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Note

Spectrofluorodensitometric estimation in thin-layer chromatography of gibberellic acid produced by solid-state fermentation

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Gibberellic acid, an important plant growth regulator, is used extensively in agriculture, nurseries, green houses, viticulture, tea-gardens, etc.[1]. It is traditionally produced by submerged fermentation by employing *Gibberella fujikuroi* or *Fusarium monoliforme*[2]. Spectrophotometric, fluorometric and bioassay methods are routinely used for the estimation of gibberellic acid in fermentation broths or in crude forms[3–5], though a variety of other methods are available for its estimation[6–10]. Recently, the potential of solid-state fermentation for production of gibberellic acid was established[11]. Preliminary studies indicated that the above routine analytical methods give poor responses unless the crude extract of mouldy bran obtained by solid-state fermentation is extensively purified. Since microbial products, such as mycotoxins[12–18], can now be estimated by spectrofluorodensitometry with greater accuracy[19] without resorting to extensive purification, we studied the application of this technique to the estimation of gibberellic acid in crude extracts of mouldy bran obtained by solid-state fermentation.

EXPERIMENTAL

Fermentation

The methodology adopted for solid-state fermentation was as described by Sreekantiah[20]. The dry mouldy bran was extracted with ethyl acetate (1:6) at 30°C for 30 min. In case of submerged fermentation, Czapek Dox liquid medium was used and the product was extracted with ethyl acetate at pH 2.5. The extracts were concentrated under vacuum.

Spectrofluorodensitometry

Thin-layer chromatographic (TLC) plates, coated with silica gel G, (46% suspension in water, Glaxo Labs.) to a thickness of 300 μm, were activated at 110°C for 1 h. A 10-μl volume of concentrated extract and of standard gibberellic acid solutions (Sigma) were spotted. The plates were eluted with chloroform–ethyl acetate–acetic acid (5:4:1), then sprayed with concentrated sulphuric acid containing 5% ethanol, heated at 100°C for 30 min and finally observed under UV light at 254 nm. The intensity of the fluorescence of the spots was measured by an automatic recording spectrofluorodensitometer (Model SD 3000; Schoeffel, F.R.G.).

Comparative accuracy

Standard solutions of gibberellic acid and concentrated crude extracts obtained by submerged and solid-state fermentations were analyzed by spectrophotometric[3], fluorometric[4], bioassay[5] and spectrofluorodensitometric methods. Gibberellic acid, eluted from a TLC plate spotted with crude extract, was also analyzed by these methods. Two-dimensional TLC was also carried out using ethyl acetate–chloroform–acetic acid (4:5:1) followed by benzene–acetone–acetic acid (13:6:1).

RESULTS AND DISCUSSION

Characteristics of crude extract

Among the 5–7 distinct spots having different R_F values on the chromatogram of the crude extract from solid-state fermentation, 2–3 spots emit fluorescence at the wavelength used for estimation of gibberellic acid, but only one of these corresponds to authentic gibberellic acid. The particular strain employed is also known to co-produce plant growth regulators other than gibberellic acid[21]. In addition, the constituents of wheat bran, a substrate used in solid-state fermentation, are also co-extracted by the solvent. These compounds interfere with the accurate estimation of gibberellic acid when methods based on fluorescence or plant-growth-promoting activity are used. They also have similar solubilities to gibberellic acid in solvents used for its extraction from mouldy bran. The complete separaton of gibberellic acid from other constituents was not possible with various solvent systems such as chloroform–methanol–acetic acid–water (70:20:3:2) and ethyl acetate–chloroform–acetic acid (15:5:1)[22,23].

Reliability of spectrofluorodensitometry

The spectrofluorodensitometric analysis of standard solutions of gibberellic acid, varying in concentration from 2 to 10 μg per 10 μl, gave a linear relationship: $Y = 0.998X$. The sum of the squares of the deviation from this regression equation[24] was 0.002, while the standard error was 0.0265. The sensitive range for gibberellic acid lies between 2 and 10 μg per 10-μl spot at 0.1 absorbance unit of the spectrofluorodensitometer and it can be enhanced to 5–40 μg per 10-μl spot, without affecting the reliability, if 2.0 absorbance units are used. The percentage error is less than $\pm$ 2 in both cases.

TABLE I

CO-SPECTROFLUORODENSITOMETRIC RESPONSE

Sample spotted	*Gibberellic acid (ppm)*	
	Spectrofluoro-densitometry	*Probable range**
Standard solution (1000 ppm)	1000	980–1020
Unknown sample	393	401–385
Unknown sample + standard solution (1000 ppm)	1379	1351–1407

* Based on error of ± 2%.

TABLE II

RESULTS OF THE ANALYSIS OF A CRUDE EXTRACT OF DRY MOULDY BRAN BY SPECTROFLUORODENSITOMETRY OF FOUR REPLICATES

S.D. = Standard deviation; C.V. = coefficient of variation.

