

Studies on The N₂-Fixing Bacteria Association with Vetiver

- 1) Biosynthesis of Plant Growth Hormone by *Azospirillum*.
- 2) Use of the *gusA* Gene to Study *Azospirillum*.

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ABSTRACT

1. The N₂-fixing bacteria (*Azospirillum*) was isolated from soil on vetiver root zone. This Bacteria fix N₂ and also produce plant growth hormone, indole-3-acetic acid (IAA). Then IAA could be produced in culture media in the laboratory. By inoculating *Azospirillum* into malic-yeast broth media and grown for 5 days, IAA concentration was determined by the method of Gordon and Weber (1951), and this supernatant was verified by HPLC analysis. The culture was inoculated on young vetiver grown in N-free media. The results indicated that IAA was produced at 30-45 µg/ml in broth media grown *Azospirillum* plus tryptophan. Plants inoculated with this culture induced root elongation, increased plant height and biomass.

2. The *gusA20* from *E.coli* was inserted into the genome of *Azospirillum*. Plate mating was carried out. The transconjugant colonies were expressed by blue colonies and resistance to the antibiotics on the agar plates. The transconjugant was used to inoculate the vetiver root. The results indicated that *Azospirillum* grew

well outside and insides the roots.

Key words : *gus A20*, *Azospirillum*

Introduction

The most important auxin produced by plants is indole-3-acetic acid (IAA). It plays important roles in a number of plant activities including root initiation, apical dominance, fruit development and abscission etc. IAA can be synthesized by bacteria and the highest IAA concentrations were found in *Rhizobium* culture supernatants containing the highest concentration of added tryptophan. (Badenoch-Jones *et al.*, 1982). Minamisawa and Fukai (1991) reported that fraction from USDA 94 culture was IAA as proved by UV spectrophotometry, mass spectrometry and HPLC analysis. This study was conducted 1) to produce IAA in culture media using *Azospirillum*, determine the concentration of IAA in supernatants at a period of time, the effect of culture supernatant on young vetivers was also assayed and 2) to prove that *Azospirillum* can go inside the roots and fix N_2 by inoculating the vetiver root with the marker strain.

Materials and Methods

Bacterial culturing

Azospirillum and *Bradyrhizobium* were grown in Tris-YMRT plus tryptophan and in yeast-malic broth plus tryptophan in 2 liter flasks covered with aluminum foil (to prevent light). These two culture flasks were aerated with a small pump and kept at room temperature (28°C) for 5 days. *Azospirillum* cultures were also grown as above for the other purpose, autoclaved and kept in the cold room (10°C).

IAA analysis

Azospirillum culture was separated, centri-

fuged at 15,000 rpm for 5 minutes, 10 milliliter of supernatant was analyzed by HPLC (uBondapak C 18 column 3.9 x 300 mm, 10 μ) at flow rate 1 ml/min.

IAA determination

After removing the cells by centrifugation, IAA concentrations of two cultures were determined by colorimetric method (Gordon and Weber, 1951) at the fifth days. And at 1, 3, and 6 months, samples of autoclaved cultures were taken and concentrations of IAA were measured.

Bioassay and the effect of bacterial culture on vetiver

A. For the bioassay of *Azospirillum* culture, 1 milliliter of each culture grown in media without tryptophan was inoculated on a young vetiver grass grown in N-free media in glass bottles (4 treatments x 5 replications), kept in the growth room for 20 days, and harvested. Root length, height, dry weight were collected and data were analyzed by RCB design.

B. Two flasks of 50 ml. *Azospirillum* broth culture (covered flasks with aluminum foil) were grown at room temperature for 5 days, After checking for purity and concentration of IAA, one culture flask was autoclaved. Young vetiver grasses grown from tissue culture, were inoculated with 0.2 μ g IAA, 0.2 ml alive culture, 0.2 ml autoclaved culture (=2 μ g IAA) and uninoculated (4 treatments x 5 replications). After growing for 20 days, plants were harvested.

GUS conjugation and colour development

The *gusA20* was inserted to the genome of *Azospirillum* (vetiver 101) by conjugation as

described by Wilson (1995). One milliliter of the transconjugant (vetiver 101 : *gusA20*) was inoculated to the young vetiver roots grown in N-free media in growth room for 7 days. Roots were separated some were washed and soaked in GUS extraction buffer plus X-glc for 24 hours, another parts of the roots were cut in small pieces, put on a slide. Pictures of bacteria, colonization and infection were taken by the fluorescence microscope.

Results and Discussion

Indole-3-acetic acid can be produced in the laboratory by growing *Azospirillum* or *Bradyrhizobium* in liquid culture. The concentrations of IAA increased with increasing amount of tryptophan (Table 1). *Azospirillum* (vet.101) produced IAA 30-40 µg/ml after growing for 5 days in yeast-malic broth

plus various amounts of tryptophan. And after keeping this autoclaved culture in the brown glass bottle in the cold room (10°C), the concentrations of IAA remained constant. Badenoch-Jones *et al.*, (1982) grew *Rhizobium* culture for IAA production and quantitated the concentration of IAA. They found that IAA was produced and decomposed over culturing period. For this study the activity of bacteria was stopped by autoclaving, then the concentration of IAA remained constant (45 µg/ml) over 3 months. As comparing the amount of IAA produced by *Azospirillum* to *Bradyrhizobium*, *Azospirillum* produced more amount of IAA, but it is not true because some strains of *Bradyrhizobium* produced as much as 34-61 µg/ml depending on the concentration of tryptophan and different media (Minamisawa and Fukai, 1991).

