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Production of Gibberellin by different *Fusarium* species in shake flasks and bioreactor

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Thirty three strains of *G. fujikuroi* were screened to select *G. fujikuroi*-52, a strain capable of giving consistent production of gibberellin. The comparative production of GA_3 by LSF and Submerged fermentation (SmF) indicated better productivity with the later technique. Quantitative estimation of gibberellin from different strains of *Fusarium* was done by T.L.C., H.P.L.C., and spectrophotometrically which showed the presence of gibberellin in variable amounts. In time course, the production of GA_3 started from 3rd day and continued to rise up to 7th day of incubation in fermentation medium. Increase in GA_3 level from 4th to 7th day of incubation under fermentation was more or less in linear fashion.

Key Words : Gibberellic acid, *Fusarium moniliforme*, T.L.C., Spectrophotometer, HPLC

INTRODUCTION

Gibberellins are secondary metabolites of the fungus, *Gibberella fujikuroi*, perfect state of *Fusarium moniliforme*. It gives higher yields of GA_3 on a variety of media (Surulirajan and Sarbhoy, 2000) and is associated with bakane disease of rice. Gibberellic acid (GA_3) is a potent plant growth regulator and is extensively used for variety of beneficial effects. It plays an important role in hybrid rice seed production (Ahamad *et al*, 2004). It can adjust physiological and biochemical metabolism of rice plants, specially stimulating elongation of young cells. About 25-30 per cent spikelets of a panicle are inside the flag leaf sheath in most of the *indica* cytoplasmic male sterile (CMS) lines of rice than that of the *japonica* CMS lines. GA_3 has a definite role in exertion of panicle. The application of GA_3 increases seed set and seed yield in hybrid rice (Srivastava *et al.*, 2003). The gibberellins are one of major groups of growth promoting hormones which play an esential role in regulation of growth and development of angiospermic plants. *F. moniliforme* produces many types of gibberellins in culture media as well as in the inoculated plants, amongst which gibberellic acid (GA_3) has received the greatest attention. Out of which GA_3 regulates the rate of growth and expansion of internodes (Graebe and Ropers, 1978) and is being extensively used for a variety of beneficial effects (Phinney, 1983). Recently, it has also been introduced as a seed application to improve seedlings establishment in seeded rice (Carlson *et al.*, 1992). Ahamad (2003) has suggested various methods to elaborate GA_3 from different plants.

A number of microorganisms have been reported to produce GA_3 and GA like substances. Among these, the fungal cultures are able to produce GA like activities in higher yields. Its use at present is limited to high premium crops. The demand of gibberellin is increasing but the research and developmental efforts on the production of GA_3 are on a low key (Srivastave *et al.*, 2003). A critical analysis of all production aspects to understand various unit parameters and the evaluation of the possible uses of these in the production of GA_3 for higher yield and lower cost has been dealt systematically.

Fusarium are widely distributed in soil as well as inorganic substrates and can survive on wide range of substances (Tudzynski and Tudzynski, 2002). Furthermore the ability of various strains of *Fusarium* to produce GA_3 varied widely (Kumar and Lonsane, 1987; Bruckner *et al.*, 1989) and the production of bioactive metabolites is dependent both on climatic conditions and strain specificity (Voigt *et al.*,1995). Indian subcontinent provides best conditions for growth of various *Fusarium* species. The main purpose of this study is to screen different *Fusarium* species, for getting stable and high producing gibberellins and time course for GA_3 production under LSF and SmF techniques and to provide best isolates of *Fusarium* for gibberellin production to the industry so that cost effective approach for the sustainable crop production can be made.

MATERIALS AND METHODS

Thirty three isolates of *Fusarium* spp. were screened for GAs production through LSF (Liquid surface fermentation) and SmF (Submerged fermentation) techniques in this study.

Culturing of the fungus

Thirty three *Fusarium* species were tested for their potentiality to produce plant growth regulatory metabolites in their culture filtrate. These test fungi were isolated from plants and soil samples obtained from various parts of India. Twelve isolates of the fungus were obtained from ITCC (Indian Type Culture Collection), Division of Plant Pathology, IARI, New Delhi and twenty one were isolated from different soil samples and infected plant tissues (Tables 1 & 2). *Fusarium* strains were isolated from the freshly collected soils (Nash and Snyder, 1965). These species were identified using combinations of microscopic and colony character (Nelson *et al.*, 1983). However, difference between some species were subtle and these were characterized by RAPD (Mitter *et al.*, 2002). Each of test fungi was purified by single spore/hyphal tip isolation method. These were maintained in PDA in 4^0C in BOD incubator. For the secretion of plant growth regulatory fungal metabolites, each isolate was grown in Richard's broth medium containing sucrose 50 g, potassium nitrate 10 g, potassium di-hydrogen orthophosphate 5 g, magnesium sulphate 2.5 g, ferric chloride 0.02 g, distilled water 1000 ml and pH 7.0-7.5.

