Optimization of culture conditions for gibberellic acid production by *Fusarium solani* KUSF0301

ABSTRACT

The current study reports the effect of various cultural parameters on the gibberellic acid (GA) by *Fusarium* sp. isolated from an agricultural field of Murshidabad district in West Bengal, India. Gibberellin is one of the ~~principle~~principal plant growth regulators that influence the overall developmental processes in angiosperms. The *Fusarium* sp. was isolated by soil dilution plate technique on selective pentachloronitrobenzene (PCNB) medium. The isolate was identified and subsequently characterized by the routine morphological studies and molecular analysis. Various physiological parameters viz., growth media, carbon and nitrogen sources, incubation temperature and pH of the culture medium were taken into consideration for determining their effect on the phytohormone production and subsequent optimization of the culture conditions. GA production was found to be highest in CD broth. Among the carbohydrate sources used, sucrose (7300 µg/ml), dextrose (7150 µg/ml) and maltose (6650 µg/ml) were found to be most conducive for GA production (Table 4.20). Among the nitrogen sources, glycine (7360 µg/ml) and sodium nitrate (7250 µg/ml) were found to be most stimulatory in GA production. Interestingly, with the increase in pH, GA production was also found to be increased. Maximum GA production was recorded at pH 10 (7800 µg/ml). GA production was found equally well in temperature range 14-32°C (7000-7160 µg/ml).

Key Words: Gibberellin, culture medium, *Fusarium*, carbon, nitrogen, pH, temperature etc.

INTRODUCTION

A phytohormone is an organic substance synthesized in defined organs of the plant that can be translocated to other sites, where it triggers specific biochemical, physiological, and morphological responses. However, phytohormones are also active in the tissues where they are produced. The commonly recognized classes of phytohormones, regarded as the “classical five”, are: the auxins; gibberellins; cytokinins; abscisic acid; and ethylene [1]. Plant hormones are

signal molecules that act as chemical messengers to control plant growth and development. Some microorganisms also produce phytohormones. Microorganisms inhabiting rhizosphere of various plants are likely to synthesize and release GA as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non rhizospheric soils [2]. Gibberellic acid is a plant growth regulator of economic and industrial importance. Various gibberellins are available and are associated with several plant growth and development processes, such as seed germination, stem elongation, flowering, and fruit development [3].

Fungal synthesis of GA is an important aspect to ensure fertility in soil. The fungus *Fusarium* produces many types of GAs in culture media as well as in the inoculated plants [4]. Especially in developing countries like India where agricultural activities play an important role, plant growth regulators as well as fertilizers should be used to maximize production efficiency. Indian subcontinent provides best conditions for growth of various *Fusarium* species and these are distributed widely in the agricultural soil [5]. The members are abundance in soils as free-living saprophytes, pathogens or endophytes. The non-pathogenic isolates with potential plant growth promoting attributes can be explored to increase crop productivity. Furthermore, the ability of a variety of species of *Fusarium* to produce GAs varied widely [6-7] and the production of such bioactive metabolites is dependent both on culture conditions and strain specificity [8].

Especially in developing countries where agricultural activities play an important role, plant growth regulators as well as fertilizers should be used to maximize production efficiency. Therefore, synthetic plant growth regulators should be produced economically on an industrial scale. In this respect, the effects of some physiological conditions of gibberellic acid production in *F. solani* were investigated in this study.

MATERIALS AND METHODS

2.1. Collection of soil sample: A soil sample was collected from the rhizospheric region of the rice plant located in Rakhaldaspur village of Raninagar block I, Murshidabad district, West Bengal, India. The agricultural field was placed in close vicinity of Padma river near Indo Bangladesh boarder region and cultivated for several crops throughout the year where no *Fusarium* diseases were reported previously.

2.2 Isolation and identification of fungus: The soil was screened for isolation of the fungi by dilution plate technique on selective peptone PCNB agar medium [composition (g/l): peptone 15, KH_2PO_4 1.0, $MgSO_4$, $7H_2O$ 0.5, PCNB 1.0, agar 20, pH 6] supplemented with streptomycin sulphate 1.0 g/l and neomycin sulphate 0.12 g/l. The plates were incubated at 28°C for 5-7 days until visible sign of colony growth occurred. Fungal isolates were identified by observing their colony morphology, sporulation and pigmentation on Czapek's Dox agar (CDA) medium. One promising isolate was further identified based on rDNA gene analysis. For this, genomic DNA was extracted and used as template for amplification of the rDNA region using the primer pair LROR (TCCGTAGGTGAACCTGCGG) and LR5 (GCTGCGTTCTTCATCGATGC). The amplified product was sequenced and the sequence was analysed using Nucleotide BLAST function at NCBI to find similarity of the sequence with nucleotide database. Phylogenetic tree was constructed using the software MEGA6. Multiple sequences alignment was carried using CLUSTALW and the evolutionary history was inferred using the Neighbour-joining method.

