

Cytokinin-like substances and ethylene production by *Azospirillum* in media with different carbon sources

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Abstract

Production of cytokinin-like substances (CLS) from two strains and ethylene from three strains of *Azospirillum* in the presence of different carbon sources was studied. Ethylene production depended on the presence of methionine and different carbon sources. CLS were detected by the soybean callus test and gas chromatography. A strong stimulation of soybean callus was stated with *Azospirillum* No 1 and 3 in media containing malate. In culture filtrates from both strains of *Azospirillum* 2iP = 6(γ,γ -dimethylallylamino)purine was detected. All strains of *Azospirillum* produced ethylene in the presence of methionine and malate, succinate and pyruvate.

Key words: Cytokinin-like substances – ethylene – *Azospirillum* – carbon sources

Introduction

There exists a firm evidence that plant growth regulators like auxins, giberellins, cytokinins and ethylene produced by plants are essential for their growth and development. Plant growth regulators are also produced by various microorganisms living in association with plants (Tien *et al.* 1979; Ho and Trappe 1987; Gogala 1991). There is also evidence that plant growth hormones produced by bacteria can increase growth rates and improve yields of the host plants (Barea and Brown 1974). Different microorganisms, both saprophytic, symbiotic and pathogenic, have been found to be capable of producing cytokinins (Klamt 1967; Phillips and Torrey 1972; Barea *et al.* 1976; Kampert and Strzelczyk 1980; Strzelczyk and Kampert 1983 a; Evidente *et al.* 1991).

Ethylene is an endogenous plant regulator that is produced by higher plants as well as by microorga-

nisms. This gas affects plant growth and some of the biochemical processes in plants (Archer and Hislop 1975). Fungi are known to produce ethylene often in correlation with infection or pathogenesis (Archer and Hislop 1975; Graham and Linderman 1980).

Different bacteria belonging to the species *Azotobacter*, *Bacillus* and *Pseudomonas* were shown to be able of producing ethylene (Primose and Dilworth 1976).

Production of ethylene by microorganisms depends on the growth rate, composition of the growth medium and on the presence or absence of oxygen (Thomas and Spencer 1978). Also it has been shown that some soil bacteria use ethylene as a carbon source for growth and it has been suggested that these bacteria may play an important role in the removal of ethylene from the soil atmosphere (Abeles *et al.* 1971).

During recent years, *Azospirillum* attracts the attention of many researches, because it is living in associative symbiosis with many plants and mycorrhizal fungi. It is a nitrogen-fixing bacterium producing also plant growth regulators thus, affecting growth and development of the plants (Tien *et al.* 1979; Li and Hung 1987; Falik *et al.* 1989; Tilak *et al.* 1989).

Materials and methods

The following bacteria were used in our studies:

- Azospirillum* 1 – isolated from *Rhizopogon vinicolor* (ectomycorrhiza of Douglas-fir).
- Azospirillum* 2 – isolated from *Laccaria laccata* sporocarp (production of CLS was not studied)
- Azospirillum* 3 – isolated from a *Hebeloma crustuliniforme* sporocarp

Determination of cytokinin-like substances (CLS). The bacteria were grown in Dobereiner medium

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(1980). The following carbon sources were used in our experiments (5 g per liter each): malate-succinate-pyruvate-fumarate-Na. Erlenmeyer flasks containing 200 ml of the above medium were inoculated with 2 ml suspension of *Azospirillum* (3-days old culture). The inoculated flasks were incubated at 26°C for 7 days. Subsequently, the bacteria were

separated from the medium by centrifugation and biomass was determined.

Extraction was performed as follows: The supernatants were adjusted to pH 2.5–3.0 with 1N HCl and passed through a Dowex W × 8 (Merck) cation exchange column H⁺ form, 50–100 mesh. The column was washed with 500 ml of double distilled water and the CLS were eluted with 340 ml of 2N NH₄OH followed by 5N NH₄OH. The combined eluates were evaporated to dryness *in vacuo* at 60°C to remove ammonia. Two ml samples obtained from 1000 ml of the culture fluid were applied to a Sephadex LH-20 (Pharmacia, Uppsala) column (45 × 2.5 cm) and eluted with 35% ethanol. Four fractions, 10 ml each, were collected and evaporated to dryness at 50°C. Into each flask with the dry residue, 50 ml aliquots of the medium according to Miura and Miller (1969), were added and autoclaved at 117°C for 20 min. Three pieces of soybean callus (*Glycine max.* (L.) Merrill. var. Acme) – ca. 40–45 mg each – were placed in each flask. The cultures were grown in an illuminated chamber at about 60 lx at 25°C. After 25 days of growth the increase of fresh mass of the callus was recorded. The total amount of CLS produced was calculated from a standard response curve prepared for pure kinetin (Serva) and finally expressed as kinetin equivalents (E. kin. µg/g dry weight of cells). Details of gas chromatographic analysis were

