APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1995, p. 793–796 Vol. 61, No. 2
0099-2240/95/$04.00+0

A Critical Examination of the Specificity of the Salkowski Reagent for Indolic Compounds Produced by Phytopathogenic Bacteria

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Received 8 August 1994/Accepted 15 November 1994

We examined the sensitivity and the specificity of three versions of the Salkowski colorimetric technique. Two of these allowed the detection of indoleacetic acid (IAA) over a low range of concentrations (0.5 to 20 μg/ml), while the third permitted the detection of IAA over a range of higher concentrations (5 to 200 μg/ml). Overall, the three formulations reacted not only with auxin (IAA) but also with indolepyruvic acid and indoleacetamide. Therefore, these techniques appear to be specific for IAA, indolepyruvic acid, and indoleacetamide rather than for IAA alone.

Numerous plant-associated bacteria produce auxin and related indolic compounds. Among these bacteria, some are plant pathogens for which auxin production constitutes a key determinant of pathogenicity (reviewed in references 8 and 14). Assessing the production of auxin is therefore of interest to the pathologist. Several methods exist. Some are based on the biological effects of auxin upon plants, such as root hair deformation (Avena curvature test) or coleoptile elongation (wheat coleoptile or corn coleoptile section test [6]). Other techniques are based on physicochemical properties of these compounds. Thus, precise identification of auxin and related molecules can be obtained following extraction of bacterial culture supernatants, concentration of the extracts, and separation and identification of the compounds by high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (3, 7). Such methods, however, are time-consuming and cannot be used as routine assays. To circumvent these problems, various authors have used a colorimetric technique derived from that of Salkowski (4, 11, 13) for indole detection. This method has been used for years because it is simple, rapid, and cheap and allows the daily analysis of numerous bacterial supernatants. It proved to be useful for screening bacterial mutants affected in auxin synthesis (15), and a derivative of the technique was also used in assays in situ for the direct analysis of auxin production by bacterial colonies (1). The specificity of this method, however, is not clearly established. Previous available studies (4, 9) dealt with a limited number of indolic compounds and did not evaluate the specificity of the technique for compounds involved in auxin biosynthesis, such as indolepyruvic acid (IPyA) or indolelactic acid (ILA). The need for a critical evaluation of the validity of the technique led us to examine the specificity and the sensitivity of various formulations of the Salkowski reagent for a broad range of indoleacetic acid (IAA) biosynthetic intermediates such as tryptophan (Trp), indoleethanol (or tryptophol), indoleethanolamine (or tryptamine), IPyA, ILA, and indoleacetamide (IAM). Other compounds tested included the following IAA analogs: 5′-hydroxy-IAA (5′OHIAA), indolepropionic acid, indolebutyric acid, indoleglyoxylic acid, and indolealdehyde.

The first colorimetric technique (termed PC) was performed according to Pilet and Chollet (9), using reagent R1, which consisted of 12 g of $FeCl_3$ per liter in 7.9 M H_2SO_4. One milliliter of reagent R1 was added to 1 ml of the sample solution, well mixed in a 3-ml spectrophotometer cuvette, and the mixture was left in the dark for 30 min at room temperature. The second and third colorimetric methods, termed S1/1 and S2/1, were derived from that of Tang and Bonner (13). They used reagent R2, which consisted of 4.5 g of $FeCl_3$ per liter in 10.8 M H_2SO_4. One (S1/1 method) or 2 (S2/1 method) ml of reagent R2 was added to 1 ml of the sample solution, and the mixture was processed as indicated above for the PC colorimetric technique (the only difference between S1/1 and S2/1 is in the volume of R2 reagent added). Both reagents R1 and R2 were kept in the dark at room temperature. We noticed that heat was generated by the addition of sulfuric acid to the aqueous sample. However, no major difference in heat production was observed as judged by the temperature of the reaction mixtures (32 ± 4°C). The absorption spectra of the mixtures were determined at 370 to 670 nm on a Beckman DU-68 spectrophotometer equipped with a Soft-Pac Quant I module. Sample solutions and absorption blanks were obtained by using King B bacterial growth medium alone or supplemented with 2.5 mM Trp (0.5 g/liter). This medium consisted of the following: Difco Proteose Peptone no. 3, 20 g/liter; K_2HPO_4, 1.15 g/liter; $MgSO_4 \cdot 7H_2O$, 1.5 g/liter; and glycerol, 1.5% (vol/vol). This complex medium, supplemented with Trp, was chosen because it is often used to assess auxin production by phytopathogenic bacteria, especially members of the family *Pseudomonaceae* such as *Pseudomonas syringae* (2) or members of the family *Enterobacteriaceae* such as *Erwinia herbicola* (7). Our experimental conditions were therefore very similar to those used by most investigators.

