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Determination of bacterially derived auxins using a microplate method

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M. SARWAR AND R.J. KREMER. 1995. Rapid and efficient methods for determining the ability of soil and rhizosphere bacteria to produce key metabolites which are useful in growth promotion or suppression of plant growth are needed. A microplate method was developed and compared to a standard method for assays of auxin compounds produced by bacteria. The microplate method was similar to the standard method in accuracy of determination, required less chemical reagents, and considerably reduced the time required for analyses.

INTRODUCTION

Auxins, a class of plant hormones, are known to affect plant growth throughout ontogeny. Auxins are produced by plants (Okamoto *et al.* 1976; Arshad and Frankenberger 1991) and several micro-organisms including bacteria (Barea *et al.* 1976) and fungi (Dvornikova *et al.* 1970). Certain bacteria can be very prolific producers of the auxin, indole-3-acetic acid (IAA), when provided with L-tryptophan (L-TRP) as a precursor (Loper and Schroth 1986).

Detection of IAA produced by plants and micro-organisms is typically accomplished using colorimetric methods (DeVay *et al.* 1968; Loper and Schroth 1986). Determination of IAA-producing ability of micro-organisms is useful for identification and also serves as a valuable indicator of physiological roles and ecological significance of these micro-organisms in the environment (Surico *et al.* 1984). Bacteria selected for IAA production may be useful in growth promotion of crop plants (Arshad and Frankenberger 1991) or in growth suppression of weeds (Kremer and Kennedy 1995).

Current methods for quantification of IAA require analysis of aliquots from numerous samples that are generally prepared individually in test tubes. As research efforts intensify in assessing the impact of bacterial metabolites on plant growth, rapid and efficient methods for assay will be required. Adaptation of microplate readers, used primarily in clinical and microbiological laboratories for spectrophotometric analyses, require less time and reagents than most standard assay methods and should be considered.

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The objectives of this study were to: (i) determine whether a microplate method could be adapted to a routine IAA assay to reduce the time and reagent requirement and still maintain the accuracy of measurements; and (ii) verify the colorimetric detection of auxins by analysis of bacterial cultures using high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Bacteria and culture conditions

Twenty different bacterial isolates originating from rhizospheres and soil were selected (Table 1). Plants sampled for rhizosphere bacteria included giant foxtail (*Setaria faberii*), redroot pigweed (*Amaranthus retroflexus*), leafy spurge (*Euphorbia esula*) and leatherleaf fern (*Rumohra adiantiformis*). A collection of 125 bacterial isolates were previously screened qualitatively for production of L-TRP-derived auxins using an *in situ* nitrocellulose membrane assay (Bric *et al.* 1991). Those isolates showing positive reaction for auxin production were selected for the colorimetric determinations. Cultures were maintained and grown on half-strength tryptic soy agar (TSA; BBL 11768). For colorimetric IAA assays, a 24-h TSA culture of each isolate was suspended in sterile water to an O.D. of 0·5 at 500 nm. The suspension (2 ml) was added to 28 ml of growth medium in a 50-ml tube. The growth medium contained (in g l^{-1}): glucose, 5·0; yeast extract, 0·025; L-TRP, 0·204. Controls were prepared by substituting sterile water for bacterial suspension. Tubes were capped, vortexed and statically incubated in the dark at 27°C for

Table 1 Auxin production by bacterial isolates detected using standard and microplate methods