Table 1 : List of *Fusarium* species isolated from different soil samples/host

Fungus isolates	Strain No	Sources	Location
F. moniliforme	FM-I	Wheat kernels	Punjab
F. moniliforme	FM-II	Wheat kernels	Punjab
F. moniliforme	FM-III	Rice field, IARI	N. Delhi
Fusarium moniliforme	FM-V	IARI soil	N. Delhi
F. moniliforme	FM-VI	Rice field	Kerela
F. moniliforme	FM-XII	Nematology, IARI	N. Delhi
F. moniliforme var. *subglutinans*	FMS-IV	Wheat Kernels	Punjab
F. moniliforme var. *subglutinans*	FNS-XVI	Maize field, IARI	N. Delhi
F. solani	FS-VI	Katrain Veg. Farm	H.P.
F. oxysporum	FO-I	Katrain Veg. Farm	H.P.
Fusarium moniliforme	FM-VII	Rice field	Meghalaya
F. moniliforme	FM-VIII	Rice field	A.P.
F. moniliforme	FM-IX	Rice field	Kerala
F. moniliforme	FM-X	Rice field	Kerala
F. solani	FS-I	Rice field	Meghalaya
F. pallidoroseum	FP-IV	Rice field	Meghalaya
F. palidoroseum	FP-I	Rice field	A.P.
F. pallidoroseum	FP-II	Rice field	Maharashtra
F. pallidoroseum	FP-III	Cotton crop	Maharashtra
F. solani	FS-II	Maize crop	Maharashtra
F. solani	FS-III	Sorghum crop	Maharashtra

Table 2 : Cultures of various isolates of *Fusarium moniliforme* acquired from ITCC, IARI, New Delhi

Fungus/Strains	Host	ITCC Nos.
F.M-1191 *Fusarium moniliforme*	*Oryza sativa*	1191
F.M-954 *F. moniliforme*	*O. sativa*	954
F.M-2927 *F. moniliforme*	*O. sativa*	2927
F.M-2708 *F. moniliforme*	*O. sativa*	2708
F.M-2193 *F. moniliforme*	*O. sativa*	2193
FMS-1857 *F. moniliforme* var. *subglutinans*	*Zea mays*	1857
FMS-2451 *F. moniliforme* var. *subglutinans*	*Zea mays*	2451
FMS-2926 *F. moniliforme* var. *subglutinans*	*Capsicum annuum*	2926
FMS-2748 *F. moniliforme* var. *subglutinans*	*C. annuum*	2748
GF-52 *Gibberella fujikuroi*	*C. annuum*	52
GF-57 *G. fujikuroi*	*C. annuum*	57
GF-1068 *G. fujikuroi*	*C. annuum*	1068

Liquid surface fermentation

In LSF technique, incubated flasks were kept in

BOD incubator at 26°C ± 1°C for 10 days. Three replications were maintained. At the end of the incubation period, mycelial mat formed over surface of Richard's broth medium was removed through filtration by using dried, pre weighed filter paper.

Submerged fermentation

In submerged fermentation (SmF) technique, flasks after inoculation of fungal discs were kept in incubator shaker (Kuhner) at 120 rpm for 10 days. The other steps were same as that of liquid surface fermentation.

Fungal biomass estimation

At the end of liquid surface or submerged fermentation process, the fungal biomass was separated by filtration using pre weighed Whatman No. 42 filter paper. The filter paper containing fungal biomass was dried in oven at 121°C for 10 minutes and weight of fungal biomass was calculated. The pH of the culture filtrate was also determined.

RESULTS AND DISCUSSION

Preparation of GA_3 extract from culture filtrate

Richard's broth medium (100 ml) was taken in 250 ml flasks and each of the isolates was grown on Richards medium for GA_3 production under LSF/ SmF condition in the shaker (Kuhner) with a speed of 120 rpm and temperature at 28°C for 10 days in presence of light, as light is a well known stimulator of GA_3 production (Ahamad *et al.*, 2003). The cultures filtrate was separated after 10 days through Whatman filter paper No. 42. *G. fujikuroi-52* recorded the highest yield of GA_3 and and *F. moniliforme*-2451 was the next highest. Rademaker (1984) observed that capability of GA formation was variable among different microorganisms. Variations recorded in quantity of GA_3 produced by different fungi might be due to genetic make-up of the fungi. Tomasini (1997) produced 28 mg ml^{-1} of GA_3 in 5 days in production medium on NRRL-2228.