To assess the sensitivity of the PC, S1/1, and S2/1 techniques, we first measured the optical density at 530 nm (OD_{530}) as a function of the IAA concentration of the samples. This wavelength was chosen since it is routinely used in auxin colorimetric assays derived from Salkowski. As indicated above, samples were prepared in King B medium alone or supplemented with 2.5 mM Trp. The results are presented in Fig. 1. Each point plotted represents the average value calculated from eight in-

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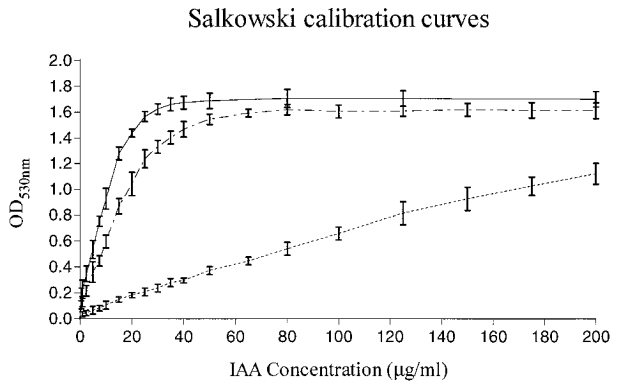

FIG. 1. Determination of sensitivities of three colorimetric techniques. OD_{530} values were measured as a function of IAA concentration. All assays were performed in King B medium supplemented with 2.5 mM Trp. ——, PC; — – —, S1/1; – – – –, S2/1.

dependent measurements. This high replication was necessary because we observed some day-to-day variations, and lot-to-lot variations in IAA, in the course of this study. Calibration curves showed that the PC reagent allows the detection of very low amounts of IAA (lower detection limit, 0.3 μg/ml; i.e., 1.7×10^{-6} M). However, this reagent cannot be used for determining IAA concentrations higher than 20 μg/ml (1.1×10^{-4} M) since a clear saturation of the colorimetric reaction was observed in these conditions. The S1/1 technique appeared a bit less sensitive than the PC technique. It allowed the detection of auxin concentrations ranging from 0.5 (2.9×10^{-6} M) to 35 μg/ml (2×10^{-4} M). Interestingly, the S2/1 technique, though less sensitive, permitted detection of IAA within a range of higher concentrations, from 5 to over 200 μg/ml (2.9×10^{-5} to 1.1×10^{-3} M). Whatever the method used, the presence of 2.5 mM Trp in the sample led to a decreased sensitivity of the assay (data not shown). It is therefore necessary to take this parameter into account when using the Salkowski colorimetric techniques.