2.3. Assay of Gibberellin (GA) production: Efficacy of gibberellin production by the *Fusarium* soil isolates was assayed by growing the isolates in Czapek's Dox broth at 28ºC for 14 days and the amount of gibberellin in the culture supernatant was determined by spectrophotometric method using phosphomolybdic acid reagent [9]. One ml culture filtrate of each *Fusarium* was taken out into 25 ml of volumetric flask, mixed with 15 ml of phosphomolybdic acid reagent and placed in a boiling water bath for 1 hour. After that, the temperature of the flask was reduced to room temperature and then final volume was made to 25 ml with distilled water. The absorbance of blue color developed was measured at 780 nm using distilled water as blank and the concentration was determined using a standard curve prepared from the standard solutions of gibberellins. Mycelial dry weights of the fungal isolates were also determined to make a correlation with the GA production.

2.4. Study of effect of different culture media on GA production: The most promising GA producer among the *Fusarium* isolates was chosen to study the effect of various culture media on its GA production. The *Fusarium* isolate was cultivated in eight different broth media, viz., Potato Dextrose broth, Potato Carrot broth, Czapek's Dox broth, Yeast Extract Mannitol broth, Sabouraud's broth, Nutrient broth, Richard's broth, and Asthana & Hawker's broth for 14 days at 28ºC. GA production was estimated using phosphomolybdic acid reagent and mycelial dry weights were also measured.

2.5. Study of effect of various carbohydrate and nitrogen sources on GA production: Different sets of modified (without carbohydrate source) CD broth were prepared supplemented

with 3% of the respective carbohydrate sources viz., dextrose, maltose, sucrose, lactose, mannitol, sorbitol and was inoculated with the selected fungal isolate. Likewise, different sets of modified (without nitrogen source) CD broths were also prepared supplemented with 0.2% of the respective nitrogen sources viz., sodium nitrate, sodium nitrite, peptone, ammonium sulphate, glycine and asparagines. Control set (without carbohydrate or nitrogen) was also prepared. Each of the media was inoculated with 5 mm mycelia disc of selected the fungal isolate and incubated for 14 days at 28ºC. GA production was estimated by phosphomolybdic acid reagent and mycelial dry weights were also recorded.

2.6. Study of effect of different pH and temperature on GA production: Seven sets of CD broths having pH range from 4-10 were prepared. The *Fusarium* isolate was inoculated in the broth medium and incubated for 14 days at 28ºC. The fungal isolate was also inoculated in four sets of CD broth and each was incubated at four different temperatures viz., 14ºC, 27ºC, 32ºC and 37ºC for two weeks. Extracellular GA production was estimated spectrophotometrically by using phosphomolybdic acid reagent and mycelial dry weights were also measured.

Results and Discussion

3.1. Isolation and identification of the fungus: On selective peptone PCNB agar medium the several fungal colonies with similar morphology had appeared. One fungal isolate, designated as KUSF0301 was selected for further study. On CDA medium the fungal isolate showed white, circular, compact, smooth, fast growing, light green pigmentation (Fig. 1). It abundantly produced straight to falcate, dorsal side more curved than ventral, medium sized (16.25-26.25 µm X 3.12-3.75 µm) macroconidia having 3-4 septa with slightly curved apical cell and foot shaped basal cell (Fig. 1). Microconidia oval, elliptical; short sized (5.25-10.50 µm X 1.75-2.25 µm) having 0-1 septa. Chlamydospores were lacking. Molecular identification of the fungal isolate KUSF0301 was performed based on rDNA sequence analysis. 518 bp amplicon of rDNA region of the isolate was observed on agarose gel and a stretch of 315 bp had been sequenced. Search for sequence homology through nucleotide BLAST function in NCBI database was performed and maximum identity (100%) was found with the rDNA sequence of *F. solani*. When a phylogenetic tree was constructed by Neighbour-joining method based on rDNA sequence of KUSF0301 and other similar sequences obtained from BLAST search and one outgroup as *Alternaria* sp., relatedness of KUSF0301 with related fungal species was observed and it belonged to the same evolutionary branch with *Fusarium solani*.