Table 1. Production of cytokinin-like substances (CLS) by *Azospirillum* depending upon the carbon source

Strain No	Carbon source	Quantity of CLS (E. kin µg/g dry mass)*	Fraction No
<i>Azospirillum</i> 1	malate	0.5564	0–40
		0.3019	16–40
<i>Azospirillum</i> 1	fumarate	0.0236	0–12; 20–28
		0.0225	16–40
<i>Azospirillum</i> 1	succinate	0	–
		0	–
<i>Azospirillum</i> 1	pyruvate	0	–
		0	–

* Equivalents of pure kinetin

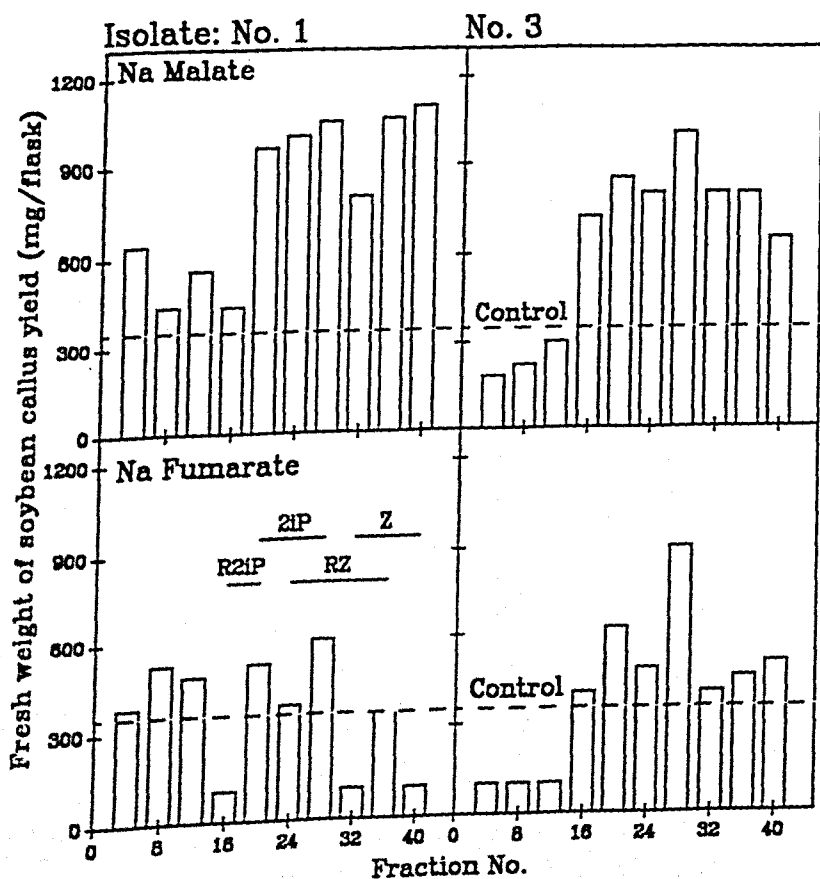


Fig. 1. Column chromatographic analysis of CLS produced by *Azospirillum*.

- 2iP — 6(γ,γ-dimethylallylamino)-purine
- R2iP — 6(γ,γ-dimethylallylamino)-purine riboside
- Z — zeatin
- RZ — zeatin riboside