To evaluate the specificity of the three techniques described above, we first determined the OD_{530} of colorimetric mixtures prepared with samples of different indolic compounds, all from commercial sources (Sigma Chemical Co.). For each colorimetric technique, two different concentrations of indolic compounds were retained. The values were chosen by examining the calibration curves obtained with IAA (Fig. 1). For the PC and S1/1 methods, the higher retained concentrations corresponded to the saturation phase of the techniques. These were, respectively, 2.9×10^{-4} M (50 μg of IAA equivalent per ml) and 3.7×10^{-4} M (65 μg/ml). The lower values were chosen as the concentrations yielding half of the maximum OD_{530} obtained with these techniques, e.g., 5.1×10^{-5} M (9 μg/ml) for PC and 8×10^{-5} M (14 μg/ml) for S1/1. For the S2/1 technique, no saturation phase was observed in the range of concentrations studied (Fig. 1). Consequently, we chose a low and a high value, i.e., 3.7×10^{-4} M (65 μg/ml) and 8.6×10^{-4} M (150 μg/ml), respectively. Sixteen compounds were tested (with or without Trp in the sample). The results are given in Table 1. To facilitate data analysis and to reproduce closely the routine experimental procedures (2, 3, 7), we have only reported the values obtained in the presence of Trp. As noted above, the OD_{530} values measured in the absence of Trp were slightly higher than those observed in its presence (data not shown). Whatever the colorimetric technique used, IAA, IPyA, and IAM clearly reacted (Table 1). However, the three formulations exhibited different specificities for IAA and indolic compounds. The PC method was the most specific since it essentially reacted with IAA and, to a lesser extent, with IPyA and IAM. None of the other compounds assayed reacted strongly. As observed in the studies on the sensitivities of the

TABLE 1. Reactivity coefficients of various indolic compounds upon assay with the techniques modified from Pilet and Chollet (9) and Tang and Bonner (13)

Compound[b]	Reactivity coefficient[a] (%) by:					
	PC technique		S1/1 technique		S2/1 technique	
	5.1×10^{-5} M[c]	2.9×10^{-4} M	8×10^{-5} M	3.7×10^{-4} M	3.7×10^{-4} M	8.6×10^{-4} M
Trp	—[d]	—	—	—	12	13
IPyA	39	≥100	75	85	209	≥133
ILA	—	—	—	—	—	6
IEt	—	—	—	—	—	4
INH2	—	—	—	2	14	13
IAM	17	20	64	≥100	≥307	≥133
IAA	78	≥100	78	≥100	118	110
IAld	—	—	—	—	—	—
5′OHIAA	3	5	4	8	17	13
IPA	—	1	—	2	11	12
IBA	—	1	—	2	11	7
IGA	—	1	—	2	11	11
Pyruvic acid	—	—	—	—	—	—
Pro	—	—	—	—	—	5
Phe	—	—	—	—	—	—
Indole	—	1	—	5	21	32

[a] For each compound, the average OD_{530} values were calculated from three independent measurements and converted to equivalent IAA concentrations, using the calibration curve shown in Fig. 1. These concentrations, termed [Eq. C.] (equivalent IAA concentrations), were then compared with those of theoretical input IAA ([IAA]) to determine the reactivity coefficient (RC) of the compounds given in the table according to the following formula: RC = 100 × ([Eq. C.]/[IAA]). A reactivity coefficient of 12% indicates that 3.7×10^{-4} M Trp (65 μg/ml) is detected as 3.7×10^{-4} M × 12%, i.e., as 4.4×10^{-5} M IAA (7.8 μg/ml).

[b] IEt, indoleethanol; INH2, indoleethanolamine; IPA, indolepropionic acid; IBA, indolebutyric acid; IGA, indoleglyoxylic acid; IAld, indolealdehyde.

[c] Concentration of compound.

[d] —, OD_{530} values below the detection limit, i.e., not different from zero. Values were 0.08 OD_{530} unit (corresponding to 0.3 μg of IAA per ml) for PC, 0.10 OD_{530} unit (0.5 μg of IAA per ml) for S1/1, and 0.07 OD_{530} unit (5 μg of IAA per ml) for S2/1.

