Symbiosis, 2 (1986)
Balaban Publishers, Philadelphia/Rehovot

A Fast Screening Method for Bacterial Isolates Producing Substances Affecting Root-Growth*

SIEBE C. VAN DE GEIJN and JOSÉ M.M. VAN MAAREN

Research Institute ITAL, P.O. Box 48, 6700 AA Wageningen, The Netherlands
Tel.(0)8370-91911 Telex 45856(nl)

Abstract

A method has been developed to test bacterial isolates and their metabolites for *in situ* effects on seedling root development. Surface-sterilized seeds of tomato were germinated on agar plates, and root tips were subsequently exposed to the substance to be screened, contained in a small disc of agar. Root elongation, secondary root development and morphological changes could be recorded. Bacterial isolates tested, including actinomycetes, varied widely in their production of effective substances. Effects ranged from complete inhibition to normal root development. The most active compounds were found in the non-polar acidic fraction, in which also IAA would be recovered. However, poor correlation was found between root elongation inhibition and the height of HPLC-peaks at the retention time of authentic IAA.

Keywords: bacterial metabolites; plant growth-promotion; rhizosphere; root growth

Abbreviations: HPLC: high performance liquid chromatography, IAA: indoleacetic acid

*Reviewed

0334-5114/86/$03.00 © 1986 Balaban Publishers

and siderophore producing *Pseudomonas* spp. (Kloepper et al., 1980; Geels and Schippers, 1983) have been studied intensively. However, mechanisms are not always clear, and the interference of unidentified metabolic intermediates and plant growth substances is often postulated.

Root growth, root elongation and branching is reported to be under the control of a complex balance of phytohormones (Davies et al., 1976; Wightman et al., 1980). Indeed, many of the rhizosphere bacteria do produce plant growth substances in culture (phytohormones, antibiotics, toxins) and high-producing strains have been isolated (Hartmann et al., 1983).

Exogenous application of concentrations of these growth regulators to roots to investigate possible effects does not always produce the expected results. Rhizosphere bacteria, however, having an intimate contact with the root and producing these substances *in situ* may be more effective. Moreover, the stage of development of the root section is a major factor, determining not only the intensity of bacterial colonization (Van Vuurde and Schippers, 1980), but also the sensitivity towards endogenous and exogenous phytohormones (Davies et al., 1976).

In this respect exposure of the whole root system to external concentrations of growth regulators is an oversimplification. We designed a method to expose plant roots locally and in a reproducible way to metabolites of microorganisms or other test substances. First results will be reported here, concentrating on the effects of *Pseudomonas* spp. and actinomycetes on seedling root elongation.

2. Material and Methods

Bacterial isolates

Bacteria were isolated from the rhizosphere of tomato plants of about 3 months age by blending samples of roots and adhering soil, and plating dilutions on a selective medium (P-1) for fluorescent *Pseudomonas* strains (Katoh and Itoh, 1983). Isolates Ps6 and Ps7 were identified (API 20 NE) as being *Pseudomonas fluorescens*. An isolate of *Pseudomonas aeruginosa* was also used.

A second series of isolates was obtained by taking a random selection of actinomycetes from a larger collection, established for different purposes. They were isolated from arable soil (silty loam).

Bacterial cultures and preparation of test substances

Bacterial isolates were grown for 4–5 days in a liquid nutrient medium (Nutrient Broth, Oxoid) or in a mineral medium supplemented with 0.2% glucose (in mg/l: KH_2PO_4: 500; NH_4Cl: 1000; $MgSO_4 \cdot 7H_2O$: 200; $CaCl_2 \cdot 2H_2O$: 10; $FeCl_3$: 1.35 and micronutrients) and various additions (one of proline: 500; methionine: 250 and tryptophane: 100 mg/l respectively). Suspensions were centrifuged, and the supernatant filter sterilized (0.2μm).

