

Isolation and Characterization of Gibberellic Acid 3 Producing Fusarium Sp. from Belgaum Agriculture Land And its Impact on Green Pea and Rice Growth Promotion

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Abstract

Worldwide ultimate aim of any agriculture sector or farmer is to take maximum yield. Sufficient supply of nutrients and fertilizer are not able to give maximum yield. There are numerous factors which are responsible for low yield, among that one is the environment stress or the unstable climate conditions. To increase the yield there are numerous approaches like use of genetically modified crops, but in India it is controversial approach and another approach is the use of multifunctional plant hormone like Gibberellic Acid₃ (GA₃). This research mainly involves the production of GA₃ from fungal species and to apply it on crop plants. *Fusarium* species were isolated from Belgaum agriculture soil and screened for GA₃ production under submerged fermentation. Strain showing maximum GA₃ yield (strain M104) was chosen to study the effect of various parameters on GA₃ production, like incubation time (1-12 days), initial pH (5.0-8.0), incubation temperature (20-50°C), pH (5.0 - 8.0), and carbon and nitrogen sources. The maximum production of GA₃ was observed on day 8 at 30°C, and pH 5.5 with glucose and ammonium chloride as good carbon and nitrogen sources, respectively. After optimization, a

5.56-increase in GA₃ production was observed. The GA₃ production was confirmed by thin layer chromatography. The GA₃ crude extract obtained using submerged fermentation was then used to study its effect on germination and growth of green pea plant and paddy crops. It was observed that GA₃ treated crops showed uniform growth and they were taller than non-treated plants, suggesting its application in increasing the crop plant harvests.

Keywords: *Fusarium* sp; Isolation; Gibberellic Acid; Optimization; Submerged Fermentation; Crop Plants

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Introduction

Gibberellic acids, also known as gibberellins, are the complex organic molecules acting as plant growth hormones. They are chemically known as diterpenoid acids having molecular formula C₁₉H₂₂O₆. They regulate the functions like cell division, cell elongation, sex expression, seed germination,

breakdown of seed dormancy and flowering etc. In microorganisms such as bacteria and fungi, gibberellic acid 3 is the principal product of gibberellins, and acts as secondary metabolite [5, 11]. Till now, 136 gibberellins were isolated from various plants, and among that gibberellic acid 3 shows maximum biological activity [19]. The use of GA₃ has been approved by Food and Drug Administration (FDA) because of its tremendous application and nontoxic properties, and its safety for environment and human was confirmed by Material Safety Data Sheet (MSDS) [19].

In counties under development where mainly the economy relies on agriculture, the farmers have to use fertilizers and plant hormones to increase production. As most of fertilizers are associated with environmental pollution, plant growth hormones like gibberellic acid 3 have to be produced cost-effectively in huge amounts in order to enhance the quantity of agricultural products [4]. Three routes to obtain GA₃ have been reported, viz. extraction from plants, chemical synthesis and microbial fermentation. Among this the third method is the most common method to produce GA₃ [18]. GA₃ is industrially produced by *Gibberella fujikuroi*/*Fusarium moniliforme* under submerged [20, 11]. It is also produced by several other fungal species such as *Aspergillus niger* and *Fusarium* species and some bacteria such as *Pseudomonas*, *Rhizobium*, *Azobactor*, and *Azospirillum* species [16]. All above species produced very low yield of GA₃ except *Fusarium* species in which most of the strains show the highest yield of GA₃ than any other microbes [17]. The search for new fungal species like *Fusarium* species capable of producing an important amount of GA₃ is a continuous exercise. The aim of the present study was therefore to isolate and characterize a GA₃ producing *Fusarium* sp. from soil, optimize the culture conditions for maximum GA₃ production, and to evaluate its effect on green pea and rice growth promotion.

Materials and Methods

Soil sample selection

To isolate strains of *Fusarium*, the soil sample was collected from Belgaum agriculture area (Karnataka state,

India). The soil was black coloured having high water holding capacity, good fertility and also best soil for crops like paddy, all types of beans, sugarcane and all types of vegetables.