three techniques, results obtained by the S1/1 method were similar to those obtained by the PC method, though the latter appeared slightly more specific. For instance, indoleethanolamine reacted weakly at 530 nm with the S1/1 method, whereas it did not with the PC method. In contrast, the S2/1 protocol was less specific than the PC and S1/1 protocols. Several compounds reacted at both concentrations assayed with the S2/1 technique. The presence of such compounds in a bacterial supernatant may therefore alter the accuracy of the determination of IAA concentration. However, the S2/1 technique remains a suitable method for assaying the production of indole compounds by bacteria. Thus, with this technique, concentrations (3.7×10^{-4} M, i.e., 65 μg of IAA per ml) of indoleethanolamine, indolepropionic acid, and indolebutyric acid equivalent to 7 to 9 μg of IAA per ml (relative absorption, 11 to 14%) were detected, whereas indolealdehyde, indoleethanol, and ILA were not detected (Table 1).

Whatever the technique used, only five compounds gave a visually detectable color reaction, suggesting that the λ_{max} values obtained for each compound should differ. Thus, with the PC or S1/1 method, indole gave an olive-drab color; 5′OHIAA, a beige to brown color; IAM, a strong violet to purple color; and IAA and IPyA, a red color. To further analyze the specificity of the Salkowski reagents, we attempted to determine the wavelength corresponding to the peak of absorption (λ_{max}) of colorimetric mixtures obtained with each compound. Experiments were always performed in King B medium alone or supplemented with 2.5 mM Trp. Compounds were added to this medium at the two concentrations indicated above and in Table 1. Reacting compounds exhibited characteristic absorption spectra from which λ_{max} values were directly deduced. Below, we present only the results of the study performed with King B medium supplemented with Trp. On the basis of the λ_{max} values, calculated from three independent measurements, two groups of compounds can be characterized when the PC or S1/1 technique is used (Fig. 2). The first group includes most of the indolic compounds, except indole, IAA, IPyA, IAM, and 5′OHIAA. The λ_{max} values observed for compounds of the first group ranged from ca. 425 to ca. 460 nm when the PC protocol was used. The second group of compounds includes IAA, IPyA, IAM, and 5′OHIAA. With the PC method, the λ_{max} values for the last four compounds ranged from 520 to 555 nm. As judged from analysis of the absorption spectra, the sensitivity and specificity of the PC technique should be slightly increased by measuring the OD of the colorimetric complex at 540 rather than 530 nm. Results obtained with the S1/1 protocol were very close to those obtained with the PC technique. Thus, a limited shift of the λ_{max} toward a higher wavelength was observed when the S1/1 protocol was used (Fig. 2). Here also, the specificity of the technique can be improved slightly by measuring the absorption of the complex at 540 rather than 530 nm. Results observed with the S2/1 method were quite different from the previous ones. Indeed, a red shift of all λ_{max} values was observed with this technique. This shift is quite clear for compounds such as indoleglyoxylic acid, indoleethanol, ILA, and indolepropionic acid. As a consequence, and given the λ_{max} values of IAA and related compounds, the measurement of the OD of the colorimetric complex should be performed at 550 rather than 530 nm when the S2/1 technique is used. Overall, the λ_{max} values of the first group of compounds are much nearer those of the second group of compounds when the S2/1 method is used. This provides an explanation for the reduction of specificity observed when the S2/1 procedure is used. As indicated above, the latter assay is more sensitive for IAM than for IAA. The sensitivity toward IAM can even be increased by measuring OD_{590} rather than OD_{530} with the S2/1

