded from IAA standards. The isolated tentative IAA was also analyzed by MS/MS in the positive mode (Esquire LC ion trap mass spectrometer, equipped with an electrospray ion source, Bruker Daltonik, Bruker Co.). Samples were dissolved in methanol/water (1:1) and injected using a syringe pump at 1 µl/min. The scan range was 100–500 *m/z* and the results from ten mass spectra were averaged. For MS operation the ion accumulation time was 1 ms, whereas for MS/MS operation the ion accumulation time was 30 ms.

### Quantification of indole acetic acid (IAA) production

IAA production in wild-type P7014 strain was studied as previously described (Glick and Patten, 2002). GP Media

(Glucose 40/g; peptone 10/g medium) was used along with DF salt minimal medium (Glick and Patten, 2002). The concentration of IAA in each culture medium was determined by comparison with a standard curve. As well as, filtered supernatant was analyzed by using HPLC and IAA was quantified by integrating the areas under the peaks comparing to authentic standard IAA. IAA production by P7014 strain was measured in triplicate.

HPLC was carried out by injecting 10  $\mu$ l of the filtered extract onto a reverse-phase C<sub>8</sub> column (4.6  $\times$  250 mm, Brownlee, Applied Biosystems) on PerkinElmer Series 200 series HPLC (Perkin-Elmer Co., USA). Samples were eluted at a flow rate of 1 ml/min using 0.5% acetic acid and methanol (65:35 [v/v]) with an operating pressure of 140 Bar. The absorbance of the separated IAA containing fractions were measured at 280 nm and quantified by comparing IAA standards to bacterial cultures by UV detection (Perkin-Elmer 200 series, Perkin-Elmer Co.).

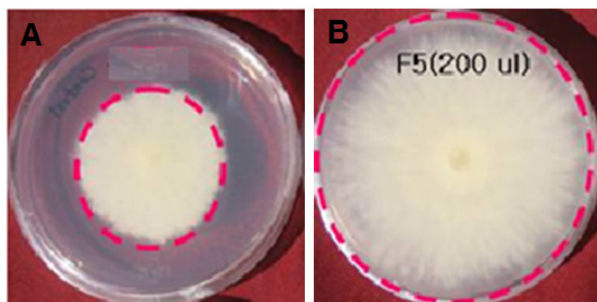
### Statistical analysis

All values are means of determinations in three independent experiments. Differences in the means of each value were determined by one-way ANOVA followed by the Tukey's multiple range tests at  $P < 0.05$  using the Statistical Analysis System software, Version 9.0 (SAS Institute, USA).

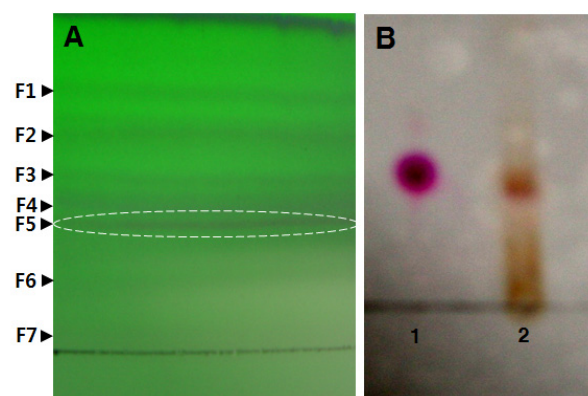
## Results and Discussion

### Identification of growth promoting compound (GPC) from *Pseudomonas* sp. P7014

Ethyl acetate fractions from a *Pseudomonas* sp. P7014 supernate exhibited a growth promoting effect on mushroom mycelia compared to control plate in reproducible manner. TLC of the ethyl acetate extract from the culture supernatant of