Isolate	Origin	Auxins produced (± S.D.) (μg IAA equivalents per ml)	
		Standard	Microplate
Rhizosphere isolates			
Enterobacter sp. 3.8.12.4	*Setaria faberii*	4·87 (0·62)	4·71 (0·59)
Enterobacter taylorae 3.8.12.7	*S. faberii*	5·16 (0·19)	4·73 (0·26)
Enterobacter sp. 3.8.12.10	*S. faberii*	1·29 (0·07)	1·26 (0·06)
Xanthomonas maltophilia 7.8.12.7	*S. faberii*	4·21 (0·34)	3·77 (0·27)
7.8.12.8*	*S. faberii*	10·94 (0·28)	9·36 (0·08)
Enterobacter sp. 7.8.12.10	*S. faberii*	3·79 (0·26)	3·72 (0·18)
Pseudomonas cepacia P30	*Amaranthus retroflexus*	4·79 (0·22)	4·71 (0·07)
Pseudomonas sp. P40	*A. retroflexus*	4·17 (0·23)	3·86 (0·29)
Alcaligenes faecalis K2	*Euphorbia esula*	3·79 (0·16)	3·52 (0·05)
Agrobacterium radiobacter N24	*E. esula*	4·87 (0·62)	4·71 (0·59)
2.6.10.19	*E. esula*	4·64 (0·45)	4·39 (0·54)
TS54	*E. esula*	4·60 (0·04)	4·07 (0·04)
TS55	*E. esula*	5·61 (0·12)	4·75 (0·23)
Ps. paucimobilis Y4	*E. esula*	4·10 (0·28)	3·66 (0·32)
Pseudomonas sp. 311K1	*Rumohra adiantiformis*	1·06 (0·08)	0·97 (0·15)
Pseudomonas sp. 32K	*R. adiantiformis*	4·17 (0·13)	3·73 (0·22)
Soil isolates			
Ps. fluorescens SD8	Marshall county, South Dakota	0·38 (0·02)	0·42 (0·06)
Ps. aureofaciens SD10	Marshall county, South Dakota	0·60 (0·04)	0·69 (0·07)
Ps. paucimobilis SD13	Marshall county, South Dakota	0·36 (0·05)	0·47 (0·01)
Pseudomonas sp. 311K2	Volusia county, Florida	0·50 (0·10)	0·60 (0·09)

* Accession numbers alone indicate inability to identify to genus.
IAA, Indole-3-acetic acid.

72 h. Prior to analyses for auxins, individual cultures were adjusted to 10^8 cells ml^{-1} at 500 nm absorbance with sterile water and filtered through 0·2-μm membranes. Triplicate tubes of each isolate were used for the assays.

Assay methods

Samples were assayed for production of auxins (IAA equivalents) using two methods. The Standard method was that of Gordon and Weber (1951) in which auxin present in the culture filtrate (3 ml) was reacted with Salkowski reagent (2 ml) to yield a pink-coloured product after 30 min incubation, which was quantitatively measured on a spectrophotometer (Pharmacia Ultrospec III) at 530 nm. The Microplate method was a modification of the Standard method where 150 μl of culture filtrate were dispensed into wells of 96-well microplates (Corning 25855) followed by addition of 100 μl of Salkowski reagent, allowed to react 30 min, and colour intensity measured at 530 nm on a microplate reader (Dynatech MR 5000). Each IAA standard and culture filtrate were dispensed in three replicate wells within a 96-well microplate, which constituted one experiment. Each experiment consisted of microplates prepared with newly dispensed reagents and filtrates. Comparative experiments were then repeated twice. Results were subjected to regression analysis and analysis of variance procedures.

Identification of auxins by HPLC

Production of IAA and other auxin derivatives in bacterial cultures was confirmed by HPLC analysis. Cultures were filtered through 0·2-μm membranes and extracted with ethyl acetate according to Frankenberger and Brunner (1983). Auxin derivatives were separated using an isocratic method on a Beckman Model 338 HPLC system (Beckman Instruments, Inc., CA). The system consisted of two Model 110B pumps operated at a 1 ml min^{-1} flow rate, a Model 507 autosampler with a 100-μl sample loop, and a Model 166 variable wavelength u.v. detector set to 280 nm. An ultrasphere octadecyl (C-18) reversed-phase column (ODS, 5 μm, Beckman) with dimensions of 250 × 4·6-mm

(i.d.) was used. The mobile phase was 35% methanol : water (pH 2·53) at an ambient temperature of 26°C. Sample peaks were identified by comparing their relative retention times with that of the standard chromatogram using indole-acetonitrile (IAN) as a reference peak.

RESULTS AND DISCUSSION

The calibration curves (based on known concentrations of IAA) for both methods were linear (Fig. 1) with O.D. highly correlated with IAA concentration ($R^2 = 0{\cdot}999$) at 530 nm. This suggested that the Microplate method could be used to detect levels of IAA similar to the Standard method.

A comparison was made of auxin (IAA equivalents) production between the two methods using 20 different bacterial isolates (Fig. 2). Auxin production as measured by both methods was statistically correlated ($R^2 = 0{\cdot}991$). Thus, the Microplate method appeared to be as accurate as the Standard method in detecting levels of auxins among different genera of bacteria originating from various environmental sources.

Comparison of auxin production determined for the 20 bacterial isolates (Table 1) revealed there was no significant difference ($P = 0{\cdot}01$) between the two methods according to analysis of variance. Both methods detected differences in auxin production apparently related to environmental origin of the isolates. In general, isolates originating from plant rhizospheres were more prolific auxin producers than those from bulk soil samples. For example, auxin production by isolate no. 7.8.12.10, originating from giant foxtail rhizosphere, was > 30 times higher than isolate no. SD13, originating from bulk soil. Both methods showed that the collection of rhizobacteria from giant foxtail exhibited a wide range of auxin production whereas rhizobacteria from leafy spurge all produced nearly the same levels of auxins. The variability in auxin production by rhizosphere bacteria has been reported previously (Prikryl *et al.* 1985; Mordukhova *et al.* 1991).