Experiment	*Gibberellic acid (ppm) after incubation for period*		
	5 days	*6 days*	*7 days*
1	139.5	247.0	355.0
2	146.2	251.6	352.0
3	145.4	237.4	348.0
4	148.4	248.0	364.0
Mean ± S.D.	144.9 ± 3.80	246.0 ± 6.06	353.5 ± 8.66
C.V. (%)	2.62	2.46	2.45
Mean ± 2 S.D.	137.3–152.5	233.9–258.1	336.2–370.8

The results of the co-spectrofluorodensitometric analysis of a mixture of a crude extract of unknown concentration and of standard gibberellic acid solution were similar to those of individual spectrofluorodensitometric analyses of these samples (Table I). Even two-dimensional TLC showed a single spot corresponding to gibberellic acid, thereby confirming the purity of the spot. The results and corresponding statistical analysis for the crude extract of mouldy bran, performed as four

TABLE III

COMPARATIVE RESPONSES OF VARIOUS METHODS FOR ASSAYING PURE GIBBERELLIC ACID IN STANDARD SOLUTIONS

Figures in parentheses are the per cent errors.

Pure gibberellic acid concentration (ppm)	*Estimated value (ppm)*			
	Spectrofluorodensitometry	*Fluorometry*	*Spectrophotometry*	*Bioassay*
1	–	1.02 (2.0)	–	–
2	1.96 (2.0)	1.99 (0.5)	–	1.88 (6.0)
4	3.96 (1.0)	3.98 (0.5)	–	–
5	5.00 (0)	4.95 (1.0)	–	4.89 (2.2)
6	5.97 (0.5)	–	–	–
8	7.93 (0.9)	–	–	–
10	10.14 (1.4)	–	–	10.80 (8.0)
20	20.19 (1.0)	–	–	–
25	–	–	–	25.63 (2.5)
30	30.14 (0.5)	–	–	–
40	40.09 (0.2)	–	–	–
50	–	–	51.25 (2.5)	–
100	–	–	102.50 (2.5)	–
200	–	–	209.10 (4.6)	–
400	–	–	403.90 (1.0)	–
600	–	–	590.40 (1.6)	–

TABLE IV

COMPARATIVE RESPONSES OF CHEMICAL AND BIOLOGICAL ASSAYS OF GIBBERELLIC ACID IN CRUDE EXTRACTS FROM SOLID-STATE AND SUBMERGED FERMENTATIONS

Fermentation technique	*Volume of crude extract processed for spotting (l)*	*Estimated value (mg) in crude extract processed*				
		Spectro-fluoro-densitometry	*Fluorometry*	*Bioassay*	*Spectrophotometry*	*Probable range (mg)*
Solid state	0.005	1.29	1.86	2.81	–	1.26–1.32
	0.010	2.60	3.77	5.99	–	2.55–2.65
	0.015	3.87	5.70	8.49	–	3.79–3.95
Submerged	1.0 (5-day incubation)	11.50	–	31.00	17.00	11.27–11.73
	1.0 (6-day incubation)	29.20	–	76.00	44.50	28.62–29.78
	1.0 (7-day incubation)	44.50	–	92.00	60.00	43.61–45.39

* Based on error of ± 2% for spectrofluorodensitometric method.

replicates, are presented in Table II. The estimated values are within the limits of the mean ± two standard deviations.

Comparison of methods

The results of the estimation of gibberellic acid in standard solutions and crude extracts by different chemical and biological methods are compared to those from the spectrofluorodensitometric method in Tables III and IV. The per cent error was smaller (less than 2%) for the spectrofluorodensitometric and fluorometric methods. However, the estimated values varied widely when a crude extract of dry mouldy bran was analyzed (Table IV). The spectrofluorodensitometric method gave the lowest values. The higher values for gibberellic acid obtained by spectrophotometric, fluorometric and bioassay methods is due to the presence of fluorescent compounds other than gibberellic acid in the crude extracts. These methods are unable to distinguish gibberellic acid from these interfering compounds. On the other hand, the spectrofluorodensitometric method is specific for gibberellic acid as the interfering compounds are separated from the spot of gibberellic acid on TLC plates.

The results of the bioassay of the gibberellic acid spot from a crude extract of mouldy bran confirm the reliability of the spectrofluorodensitometric method. It gave about 80% of the value obtained for the original crude extract by spectrofluorodensitometry. The difference of 20% is due to losses of gibberellic acid[25] during scraping of the spot from the plate, extraction and transfers. On the other hand, the value obtained by bioassay was about one-half and one-fourth of those estimated by fluorometry and bioassay respectively in the original crude extract. Such large differences

are due mainly to the complete elimination of plant-growth regulatory and fluorescent compounds other than gibberellic acid in the eluted sample.

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