Production of GA_3 in SmF technique

Results showed that it was comparatively higher under SmF process as compared to LSF. Again *G. fujikuroi*-52 recorded the highest quantity of GA_3 production (0.77 gl^{-1}) followed by *F. moniliforme*-2451 (0.61 g l^{-1}). However, significant variations were observed in total quantity of GA_3 produced by different cultures (Table 4).

Table 3 : Production of GA_3/fungal biomass under liquid surface fermentation (LSF) technique

Cultures/ Strains No.	GA_3 Yield (gl^{-1})	Fungal biomass yield* g100ml^{-1}	Final pH of the medium
Fusarium moniliforme-1	0.23	0.22	6.39
Fusarium moniliforme-1191	0.07	0.16	6.67
Fusarium moniliforme-2451	0.26	0.20	6.22
Fusarium moniliforme-2708	0.13	0.20	6.43
Fusarium moniliforme-2926	0.11	0.16	6.12
Fusarium moniliforme-2927	0.10	0.15	6.11
Fusarium moniliforme-VII	0.11	0.16	6.12
Fusarium moniliforme-VIII	0.15	0.13	6.02
Fusarium moniliforme-X	0.16	0.16	6.36
Gibberella fujikuroi-52	0.28	0.23	6.01
Gibberella fujikuroi-57	0.20	0.16	6.68
Gibberella fujikuroi-1068	0.26	0.20	6.42
Fusarium moniliforme var. *subglutinans*-2193	0.24	0.24	6.43
Fusarium solani	0.10	0.15	6.00
Fusarium pallidoroseum	0.06	0.17	6.38
S. Ed.	0.02	0.02	0.04
C.D.(P=0.05)	0.04	0.04	0.09

*Expressed on dry weight basis.

Table 4 : Production of GA_3/fungal biomass under submerged fermentation (SmF) technique

Cultures/ Strains No.	GA_3 Yield (gl^{-1})	Fungal biomass yield* g100ml^{-1}	Final pH of the medium
Fusarium moniliforme-1	0.48	0.22	6.56
Fusarium moniliforme-1191	0.22	0.16	6.49
Fusarium moniliforme-2451	0.61	0.18	6.78
Fusarium moniliforme-2708	0.23	0.13	6.19
Fusarium moniliforme-2926	0.34	0.19	6.38
Fusarium moniliforme-2927	0.32	0.13	6.26
Fusarium moniliforme-VII	0.20	0.12	6.36
Fusarium moniliforme-VIII	0.33	0.12	6.23
Fusarium moniliforme-X	0.28	0.13	6.62
Gibberella fujikuroi-52	0.77	0.20	6.75
Gibberella fujikuroi-57	0.35	0.13	6.90
Gibberella fujikuroi-1068	0.46	0.13	6.13
Fusarium moniliforme var. *subglutinans*-2193	0.54	0.20	6.79
Fusarium solani	0.34	0.14	5.62
Fusarium pallidoroseum III	0.34	0.19	6.33
S. Ed.	0.03	0.02	0.05
C.D.	0.06	0.04	0.10

*Expressed on dry weight basis.

In the past microbial synthesis of gibberellin by using various strains of the fungus *F. moniliforme*

(Ahamad and Agarwal, 2002), *G. fujikuroi, Sphaceloma manihoticola, Phaeosphaeria* sp., *Neurospra crassa* (Ahamad 2003) and *Aspergillus niger* (Cihangir and Aksoz, 1993) were studied. Among these, *F.moniliforme* was found to be the most effective fungus for the production of gibberellin.

Kumar and Lonsane (1987) recorded GA_3 yield of 0.218 g l^{-1} in wheat bran (WB) medium under SmF condition. In the present study the GA_3 yield 0.77 g l^{-1} was recorded higher. This might be due to the chemical nature of the medium used as well as better strains. Eventhough the highest GA_3 yield i.e. 1 g l^{-1} was obtained in SmF by using *G.fujikuroi* ACC-917 (Bruckner *et al.,* 1989) and more recently, a Russian patent claimed an increased GA_3 yield of approximately 2 g l^{-1} by using improved strain and media under SmF condition (Qian *et al.*, 1994). The present findings are in accordance with the earlier findings (Mitter *et al.,* 2002).

Spectrophotometric analysis showed presence of gibberellin in the extract of different strains in variable amounts under LSF and SmF conditions. Range of gibberellin production varied from 0.06 to 0.77 gl^{-1} dry weight of the mycelium.