To obtain different fractions of the cell-free culture liquid, the method of Tien (1980) was slightly modified (Fig. 1). Ten ml of the cell-free culture liquid was pH-adjusted and passed through successive C-18 cartridges (Millipore) to retain the non-polar fractions.

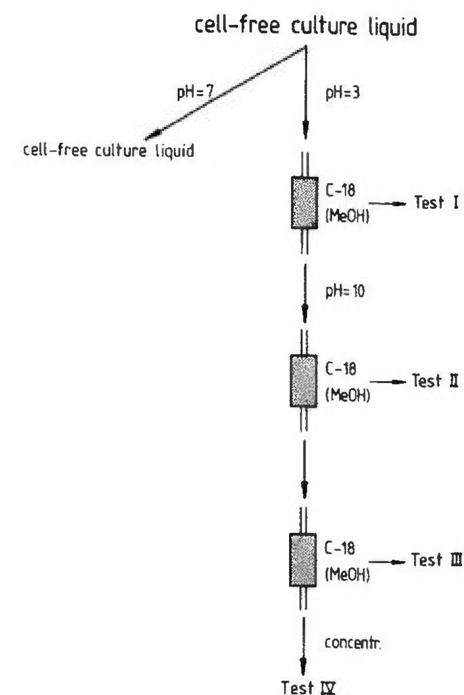

Figure 1. Procedure used in partitioning the cell-free culture liquid using reversed phase cartridges (C-18). MeOH = elute with 1 ml methanol.

Cartridges were eluted with 1.0 ml of methanol, and the liquid evaporated to dryness at room temperature in a gentle stream of air. The residue was dissolved in 5 ml distilled water (pH = 7).

Test solutions were passed through a 0.2μm filter and mixed (1:2 v/v) with sterile molten (45°C) agar (Technical agar, Oxoid; 2.25%, pH = 7), and solidified in a petri dish. Small discs (about 3 mm diam., 1.5 mm thick) of this agar were obtained using a cork bore of the appropriate diameter.

Seedlings

Seeds of tomato (*Lycopersicon esculentum* Mill cv. Marathon) were surface sterilized using 70% ethanol (30 sec) followed by 20 min 1.5% sodium hypochlorite + 1% tween 80, and 5 × 5 min rinsed in sterile demineralized water.

Assay method

Petri dishes (14 cm) with a 1.5% Technical agar in a 10 mM phosphate buffer at pH = 7 were prepared, and three seeds per plate carefully positioned on the agar. The plates were put at a slope of about 60°to have the radicle grow along the surface. After three days of germination (dark, 23–25°C) a small square of cellophane was gently slided underneath each of the root tips, and the position of the tip marked. A small disc of agar, containing the metabolites or other test substances was positioned at about 1 mm in front of the root tip, onto the cellophane. Root elongation was recorded daily.

3. Results

In each of the series of isolates tested effects ranged from complete inhibition to unaffected development (Fig. 2 and 3). The tested isolates were not pathogenic, as indicated by a test on potted plants in normal horticultural potting soil (Dr. B. Gerhardson, pers. comm.).

The bioassay turned out to be very reproducible, and apparently not very sensitive to the relatively small variation in the length of the radicle at the time of application. In the control treatment, using a disc of technical agar at pH = 7, a root extension growth of 25±2 mm/day was observed. Retardation or inhibition of root elongation always stimulated the development of lateral roots. In addition, the local density of root hairs increased considerably, supporting the view that at first cell-division did not stop, but cell-elongation was inhibited (Fig. 4c).

Application of the bioassay without interposed sheets of cellophane resulted in a rapid loss of effects, clearly indicating that substances were lost and diffused into the supporting agar (Fig. 4a,b).

Autoclaving (20 min, 120°C) did not decrease the effectiveness of the substances, and results were not dependent on the pH of the supporting agar.