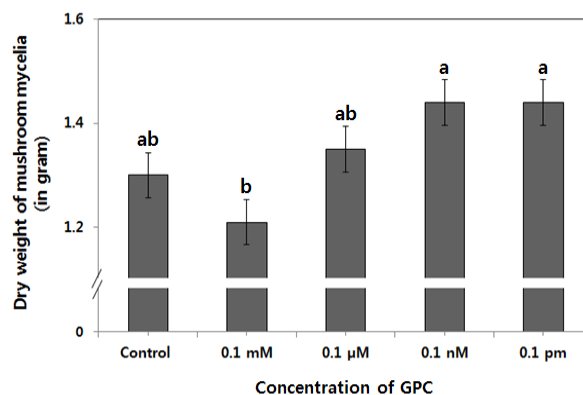


**Fig. 2.** Effect of *P. ergyii* mycelium growth by the treated growth promoting compound (GPC). (A) Control and (B) ethyl acetate fraction No. 5 (F5). F5 was scratched out of the thin-layer chromatograms, and the silica gel material was extracted with methanol. The extractions were added approximately 200  $\mu$ l on PDA and dropped on *P. ergyii* mycelium.



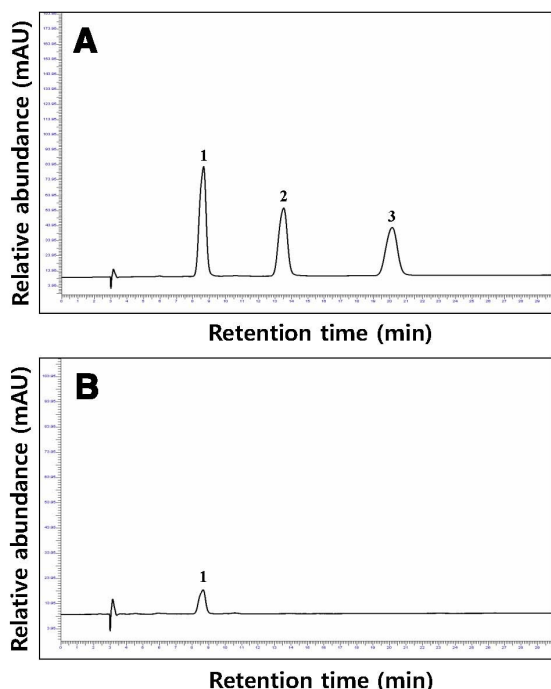
**Fig. 1.** TLC patterns of ethyl acetate extraction and fraction No. 5 (F5). (A) TLC patterns of ethyl acetate extraction and (B) TLC patterns of IAA standard and ethyl acetate fraction No. 5 (F5) by the treated with Salkowski's reagent. 1, IAA standard; 2, fraction No.5. The fraction was characterized by clear reddish pink spot at the  $R_f$  0.34 corresponding to IAA.

*Pseudomonas* sp. P7014 strain consistently showed seven spotting band (Fig. 1A), and interestingly band No.5 (F5) was characterized by a clear reddish pink spot at the  $R_f$  0.34 corresponding to IAA, when the chromatogram was treated with Salkowski's reagent (Fig. 1B). The F5 fraction of ethyl acetate exhibited the most impressive positive effect on mycelium growth (Fig. 2), whereas direct addition of extract caused growth inhibition. GPC inhibited mycelium growth in the mM concentration range, but promoted growth in the nM concentration range (Fig. 3). Meanwhile, *P. eryngii* mycelia grown in PD broth in the presence of bacterial GPC also increased in dry mass at nM concentration. The dry mass of mycelia grown in the flask is shown in Fig. 3. *P. eryngii*



**Fig. 3.** Growth effect of *P. ergyii* mycelium by the different concentrations of growth promoting compound (GPC). All values are means of determinations in three independent experiments. Means with different lowercase letters (a and b) indicate significant differences of fermentation times by Tukey's multiple range test ( $P < 0.05$ ).

mycelia grown in PD broth in the presence of bacterial GPC might have a significant improvement in biological efficiency. An economically attractive species of commercial strains such