Comment [r1]: Selection criteria - What was the criteria used in selecting one (KUSF0301) of the "several fungal colonies with similar morphology"? Or in other words, why the other isolates were reject?

Based on all these key specifics, the fungal isolate was identified as *Fusarium solani.* The partial rDNA region of the fungal isolate KUSF0301 had been submitted to the genebank under the accession no. MF136401.

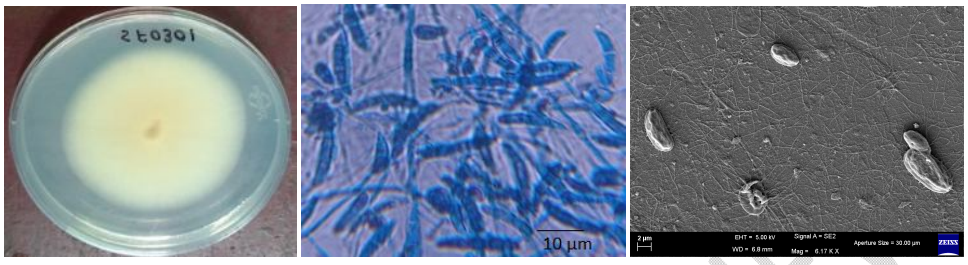


Fig. 1: Reverse side of growth of *Fusarium sonali* MF136401 on CDA medium after 7 days (left); Microscopic field of *Fusarium solani* MF136401 showing macroconidia stained with cotton blue (middle); SEM photograph of a single macroconidium of *Fusarium solni* MF136401 (right)

3.2. Effect of different culture media on GA production: Isolate SF0301 was tested for its GA production in eight different culture media. Maximum GA production (7200 µg/ml) was observed in Czapek's Dox broth and minimum GA production (1140 µg/ml) was observed in Potato Carrot broth (Table 1; Fig. 2). In all cases, GA production was directly related to mycelial growth.

3.3. Effect of various carbohydrate and nitrogen sources on GA production: Isolate SF0301 was tested for its GA production in modified CD broths containing any of the seven carbohydrates and six nitrogen sources. Maximum GA production was observed in broth containing sucrose as carbohydrate and glycine as nitrogen source (Table 2; Fig. 3). Minimum GA production was found in broth containing starch or peptone. In these cases, growth of the fungal biomass may influence the GA production of the isolate.

Table 1. GA production of SF0301 in different culture media*

Sl no	Culture media	Mycelial dry wt. (g)	GA production (µg/ml)
1.	**Potato Dextrose Broth**	0.176	1260
2.	**Potato Carrot Broth**	0.067	1140

Fig. 2. Effect of different culture media on GA production of SF0301

3.	**Czapek's Dox Broth**	0.188	**7200**
4.	**Yeast Mannitol Broth**	0.087	4100
5.	**Sabouraud's Broth**	0.199	4300
6.	**Nutrient Broth**	0.089	2220
7.	**Richard's Broth**	0.177	2560
8.	**Asthana & Hawker's Broth**	0.192	3780

**data taken after 14 days of incubation*

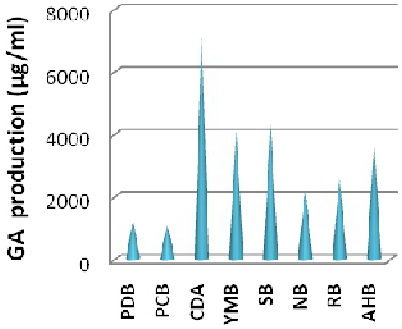
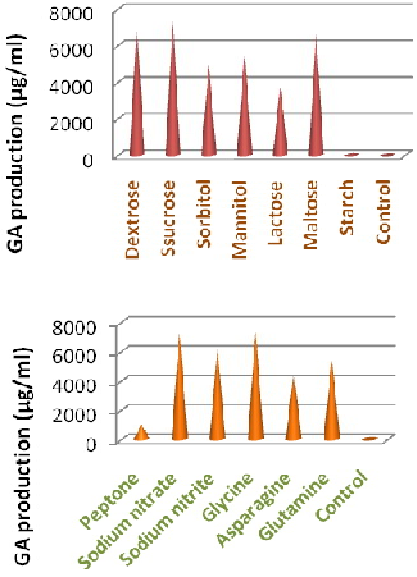