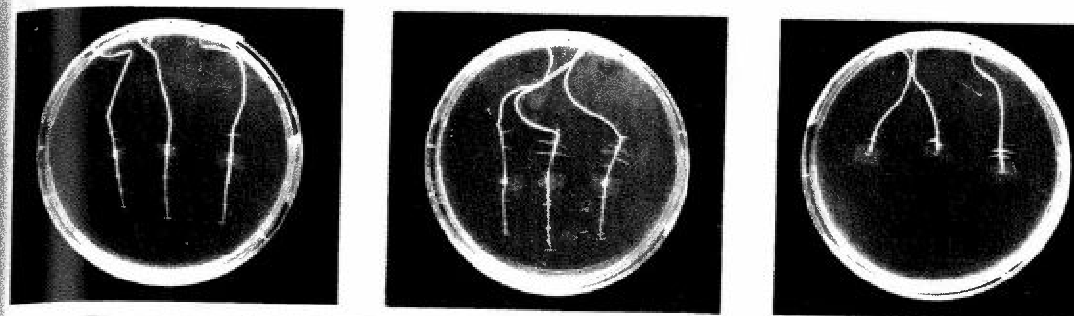

Figure 2. Result of the bioassay of the cell-free culture liquid of *Pseudomonas* isolates. (a) Control (Nutrient Broth); (b) Ps7; (c) Ps6. Small white marks indicate daily growth.

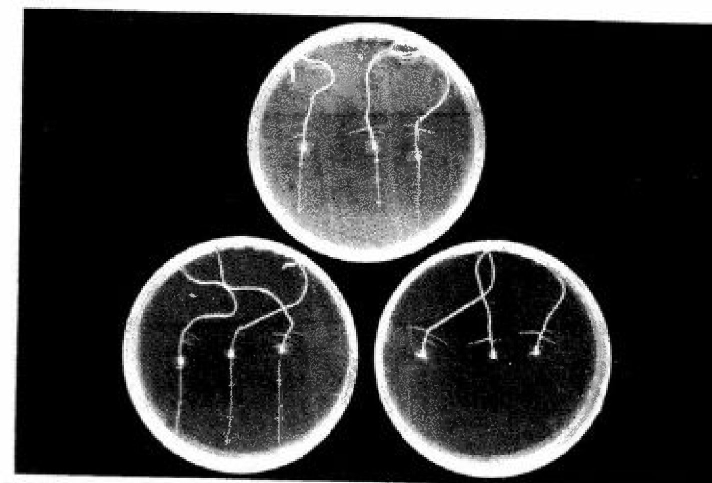

Figure 3. Result of the bioassay of the cell-free culture liquid of actinomycete isolates. top: SR 5-73, left: SR 5-24, right: SR 5-75

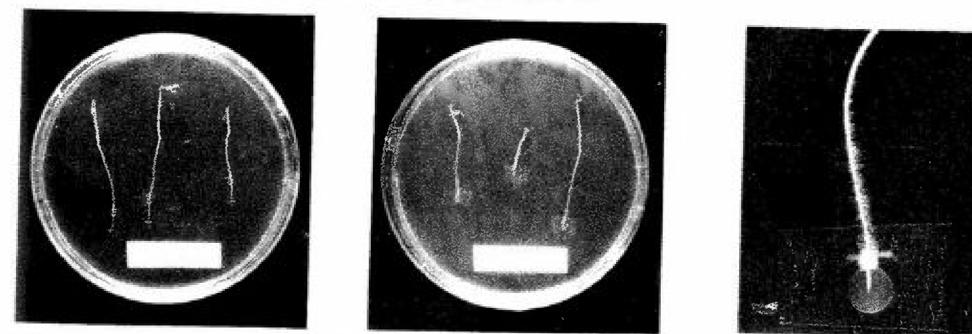

Figure 4. Bioassay without (a) and with (b) interposed sheet of cellophane. Note the increased density of root hairs (c) near the root tip.

However, a marked difference in lateral root growth was observed, being much better at pH = 7 than at pH = 5 (results not shown).