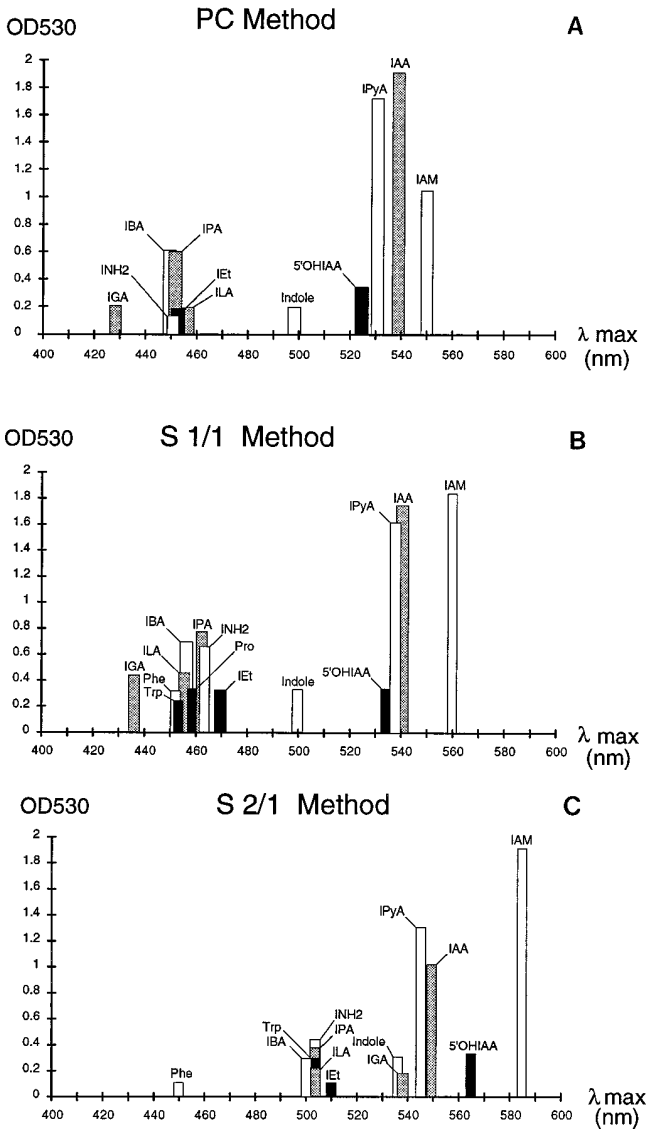

FIG. 2. Determination of specificities of the colorimetric techniques PC (A), S1/1 (B), and S1/2 (C). OD values at λ_{max} are given for each reacting compound. IEt, indoleethanol; INH2, indoleethanolamine; IPA, indolepropionic acid; IBA, indolebutyric acid; IGA, indoleglyoxylic acid; IAld, indolealdehyde.

method. Interestingly, determination of the λ_{max} of the colorimetric mixtures may also give some clues to the nature of the compounds produced by the bacteria. Whatever the method used, proline, phenylalanine, and pyruvic acid did not exhibit characteristic absorption spectra, although a limited absorption of the colorimetric mixture was observed with Phe, Trp, or Pro when the S1/1 and S2/1 methods were used.

When a similar study was performed with King B medium without added Trp, we first observed a limited blue shift of the λ_{max} values (data not shown). We also observed that, whatever the technique, the colorimetric methods were more specific for indolic compounds when performed in the absence of Trp. This phenomenon probably results from the limited reaction of Trp with the colorimetric reagents. We also performed Salkowski colorimetric assays with PC and S2/1 reagents prepared with perchloric acid (1, 4, 11) instead of sulfuric acid (same molar concentration). In our experimental conditions,

TABLE 2. Detection of indolic compounds in actual spent bacterial cultures

P. syringae pv. *savastanoi* strain[a]	Growth medium[b]	Concn of Salkowski-positive compounds (μg of IAA per ml)[c]		Compounds detected[d]		
		PC	S2/1	IAA	IPyA	ILA
CFBP2088	KB	1	≤5	+	+	+
	KB + Trp	≥20	123	+++	+++	+++
CFBP2169	KB	≤0.3	≤5	−	−	−
	KB + Trp	0.5	11	+/−	−	+
CFBP1670	KB	4.5	20	++	−	−
	KB + Trp	≥20	145	+++	−	−

[a] These strains were obtained from the Collection Française des Bactéries Phytopathogènes, Institut National de la Recherche Agronomique, Angers, France.

[b] KB, King B medium without Trp; KB + Trp, King B medium supplemented with 2.5 mM Trp.