Isolation of *Fusarium* species

The soil sample collected from Belgaum agriculture land was taken, serially diluted in distilled water and inoculated in a Malachite Green Agar (MGA). Petri plates containing 15 g of peptone, 0.01 g of Malachite Green (triaryl methane dye), 1 g of potassium dihydrogen phosphate, 0.5 g of magnesium sulphate, and 20 g of agar per 1000 ml of distilled water were prepared. The incubation was carried out at 30°C for 5 days [6]. After incubation period, the different colonies obtained were picked up, streaked on Potato Dextrose Agar (PDA) plate, and incubated for a week for secondary pigmentation. The colony with different morphology and colour pigmentation were subcultured on PDA slants and labelled [21]. The lactophenol cotton blue technique was used to study the characteristics of the fungal isolate [26].

Screening of the isolates for GA₃ production under submerged fermentation

The Czapek Dox media (CD broth) containing sucrose (30 g), sodium nitrate (3 g), dipotassium hydrogen phosphate (1 g), potassium dihydrogen phosphate (0.5 g), magnesium sulphate (0.5 g), potassium chloride (0.5 g) and ferrous sulphate (0.1 g) per 1000 ml of distilled water was used. The CD broth was prepared in conical flask and adjusted the pH to 7.0, and sterilised in an autoclave for 20 min at 15 psi. After cooling the medium, it was aseptically inoculated (1×10^8 spores/ml) with individual isolated strains. The fermentation flasks were kept on a rotary shaker (100 rpm) at 30°C for 12 days [10, 17].

GA₃ pre-treatment, extraction and Stimulation

The fermented broth was taken and centrifuged at 13200 rpm for 10 min and the supernatant was taken and acidified to pH 2-2.5 using 1N HCl. GA₃ was extracted thrice using equal amount of ethyl acetate/NaHCO₃ [27]. The ethyl

extract was kept on hot air oven at 50°C overnight to remove ethyl acetate and obtain crystals of GA₃ [10, 11, 4, 17]. It was estimated by Berrios et al. (2004) spectrophotometric method and absorption was read at 254nm in UV-VIS spectrophotometer (Elico, SL-159 model, India).

Confirmation of GA₃ by Thin Layer Chromatography (TLC)

The slurry of silica gel was poured on a TLC plates, air dried, and the matrix was activated by keeping the plates on hot air oven at 80°C for 1 h. GA₃ dissolved in ethanol was added as a spot and plates were run using mobile phase containing isopropanol: ammonia: water (10:1:1) for 2 h. The plates were removed, sprayed with 3% sulphuric acid containing 50 mg FeCl₃ and heated in oven at 80°C for 10 min. The GA₃ appeared as greenish black/spot fluorescence under UV light [7, 21].

Optimization of culture conditions for maximum GA₃ production by *Fusarium* sp. (Isolate M104)

The incubation time for GA₃ production by the fungal isolate under submerged fermentation at 30°C and at initial pH 7.0 was analysed by inoculating CD broth with 1 ml of fungal spores and incubating on a rotary shaker (100 rpm) for 12 days. The sample was taken every day as the fermentation proceeds in order to find the most suitable incubation time for GA₃ production. The effect of pH on GA₃ production was studied by adjusting CD broth at different pH, viz. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The cultivation flasks were inoculated with 1.5% (v/v) inoculum, and incubated for 8 days on rotary shaker (100 rpm) at 30°C. The effect of temperature on GA₃ production was investigated by inoculating 1 ml of the fungal spores in CD broth of pH 7.0 and by incubating at four different temperatures (20, 30, 40, and 50°C). The effect of carbon sources on GA₃ production was analysed by replacing the sucrose in the CD broth of pH 5.5 by dextrose, glucose, mannitol, and starch, and by incubating at 30°C for 8 days. The effect of nitrogen sources on GA₃ production was studied by replacing the sodium nitrate

in the CD broth of pH 5.5 by glycine, ammonium chloride and ammonium sulfate, and incubating at 30°C for 8 days.