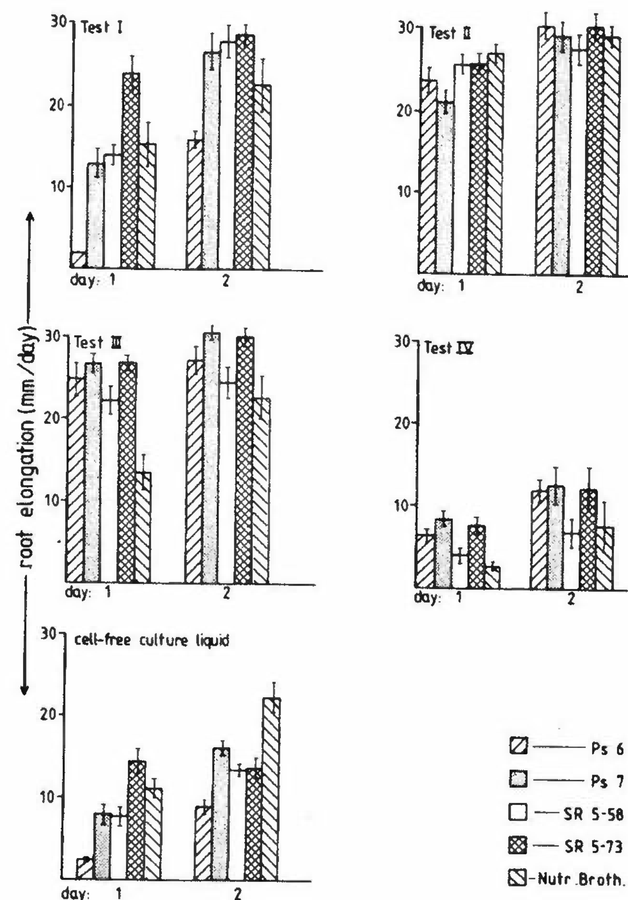

Figure 5. Bioassay of partitioned cell-free culture liquid. Fractions as indicated in Fig. 1. Root elongation in mm/day on two successive days. Ps6, Ps7: *Pseudomonas fluorescens*; SR5-58, SR5-73: actinomycetes.

Results of the bioassay of the fractions of the cell-free culture liquid prepared according to the scheme in Fig. 1 are given in Fig. 5. Special attention should be given to fractions I and III. Fraction I represents the non-polar acidic fraction retained in the cartridge at pH = 3. According to Tien (1980), and confirmed by our own experiments the auxin IAA will also appear in this fraction.

The assay with fractions III and IV shows for isolate Ps6 as well as for the other isolates a stimulation as compared to the control. This may be due to the presence of promoting substances, or the removal or inactivation of inhibitors present in the fresh Nutrient Broth (cf. growth of control: 25 mm/day).

Further analysis and comparison with results obtained with chemically defined culture media should further clarify this.

In the bioassay using a mineral medium supplemented with glucose and tryptophane, methionine or proline differences between isolates Ps6 and Ps7 (Fig. 5) had almost disappeared, and inhibition by Ps6 was only small (87%; 103%; 94% growth for Ps6 as compared to Ps7 for the three compounds tested).

The effects have been compared with authentic IAA, at various concentrations. An inhibition, comparable to that shown for Ps6 (Fig. 2) was obtained at concentrations of about 5 mg/l (50μM). However, preliminary tests on HPLC failed to show such high concentrations in the cell-free culture liquid. In general correlation between HPLC peak-height at the corresponding retention time and inhibition of root elongation was poor.

4. Discussion and Conclusions

The results obtained in the reported experiments prove the method to be very reproducible and easy to apply. The assay is very convenient, especially for the study of root affecting substances at the subtoxic level (cryptic effects) and in cases where *in situ* variations in the sensitivity of the various root parts are expected. Such variations were observed in experiments where the disc of agar was put at various positions along the root (results not shown). Primarily the root tip and elongation zone appeared to be sensitive.