Microbial synthesis of gibberellin by using various strains of *F.moniliforme* was studied in the past and correlations were found between efficiency of gibberellin production and different fungal strains (Ahamad and Agarwal, 2002).

In previous investigations also, the isolates of *G.fujikuroi* were analyzed for their ability to produce gibberellin and two groups were identified on the basis of their gibberellin production. One group had the potential to produce high level of GA_3 and the amount showed low amount of gibberellin (Rademacher, 1994). The present data are consistent with the previous findings.

Variability was observed for gibberellin production by the different strains of *F.moniliforme.* In this study it was possible because metabolic pathway of gibberellin production established by the enzyme preparations from the fungus *G.fujikuroi* (West, 1973) emphasized that GA_{12}7-aldehyde is a branch point to the various GAs and several pathways have been established from this branch point. These pathways differ mainly in the position and sequence of hydroxylation and more than one pathways from GA_{12}-7-aldehyde can occur in the same plant that has been observed in *G.fujikuroi* (Bearder *et al.,* 1975 ; Tudzynski, 1999; Rajas *et al.,* 2001) and this could be one of the main reasons for variability in gibberellin production and which was also related to the geographic source of the isolates (Carter *et al.* 2000).

Chemical detection of GAs by T.L.C. and H.P.L.C.

Thin layer chromatographic separation of GA like substance confirmed the presence of GA_3 and GA_7 in the sample. It was observed that R_f values of GA_3 and GA_7 (Sigma make) were 0.79 and 0.85, respectively close to R_f values obtained in case of all isolates. Whereas GF-1068 (R_f value 0.90) also confirmed presence of GA_5 with Sigma make GA_5 (R_f value 0.90) (Table-5). The present study is in accordance with Cavell (Cavell *et al.,* 1967). TLC

Table 5 : Separation of GA like substances from five isolates by TLC.

Standard/Sample	R_f values
GA_3(Sigma)	0.79
GA(China)	0.73
GA_5(Sigma)	0.903
GA_7(Sigma)	0.85
GF-52	0.85
	0.73
	0.67
	0.11
FM-2451	0.85
	0.73
	0.62
	0.56
	0.11
FMS-2193	0.85
	0.73
	0.63
	0.11
GF-108	0.85
	0.73
	0.90
	0.65
FM-1	0.85
	0.73
	0.55
	0.11

separation of gibberellin confirmed the presence of GA_3, GA_4 and GA_7 in the sample. To confirmed the results obtained from spectrophotometric and TLC analysis, the gibberellin extracted from various strains of *Fusarium* were subjected to HPLC. The analysis of the gibberellin contained in extracts showed one major peak (Retention time 2.4 min) of GA_3 in all the strains. The similar trend was observed by Berendse *et al.* (1980).

Table 6 : Time course for gibberellin production in Biofermenter

Date	Time	Temp. (°C)	pH	Do	RPM	GA(mg g^{-1}d.wt)
6. 1. 2000	3 pm	26	6.26	5.8	190	0.00
7. 1. 2000	9 am	26	5.10	1.4	176	0.00
7. 1. 2000	3 pm	26	2.93	0.8	177	6.00
8. 1. 2000	9 am	26	2.46	0.7	173	9.50
9. 1. 2000	11 am	26	2.60	0.5	173	14.00
10. 1. 2000	11 am	26	5.21	0.6	177	24.00
10. 1. 2000	3 am	26	5.12	0.6	180	38.32
10. 1. 2000	11 pm	26	5.43	0.6	179	40.89
11. 1. 2000	9 am	26	5.96	0.6	178	46.20
11. 1. 2000	3 pm	26	6.10	0.6	187	46.20
12. 1. 2000	9 am	26	6.32	0.6	183	58.30
12. 1. 2000	3 pm	26	6.35	0.6	184	61.00
13. 1. 2000	9 am	26	6.22	0.6	183	61.00
13. 1. 2000	4.20 pm	26	6.36	0.6	183	61.00

Time course for gibberellin production in biofermentor

In the present case, the highest quantity of GA_3 i.e. 61.0 mg g^{-1} dry weight of mycelium was recorded at the end of 6 days of incubation period and thereafter the level remained unchanged for the next 72 h (Table 6).

This character is of considerable importance, as it takes considerable time to extract GA_3 from DMB and to estimate its concentration. The production of GA_3 started from 3rd day and continued to rise up to 7th day of incubation in fermentation medium. Increase in GA_3 level from 4th to 7th day of incubation under fermentation was more or less in linear fashion. Kumar and Lonsane (1987) recorded a linear increase in GA_3 production upto 7 days.

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