Effect of GA₃ on pea plant and Paddy crops

Seeds of pea plants were soaked in 200 ppm of GA₃ fermented filtrate for 12 h and then sown in autoclaved soil. After an incubation period of 8 days, 100 ppm of GA₃ was sprayed on the plant for each alternative day for another 8 days. The control was soaked in water and sown in autoclaved soil and sprayed with distilled water only. The growth of both the control and test pea plants was monitored over a period of 15 days. 10 paddy seeds were soaked in 300 ppm of GA₃ solution for 2 days and sown in soil. They were sprayed with 200 ppm of GA₃ after growth. The control seeds were soaked in water for the same period and sprayed with only water. The observation was carried out for 25 days [23, 22].

Statistical Analysis

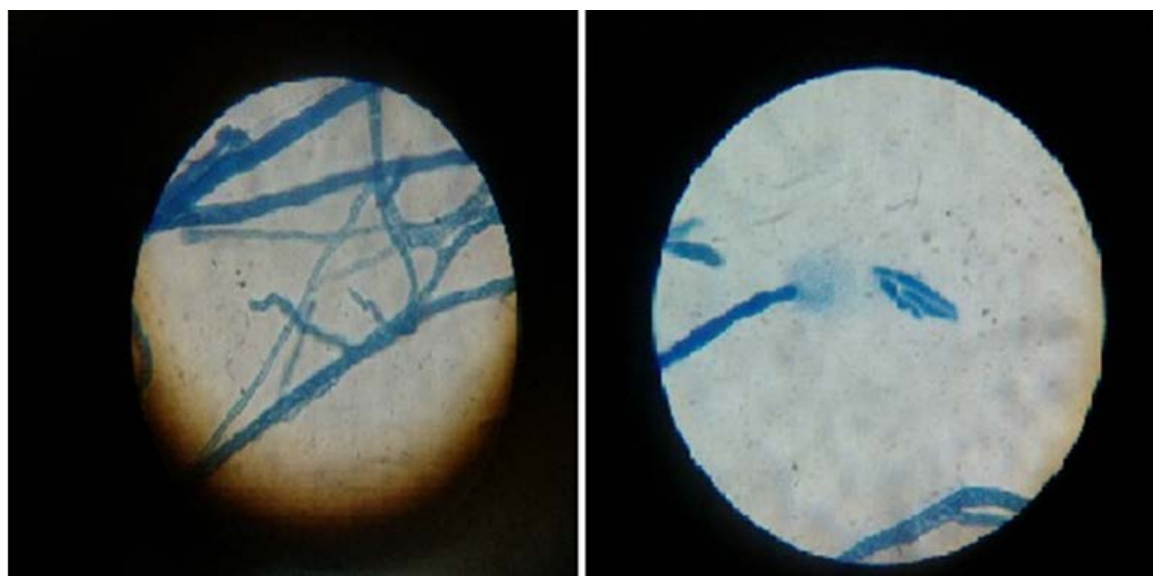
The experiments were carried out in triplicate. ANOVA and DMRT at 5% significance level were used to give the differences between mean values, using SPSS statistical software (PASW Statistics 18).

Results and Discussion

Isolation of *Fusarium* species

Four strains of *Fusarium* species were isolated from agriculture soil sample and labelled as M101, M102, M104 and M110. The present labelling was based on following pigmentation black, grey, blue and red, respectively. All strains had cottony growth appearance which is one of the important morphological characteristic of the *Fusarium* species. By staining the fungi with lactophenol cotton blue dye, it was observed that they had non septate hyaline mycelium/ hyphae as shown in Figure 1a. The macrospores of banana shape were re-septated which is a unique microscopic feature of *Fusarium* species as shown in the Figure 1b. The isolation medium containing malachite green was chosen since malachite green inhibits the radial colony growth of the saprophytes and allows only the growth of *Fusarium* species [6].

Figure 1: Lactophenol cotton blue staining of the fungal isolate M104: non septate hyphae (1a) and septated macrospores (1b).