Fig. 3. Effect of carbohydrate (upper) and nitrogen (lower) sources on GA production of SF301

Table 2. GA production of SF0301 in presence of various carbohydrate and nitrogen sources

Sl no	**Carbohydrate source**	Mycelial dry wt. (g)	GA production (µg/ml)
1.	Dextrose	0.175	7150
2.	Sucrose	0.185	**7300**
3.	Sorbitol	0.133	4960
4.	Mannitol	0.154	5450
5.	Lactose	0.176	3740
6.	Maltose	0.188	6650
7.	Starch	0.033	110
8.	Control	0.011	-
	Nitrogen source	Mycelial dry wt. (g)	GA production (µg/ml)
1.	Peptone	0.139	1000
2.	Sodium nitrate	0.180	7250
3.	Sodium nitrite	0.184	6200
4.	Glycine	0.160	**7360**
5.	Asparagine	0.137	4600
6.	Glutamine	0.125	5380
7.	Control	0.021	-



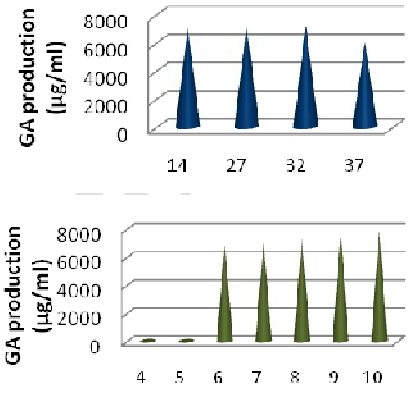
3.4. Effect of different temperature and pH on GA production: Isolate SF0301 was tested for its GA production in CD broths incubated at different temperatures and in varying pH range.

Maximum GA production was observed in cultures incubated at 32°C and 27°C (Table 3; Fig. 4). GA production by SF0301 was found to be highest in broth having alkaline pH (pH 10) although growth was comparatively lesser at the condition. At pH 8 maximum growth was observed and GA production was comparatively better than that at pH 7. Hence, the growth of the fungal biomass may affect GA production by the isolate.

Table 3. GA production of SF0301 at varying temperature and pH

Growth conditions		Mycelial dry wt. (g)	GA production (µg/ml)
Temperature (°C)	14	0.128	7000
	27	0.179	7100
	32	0.181	**7160**
	37	0.168	5980
pH	4	-	-
	5	-	-
	6	0.189	6800
	7	0.184	7000
	8	0.191	7300
	9	0.167	7450
	10	0.154	**7800**

Fig. 4. Effect of temperature (upper) and pH (lower) on GA production of SF0301



Determination of carbon-nitrogen ratio is very important for the production of secondary metabolites by microbes [10]. Among the carbohydrate sources used, sucrose (7300 µg/ml), dextrose (7150 µg/ml) and maltose (6650 µg/ml) were found to be most conducive for GA production. Among the nitrogen sources, glycine (7360 µg/ml) and sodium nitrate (7250 µg/ml) were found to be most stimulatory in GA production. Our results did conform to the findings where *F. fujikuroi* isolate SG2 produced gibberellins (1175 µg/ml) in modified medium having low concentration of nitrogen and carbon [11]. Interestingly, with the increase in pH, GA production was also found to be increased. Maximum GA production was recorded at pH 10 (7800 µg/ml). GA production was found equally well in temperature range 14-32°C (7000-7160 µg/ml). With further increase in temperature the GA production declined. *F. moniliforme* NCIM 1100 produced highest GA_3 at 30°C and pH 7 [12]. However, decreased GA_3 production was recorded in *F. fujikuroi* when the pH of the media was

beyond the range of 3.0-5.5 in a stirred culture [13]. As with other bioactive metabolites microbial phytohormone production is dependent both on climatic condition and strain specificity [14].

Conclusion

Commercial production of gibberellins could help in boosting agriculture. The present study revealed higher GA ~~productonproduction~~ efficiency of *Fusarium solani* MF136401 which could be used as potential biofertilizer candidate for promoting growth of different agricultural crops. This work proved that by adjusting culture conditions, the yield of GA can be augmented.

Comment [r2]: The authors are proposing the release/use in the field of *Fusarium solani* MF136401 as a biofertilizer; or are they proposing the use of GA produced by *F. solani* as fertilizer? Please clarify!

I presume it's the second option, as the first would likely introduce potential pathogenicity or biodiversity imbalance in the local microbial communities.

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