Table 1. Indole-3-acetic acid production (µg/ml) by bacteria grown in liquid media.

Culture	Tris - YMRT + tryptophan	Media			
		Malic-yeast+tryptophan			
		5 days	1 month	3 month	6 month
<i>Azospirillum</i> (vetiver 101)	2.5-12.0	30.0-40.0	45	45	45
<i>Bradyrhizobium</i> (USDA 76)	1.5-9.0	9.0-15.0	ND	ND	ND

IAA concentration determined by Gordon and Weber Method (1951)

ND = not done

Table 2 Effect of *Azospirillum* culture on growth of vetiver grass (var.KPP) grown in N-free media for 20 days.

Treatment	Root length (cm)	Height (cm)	Shoot & Root Dry Weight (mg/plant)
<i>Azospirillum</i> (vet. 101)	12.62 a	22.625 a	109 a
<i>Azospirillum</i> (vet .103)	8.125 ab	21.500 a	90 a
Azo.101 :gusA20	8.750 ab	21.500 a	100 a
Control	6.250 b	15.000 b	33.5 b
Mean	8.938	20.156	83
C.V. (%)	30.2	17.6	38.9

Means followed by a common letter are not significantly different at 5% level by DMRT.

Table 3 Effect of *Azospirillum* culture supernatant on growth of vetiver grass (var.SRT) grown in N-free media for 20 days.

Treatment	Root length (cm)	Height (cm)	Shoot & Root Dry Weight (mg/plant)
IAA (0.2 µg)	2.250 a	7.688 a	17.000 a
<i>Azospirillum</i> (0.2 ml)	2.750 a	6.875 ab	16.625 a
Autoclaved-Azo. (0.2 ml)	2.125 a	6.063 b	12.000 a
Control	2.000 a	5.625 b	11.500 a
Mean	2.375	6.475	14.100
C.V. (%)	22.2	14.4	25.8

Means followed by a common letter are not significantly different at 5% level by DMRT.

For determining the concentration of IAA, the colorimetric method (Gordon and Weber, 1951) gave closely amount as HPLC analysis (33 - 40 µg/ml). So we use the colorimetric method to determine the concentrations of IAA. The disadvantage of this method is the absorbancy reaches maximum at concentration of IAA at 45 µg/ml. For this study

the concentrations of IAA detected were between 1.5-45 µg/ml.

Data from Table 2 showed the effect of *Azospirillum* culture on the vetiver grass grown in N-free media in the growth room. Root length, height and dry weight of the vetiver grass increased with inoculation compared to control (noninoculated).

This culture grown without tryptophan, then the increasing affected from bacteria fix N_2 (200 η mole/plant/hr.) and also by small amount of IAA produced. Low concentration of IAA (0.1 μ M-) induced root elongation and cell division in meristematic tissue (Harrari *et al.*, 1988).

The effect of culture supernatant shown in Table 3, data of root length, biomass were not significantly different except the height. The concentration of IAA in culture supernatant used in this study was not high enough to inhibit root elongation. IAA at 2 μ M showed inhibition of alfalfa root length (Minimisawa and Fukai, 1991). However, the results indicated that there was no toxic substances in the culture supernatant, since root length, height and shoot with root dryweight of the autoclaved *Azospirillum* treatment were the same as control treatment.

To study the ecology and the association of bacteria, we can use GUS as a marker gene. The gene, *gusA20* developed by Wilson (1995) was inserted into the genome of *Azospirillum* successfully. This transconjugant (*Azospiri- gusA20*) was detected outside and inside the roots of vetiver as shown in Fig.1 and Fig.2. Wilson (1995) reported and showed the early stage of root colonization and infection by using the GUS gene fusion. Blue color in the root of vetiver (Fig. 2) showed that *Azospirillum* infected roots.

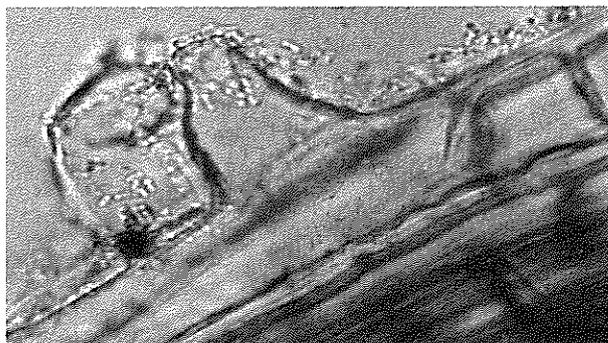


Fig. 1 Bacteria, *Azospriillum* found around roots of vetiver (colonization)

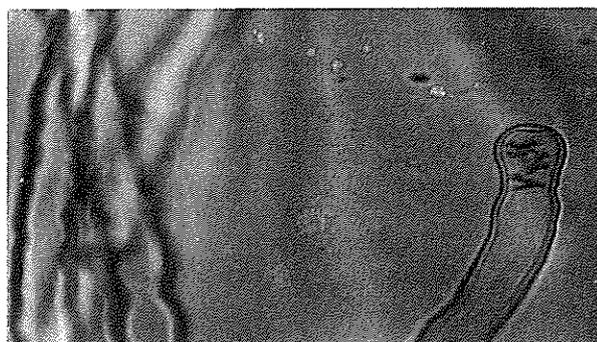


Fig. 2 Infection *Azospriillum* in vetiver roots shown by blue color when using GUS marked strain

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