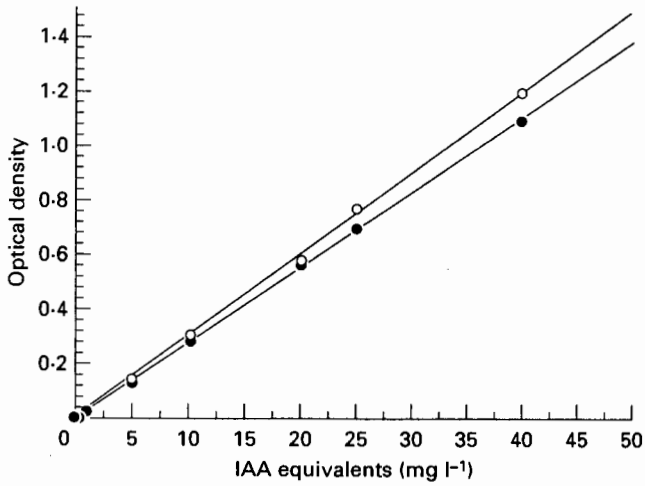

Fig. 1 Relationship between optical density and standard concentrations of indole-3-acetic acid (IAA) for two auxin assay methods. ○, Standard method; ●, microplate method

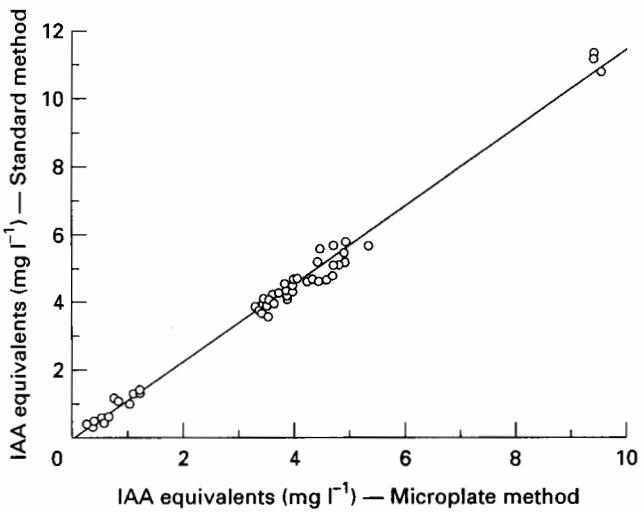

Fig. 2 Relationship between auxin production by bacterial cultures determined by two assays methods

HPLC analysis of culture filtrates examined in the comparative spectrophotometric methods confirmed that several L-TRP-derived auxins were produced by the bacterial isolates. For example, isolates 311K1 and P-30 were found to produce different levels of IAA, tryptophol (TOL), indole-3-aldehyde (IALD), indole-3-lactic acid (ILA), indole-3-acetamide (IAM) and 5-hydroxytryptophan (5-OH-TRP). This contrasts with previous reports that rhizosphere bacteria produce primarily IAA in culture (Schroth *et al.* 1984). These isolates were similarly characterized by both the Microplate and Standard methods as producing total auxins in differential amounts (Table 1).

The Microplate method resulted in 95% less sample and reagent volume used. Using triplicate wells for each sample, a 96-well microplate can be prepared to accommodate a full set (6) of IAA standards and up to 26 unknowns. It requires approximately 10 min to dispense aliquots into wells using a mechanical pipetter, about 30 s to add Salkowski reagent, 10 s for reading the microplate, and 3 min to print results. The microprocessor component of the microplate reader can directly calculate auxin (IAA equivalents) concentrations from absorbance values using readings from standards in pre-designated wells. The Standard method required at least 3 h to read the same number of samples

followed by an additional 30 min to prepare a standard curve and convert sample readings to IAA concentrations.

Using microplate instrumentation, production of L-TRP-derived auxins by bacterial cultures can be assayed rapidly and efficiently and can be readily incorporated as a key parameter in assessing plant-growth-regulating activities of bacterial culture collections, which might otherwise be limited by time-consuming methodology. The Microplate method uses less chemicals, requires drastically decreased time and labour inputs and is as accurate as the Standard method of analysis.

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