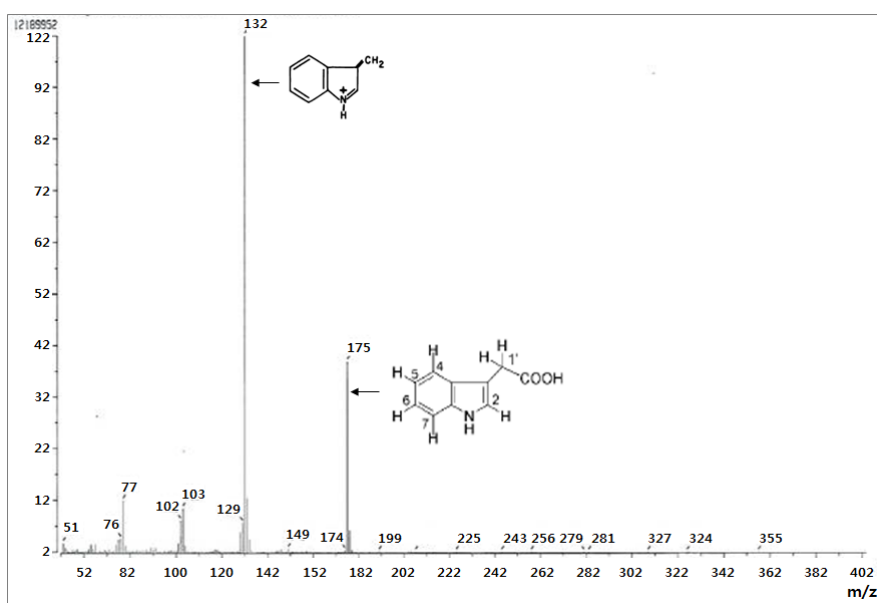
**Fig. 4.** Typical HPLC chromatograms of indole compounds. (A) Three indole compound standards as following: 1, indole acetic acid (IAA); 2, indole propionic acid (IPA); 3, indole butyric acid (IBA) and (B) Separation of IAA from ethyl acetate fraction No. 5 (F5) of P7014 strain.

as *Agrocybe cylindracea* was a significant improvement in biological efficiency because *A. cylindracea* relatively had higher yield than the dry substrate weight (Uhart *et al.*, 2008).

The GPC was followed by TLC and HPLC through extraction and purification. A HPLC chromatogram of the ethyl acetate fraction is shown in Fig. 4. The peak at 8.40 min was seen in the analytical chromatogram of the ethyl acetate extract. The level of IAA was present at concentration of 31.8  $\mu\text{g/ml}$  on F5 fraction of ethyl acetate. This indicated that the substance might be IAA. A TLC spot was extracted in methanol and compounds were purified by preparative HPLC revealing peak at 8.27 min corresponding to IAA (based on comparisons with standard IAA). This peak was collected for further analysis.

The  $^{13}\text{C}$  NMR data for the selected fractions isolated from P7014 strain was identical to the NMR data recorded for the IAA standard (data not shown). The MS-data showed an ion at  $m/z$  175 consistent with the  $m/z$  of IAA in the proton adduct form  $[\text{M} + \text{H}^+]$ . When the ion at  $m/z$  175 was subjected to MS/MS analysis, the main fragment ion formed was  $m/z$  130, consistent with the presence of an indole ring an acetate group (Fig. 5). MS/MS analysis of the IAA standard yielded similar data. Thus, both NMR and MS data confirm the presence of IAA in the strains studied.

In order to check the effect of the treated IAA on mycelial growth of *P. ergyii*, the mycelia growth at specific incubation period was very important. The mycelium could be developed as germination, elongation, saturation, and dormancy. The period between elongation and saturation on the mycelia



**Fig. 5.** MS spectrum of the HPLC purified growth promoting compound (GPC) fraction. Fragmentation of the protonated ions,  $m/z$  130 representing indole along with acetate groups and  $m/z$  175, unionized indole acetic acid.

**Table 1.** IAA Production by *Pseudomonas* sp. P7014 in the presence of various concentrations of tryptophan among DF and GP medium.