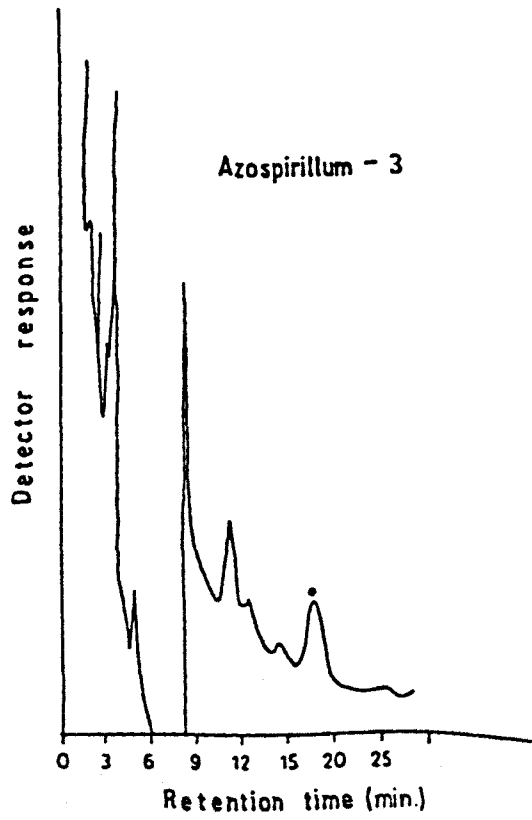
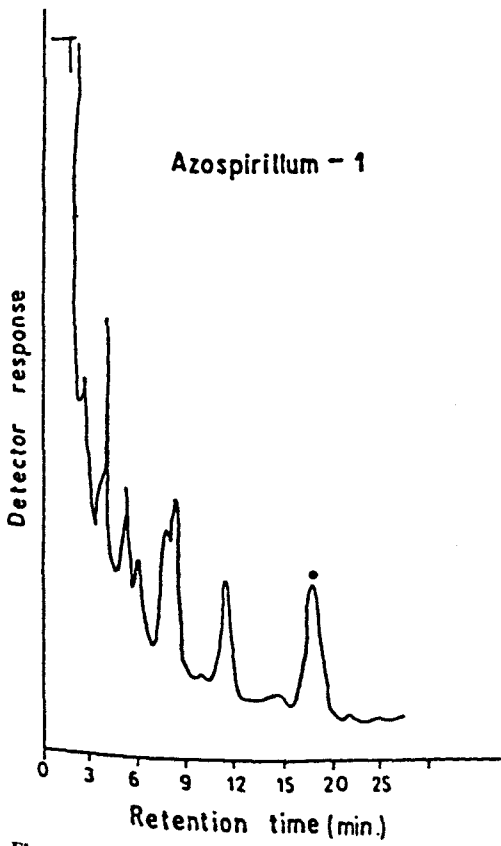
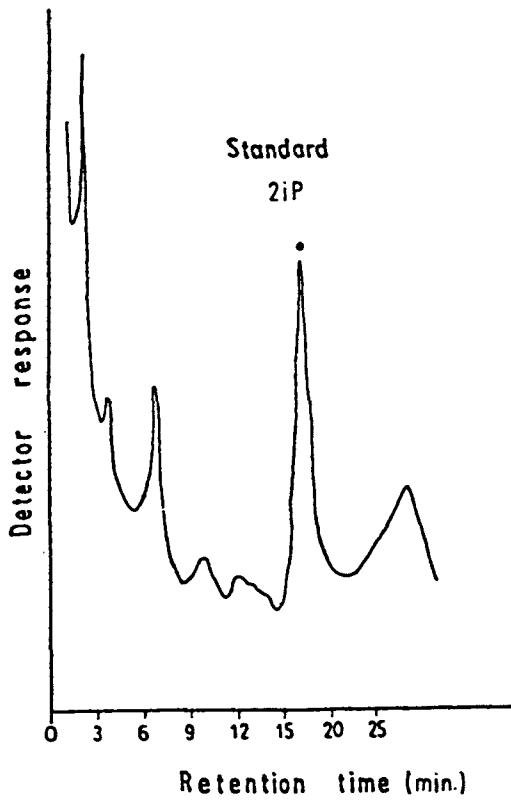


Fig. 2. Gas chromatographic analysis of CLS produced by *Azospirillum*.
 2iP - 6(γ,γ -dimethylallylamino)purine

described in a previous paper (Strzelczyk, Kampert 1983b).

Determination of ethylene. For estimation of ethylene the bacteria were grown in the Döbereiner medium (1980) with and without methionine (400 mg/l). The carbon sources were the same as in the studies on CLS production. The bottles were fitted with rubber plugs tightened with metal cowls. Each bottle was inoculated with 1 ml of *Azospirillum* suspension (72-hour old culture grown in Döbereiner medium). The bacteria were grown at 26°C for 7 days. Subsequently, chromatographic analysis has been performed. One ml of ethylene-in-air samples were withdrawn from the flasks and analysed with the use of a Gas Chromatograph GCHF 18.3-4 (Chromatron) with a 100 × 0.4 cm glass column filled with silica gel for chromatography (100–150 mesh) operated isothermally at 70°C with nitrogen as gas carrier and FID (t^0-140). Studies on ethylene production were performed in triplicate.

Pure ethylene was used as the standard during identification of this metabolite.