[c] OD_{530} values were directly converted to equivalent IAA concentrations (micrograms per milliliter), using the calibration curves shown in Fig. 1. The symbols indicate whether the values were below (≤*x*) or above (≥*y*) the detection limits ([*x*, *y*]) of the technique.

[d] These compounds were detected by HPLC of the ethyl acetate-extracted fraction of bacterial culture supernatants (3). −, not detected; +/−, trace amounts detected; + to +++, low to high amounts detected.

reagents prepared with $HClO_4$ appeared less sensitive than and as specific as those prepared with H_2SO_4 (data not shown). We therefore advise preparation of Salkowski reagents with H_2SO_4. H_2SO_4 is also cheaper and more convenient to handle.

To document the efficiency of detection of indolic compounds in actual spent cultures, we summarized results obtained with three strains of *P. syringae* pv. *savastanoi* grown in King B medium alone or supplemented with Trp 2.5 mM. The production of indolic compounds by these bacteria was investigated by using the HPLC method, essentially as described by Fett et al. (3), and two formulations of the Salkowski reagents (PC and S2/1). The results are shown in Table 2. Essentially, they confirmed the effectiveness of the colorimetric technique as a simple routine assay. Indeed, the production of Salkowski-positive compounds, even at low concentrations, was always correlated with production of IAA or IPyA. On the contrary, a strain which did not produce Salkowski-positive compounds did not produce either IAA or IPyA, as judged by HPLC analysis of the supernatant. Additionally, the values obtained with the S2/1 technique were always higher than those obtained with the PC technique. We suggest that this reflects the better specificity of the PC method versus the S2/1 method.

Our study clearly demonstrates that the three formulations of the Salkowski reagent exhibit different sensitivities and specificities for indolic compounds. It shows that the PC technique is the most sensitive and the most specific. It could be used to determine clearly whether a sample does or does not contain auxin. However, concentrations of indolic compounds detected in the culture supernatants of various soil or plant-pathogenic bacteria range from 0 to over 200 μg/ml (1.1×10^{-3} M). In spite of its reduced specificity, the S2/1 technique therefore appears to be suitable for the direct measurement of such auxin concentrations in growth media, especially when a large number of samples of unknown concentration are to be analyzed or compared. The two other techniques (PC and S1/1) might be used to quantify IAA concentrations in culture supernatants of bacterial strains producing low amounts of this compound or in diluted supernatants of bacteria producing high amounts of IAA. A common feature of the colorimetric techniques was noted: whatever the colorimetric technique used, IAA and IPyA cannot be distinguished since they yield closely related absorption spectra (even though the detection levels of these two compounds differ according to the technique used). Our results also indicate that the Salkowski reagent is specific for IAA, IPyA, and IAM rather than for IAA alone. As a consequence, production of IPyA or IAM by bacterial cells could be mistaken for production of IAA. Production of IAM, however, is often related to production of IAA since the gene controlling the synthesis of IAM (*iaaM*) is genetically linked to that encoding the conversion of IAM to IAA (*iaaH*) (2, 12). On the contrary, it is likely that IPyA could be synthesized by various bacteria as the result of nonspecific transamination of tryptophan (5, 10, 15). Even bacteria producing low amounts of IPyA could therefore be misidentified as auxin producers. Care should therefore be exercised when interpreting results obtained with these colorimetric techniques. These conclusions demonstrate the importance of our study by validation of the routine use of the Salkowski reagent. Indeed, neither IAM nor IPyA was included in previous surveys on the specificity of the Salkowski reagent.

We thank Louis Gardan (Institut National de la Recherche Agronomique, Angers, France) and Alain Delbarre and Annik Petit (Centre National de la Recherche Scientifique, Gif, France) for helpful discussions, Phil Oger for help in computer drawing, and Spencer Brown for carefully correcting the manuscript.

E.G. was supported by a fellowship from the French Ministère de la Recherche et de la Technologie.

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