Tryptophan concentration ( $\mu\text{g/ml}$ )	IAA ( $\mu\text{g/ml/OD}_{600}$ ) <sup>1)</sup>	
	DF	GP
0	0.8 $\pm$ 0.1 <sup>d</sup>	1.6 $\pm$ 0.01 <sup>d</sup>
50	16.1 $\pm$ 0.6 <sup>b</sup>	20.1 $\pm$ 0.4 <sup>b</sup>
100	24.5 $\pm$ 0.2 <sup>b</sup>	29.9 $\pm$ 0.7 <sup>ab</sup>
200	28.3 $\pm$ 0.8 <sup>ab</sup>	36.6 $\pm$ 0.3 <sup>a</sup>
500	36.7 $\pm$ 0.5 <sup>a</sup>	43.0 $\pm$ 0.6 <sup>a</sup>

<sup>1)</sup>All values are means of determinations in three independent experiments. Means with different lowercase letters (a, b, c, and d) indicate significant differences of fermentation times by Tukey's multiple range test ( $P < 0.05$ ).

growth of *P. ergyii* was about 15 days (before saturation point of mycelium) (Kim *et al.*, 2008). The mycelial growth of *P. ergyii* at incubation period for 15 days was tested and mycelia growth ratio of *P. ergyii* during incubation periods was represented on Fig. 6. During incubation period, the mycelia growth of *P. ergyii* was higher on the treated IAA than on the control.

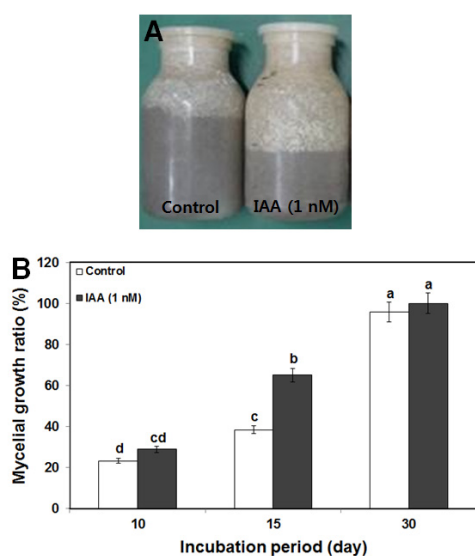
Though there are a few reports suggesting that IAA inhibits the growth of *Saccharomyces cerevisiae* (Peck and Kende, 1995). Perhaps high levels of exogenous or bacterial IAA can also inhibit growth. Meanwhile, high levels of ethylene have also been shown to inhibit root elongation (Wood and Hammond, 1977; Loper and Schroth, 1986; Barbieri and Galli, 1993; Sawar and Kremmer, 1995; Xie *et al.*, 1996; Beyerler *et al.*, 1997; Rahman *et al.*, 2001). It provided evidence of

ethylene production at the time of mushroom metabolism in *A. bisporus*. We showed that the bacterium P7014 produce a compound that exhibits a positive effect on growth. IAA produced by P7014 strain probably exhibit same kind of effect on mycelium growth at nM concentrations, but inhibitory effect at mM concentrations. In plants it is known that primary root growth is stimulated by low levels of IAA, typically between  $10^{-9}$  and  $10^{-12}$  M (Pilet and Saugy, 1987; Alvarez *et al.*, 1989; Meuwley and Pilet, 1991), and is inhibited by higher IAA concentration, likely via auxin induced ethylene (Peck and Kende, 1995). Although *Pseudomonas* sp. P7014 is capable of IAA biosynthesis, the growth effect of *P. ergyii* mycelium has slightly differences by the treatment of P7014 (Kim *et al.*, 2008) and GPC. There results are guessed that may be some other factor also involved in growth promotion.