Results

The results of our studies on CLS production are presented in Tab. 1 and Figs. 1 and 2.

It appears from Table 1 that both strains of *Azospirillum* produced CLS in the medium containing malate and fumarate as the carbon sources.

In the biotest the strongest soybean callus stimulation was obtained with *Azospirillum* 1 and 3 in media with malate. Weaker stimulation of the soybean callus was observed in the medium with fumarate (Fig. 1). The localization of the pure substances in appropriate fractions allows to suppose that the compounds produced by the bacteria studied were: riboside 2iP 6/ γ , γ -dimethylallylamino/purine, 2iP, riboside zeatin and zeatin.

However, gas chromatography allowed to identify only 2iP in two strains of *Azospirillum* (Fig. 2), other CLS were not found.

This fact indicates that the results obtained by biotest are not necessarily identical with those obtained by gas-chromatography.

The results on ethylene production are shown in Table 2. It was found that all strains of *Azospirillum*

produced ethylene in the media with methionine and malate, succinate and pyruvate. Only *Azospirillum* 1 produced ethylene in media with fumarate and methionine.

All three strains of *Azospirillum* produced ethylene in media without methionine and in the presence of pyruvate, but the amount of ethylene was smaller than in the media with methionine (Table 2).

Discussion

In the interactions between plants and microorganisms the role of biological active substances is considered to be of importance (Garbaye 1991; Gogala 1991).

Physical and chemical factors influence the production of these substances (Kampert and Strzelczyk 1984; Gay 1986; Strzelczyk and Pokojaska 1987, 1989). The production of plant growth regulators by *Azospirillum* concerned mainly auxins and gibberellin-like substances production (Tien *et al.* 1979; Crozier and Arnold 1988).

Papers on the production of cytokinin-like substances are scarce. Muralidhara and Rai (1986) found cytokinin-like substances in *A. lipoferum*.

Tien *et al.* (1979) detected in ten days old cultures (without N) of *A. brasilense* besides IAA and gibberellins also cytokinins, which were identified as zeatin and riboside zeatin. In our work the CLS production and ethylene were studied in media with different carbon sources. Carbon sources significantly affect the growth of *Azospirillum* (Westby *et al.* 1983; Różycki *et al.* 1993), therefore they might also affect the production of CLS and ethylene.

It is known that methionine is a precursor of ethylene formation in plants and microorganisms. However, some other compounds, like sugars (especially glucose) phenolic compounds, some amino acids like α -alanine, aspartic acid, serine and glycine as well as some factors like pH, age of the culture, temperature and redox potential affect the production of ethylene (Arshad and Frankenberger 1989; Strzelczyk *et al.* 1987).

In our studies the production of ethylene was determined in the presence of methionine and some organic acids salts used as the carbon sources. We found that the production of ethylene depended upon

Table 2. Effect of different carbon sources on the production of ethylene by *Azospirillum* ($\mu\text{M/g}$ dry mass)

Strain No.	Malate-Na		Succinate-Na		Pyruvate-Na		Fumarate-Na	
	with methionine	without methionine	with methionine	without methionine	with methionine	without methionine	with methionine	without methionine
1	0.1725	0	0.0695	0	0.0638	0.0066	0.0109	0
2	0.1049	0	0.0536	0	0.1371	0.0152	0	0
3	0.0559	0	0.0396	0	0.0450	0.0074	0	0

the presence of methionine, when the sources of carbon were malate and succinate. But the production of ethylene in media with and without methionine was stated only in the pyruvate — Na containing media (Tab. 2). Thomas and Spencer (1989) noted that pyruvate considerably inhibited ethylene production in *Saccharomyces cerevisiae*. However, pyruvate profoundly stimulated ethylene production in *Penicillium digitatum* (Gibson and Young 1966). According to these authors pyruvate may serve as an ethylene precursor in *Penicillium digitatum*. On the basis of the results obtained in our work we think that carbon sources may affect the production of CLS, but the production of ethylene by *Azospirillum* depended on the presence of methionine when the carbon sources were malate and succinate.

It is assumed that in some cases pyruvate can be considered as a precursor of ethylene production in *Azospirillum* similarly to the situation in *Penicillium* (Gibson and Young 1966).

Inoculation of plants with bacteria of the genus *Azospirillum* has been tested world-wide (Bashan and Levanony 1988). Among the mechanisms involved in this plant bacterial interaction and hormonal effects are suggested (Bashan *et al.* 1989). More knowledge is required on the physiology and factors affecting the production of plant growth regulators by *Azospirillum* before this bacterium can be applied commercially.

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