In each of the series of tested isolates a whole range of effects was observed, indicating that rhizosphere populations count many species capable of modifying root growth. Root systems of inoculated plants have been shown to have mostly a more stunted appearance than axenically grown plants (Kloepper and Schroth, 1980). Such changes may be of significance for growth in extreme conditions, like in the case of environmental stress (drought, nutrient limitations) but also for growth of e.g. horticultural crops in soil-free culture systems. In their review on mineral nutrition of plants Clarkson and Hanson (1980) discuss the effect of endodermis development on the uptake of Ca^{2+} and Mg^{2+} as compared to K^+, NO_3^- and phosphate.

Branching and enhanced formation of young roots may accordingly change the total and differential capacity for uptake of these ions.

In our studies the bioassay will be used to select bacterial isolates or metabolites capable of causing a change in root architecture and to investigate the consequences of these for plant nutrition and plant growth.

The present results do not indicate that IAA is playing a major role in the observed interaction. However, further tests, using strains capable of producing high amounts of IAA and other phytohormones should be done to come to a firm conclusion. The significance of this bioassay for the selection of plant growth promoting rhizobacteria has also to be established further.

Acknowledgements

The isolates of the actinomycetes were obtained from Dr. J.A. van Veen and Miss E.H. Nijhuis (ITAL). Dr. B. Gerhardson (Agricultural University, Uppsala, Sweden) is gratefully acknowledged for performing the test on pathogenicity of the isolates.

REFERENCES

Clarkson, D.T., and Hanson, J.B. 1980. The mineral nutrition of higher plants. *Ann. Rev. Plant Physiol.* **31**, 239–298.

Davies, P.J., Doro, A.J., and Tarbox, A.W. 1976. The movement and physiological effect of indoleacetic acid following point applications to root tips of *Zea mays*. *Physiol. Plant.* **36**, 333–337.

Gaskins, M.M., Albrecht, S.L., and Hubbell, D.H. 1984/1985. Rhizosphere bacteria and their use to increase plant productivity: a review. *Agric. Ecosyst. Environm.* **12**, 99–116.

Geels, F.P., and Schippers, B. 1983. Selection of antagonistic fluorescent *Pseudomonas* sp. and their root colonization and persistence following treatment of seed potatoes. *Phytop. Zeitschr.* **108**, 193–206.

Hartmann, A., Singh, M., and Klingmuller, W. 1983. Isolation and characterization of *Azospirillum* mutants excreting high amounts of indoleacetic acid. *Can. J. Microbiol.* **29**, 916–923.

Kapulnik, Y., Okon, Y., and Henis, Y. 1985. Changes in root morphology of wheat caused by *Azospirillum* inoculation. *Can. J. Microbiol.* **31**, 881–887.

Katoh, K. and Itoh, K. 1983. New selective media for *Pseudomonas* strains producing fluorescent pigment. *Soil Sci. Plant Nutr.* **29**, 525–532.

Kloepper, J.W., Leong, J., Teintze, M., and Schroth, M.N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* **286**, 885–886.

Kloepper, J.W. and Schroth, M.N. 1980. Plant growth-promoting rhizobacteria and plant growth under gnotobiotic conditions. *Phytopath.* **71**, 642–644.

Tien, T.M. 1980. *Plant growth substances produced by Azospirillum brasilense and their effects on plant growth.* Thesis, Univ. of Florida, USA.

Van Vuurde, J.W.L. and Schippers, B. 1980. Bacterial colonization of seminal wheat roots. *Soil Biol. Biochem.* **12**, 559–565.

Wightman, F., Schneider,E.A., and Thimann, K.V. 1980. Hormonal factors controlling the initiation and development of lateral roots. II. Effects of exogenous growth factors on lateral root formation in pea roots. *Physiol. Plant.* **49**, 304–314.