#### Indole acetic acid (IAA) production of *Pseudomonas* sp. P7014

Like other mushroom growth promoting bacteria, strain P7014 synthesize high levels of IAA as tryptophan concentration was increased (ranging from 0–500  $\mu\text{l/ml}$  in culture medium). Interestingly, IAA production increased in the GP medium culture, compared to production in DF salt medium (Table 1). Culture in the absence of tryptophan resulted in low IAA levels. Measurement of IAA by HPLC confirmed that high tryptophan concentrations (more than 500  $\mu\text{l/ml}$ ) have little influence on IAA production. This is in agreement with other results showing that this amino acid is simply a basic precursor for IAA synthesis in most studied IAA-producing bacteria.

Tryptophan is a precursor for bacterial IAA biosynthesis (Steenhoudt and Vanderleyden, 2000; Eckardt, 2001). As expected, higher amount of IAA production was possible with increased tryptophan concentration (0 to 500  $\mu\text{g/ml}$ ) for P7014 strain, as is the case for most of the other plant growth promoting bacteria. *Pseudomonas* sp. P7014 produced a supernatant compound which promoted the growth of mushroom mycelia. The supernatant compound was extracted with ethyl acetate and separated by TLC, and after each step a positive effect was



**Fig. 6.** Effect of the treated IAA on mycelial growth of *P. ergyii*. (A) Photograph of mycelial growth of *P. ergyii* at incubation period 15 days and (B) mycelial growth ratio of *P. ergyii* during incubation periods. All values are means of determinations in three independent experiments. Means with different lowercase letters (a, b, c, and d) indicate significant differences of fermentation times by Tukey's multiple range test ( $P < 0.05$ ).

observed. Meanwhile, structural studies were done using different analytical instruments (HPLC, NMR, and MS/MS) and the results suggested that the factor responsible for growth was an auxin compound.

IAA is well a known growth promoting phytohormone, produced by many bacteria. Likewise to plant growth promoting bacterium, tryptophan influenced strain P7014 bacteria to produce high amount of IAA in culture medium than bacteria grown without tryptophan. Likewise, IAA plays same kind of role in stimulating mycelia of mushroom. The present results, the growth of *P. ergyii* mycelium were observed by the bacterial GPC (>0.1 nM), this suggests that it might be due to the influence of IAA.

In conclusion, our findings suggest that *Pseudomonas* sp. P7014 promoted mycelium growth by producing the IAA. The data obtained unambiguously demonstrate the presence of IAA in the supernatant of strain P7014 cultures. The widely used Salkowsky reagent is not specific to IAA, but can also react with other indole derivatives such as indole-3-acetamide and indole-3-pyruvic acid, which belong to the auxins, therefore production of other auxins by P7014 strain cannot be ruled out. This possibility is also indicated by the observation that the HPLC-based estimation of IAA concentrations gave lower values compared to the colorimetric method (data not shown) (Omer *et al.*, 2004). Production of IAA in bacteria may have evolved because it was important in the bacterium-mushroom relationship. In this research we showed that bacterial IAA stimulated the development of host mushroom mycelia. The details of a potential relationship between bacteria and mushroom are stimulating for future initiatives.

## 적요

*Pseudomonas* sp. P7014 박테리아를 통한 큰느타리버섯 균사체의 생육촉진에 관한 연구가 수행되었다. 박테리아 배양액으로부터 분리한 ethyl acetate 분획물(F5)에는 성장촉진물질(GPC)이 함유되어 있음을 확인하였다. TLC, HPLC, NMR 및 MS/MS 분석법으로 확인한 바, indole acetic acid (IAA)로 확인되었다. 큰느타리버섯 균사체는 성장촉진물질(GPC)이 첨가된 PDA와 PDB 배지에서 빠른 성장을 보였다. 성장촉진물질(GPC)의 농도는 1.0 nM로 매우 낮았지만, 확인된 tryptophan은 IAA의 전구체로써 IAA가 아민화된 형태였다. 이들 결과는 박테리아에서 분리된 성장촉진물질(GPC)은 IAA이었고 큰느타리버섯 균사체의 생육촉진에 중요한 역할을 하는 것으로 확인되었다.

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