Screening for isolates for GA₃ production

GA₃ can be commercially produced under submerged fermentation using different media but the most common synthetic medium is the Czapack Dox medium (CD broth) [17]. The isolated strains M101, M102, M104 and M110 were subjected to submerged fermentation to check their ability for GA₃ production. The different amounts of GA₃ produced are given in the Table 1 and Figure 2. The strain M104 was the highest producer of GA₃ among the four isolates. Similarly, *Aspergillus niger* strains produced different amounts of GA₃ with the highest of 150.35 mg/l for *A. niger* Fursan [8]. Likewise, various amounts of GA₃ were produced by *Lentinus tigrinus* and *Laetiporus* [14].

Table 1: Amount of GA₃ produced by the isolates

Isolated Fusarium strains	Secondary pigmentation	GA ₃ produced (mg/L)
M101	Black	223 ± 1.53
M102	Grey	216 ± 5.25
M104	Blue	680 ± 22.00
M110	Red	160 ± 11.23



Figure 2: GA₃ production by the isolates.

Optimization of culture conditions for maximum GA₃ production by *Fusarium* isolate M104

The optimization of cultural parameters such as incubation time, temperature, and pH, and nutritional conditions like nitrogen and carbon sources, is necessary to produce GA₃ in a significant amount. Time course for GA₃ production by the isolate M104 was studied. GA₃ production started on day 3 and maximum production was observed at day 8, although statistically at par with day 9 and 10 (Table 2). Similar incubation period was observed for GA₃ production by *Fusarium moniliforme* [17]. 9 days was optimal time for GA₃ secretion by *Fusarium fujikuroi* SG2 [25]. and *Fusarium moniliforme* [12]. In contrast, a higher

incubation time of 12 days was observed by for *Fusarium moniliforme* [10] and *Aspergillus niger* [4]. Therefore, the optimum incubation time for GA₃ production by various fungal

species depend on the strain used. The short incubation period observed for GA₃ production by fungal isolate M104 makes the fermentation cost-effective.

Table 2: Effect of incubation time, initial pH, incubation time, and carbon source on GA₃ production. In a raw, the values with different letters differ significantly at P = 0.05.

Incubation time (days)							
3	4	5	6	7	8	9	10
17.7±2.5 ^f	69.7±5.7 ^e	137.0±15.5 ^d	398.3±52.5 ^c	637.3±37.1 ^b	728.0±24.8 ^a	721.3±36.6 ^a	691.3±20.5 ^a
Initial pH of the medium							
5.0	5.5	6.0	6.5	7.0	7.5	8.0	
1190.6±20.2 ^c	1478.2±30.1 ^a	1299.0±11.6 ^b	915.4±19.1 ^d	740.2±15.8 ^e	704.9±11.1 ^e	618.0±4.8 ^f	
Incubation temperature (°C)							
20	30	40	50				
1112.4±27.9 ^b	1489.8±26.2 ^a	1072.7±22.2 ^b	514.9±16.8 ^c				
Carbon source (3%, w/v)							
Sucrose	dextrose	glucose	mannitol	Starch			
1512.6±18.6 ^c	2576.6±27.5 ^b	3544.3±20.7 ^a	1387±38.4 ^{c,d}	1199.8±26.2 ^d			
Nitrogen source (0.3%, w/v)							
Sodium Nitrate	Ammonium sulphate	Ammonium Chloride	Glycine				
3577.1±40.1 ^b	3642.3±24.6 ^b	4775.4±53.7 ^a	1543.2±35.7 ^c				

Among all pH investigated, the pH 5.5 showed the maximum production of GA₃ which was 1478.2mg/l (Table 2). pH 5.5 was also optimum for GA₃ production by *Fusarium moniliforme* [10, 12] and *Fusarium fujikuroi* SG2 [25]. Bilkay et al. (2010) reported pH 5.0 as optimal time for GA₃ production by *Aspergillus niger*, whereas pH 7.0 was optimum for GA₃ production by *Fusarium moniliforme* [17].

The effect of temperature on GA₃ production was analysed, and maximum production was observed at 30°C (Table 2). The production of GA₃ by various fungal species was also observed at an optimum temperature of 30°C [4, 25, 17, 12]. 25°C was also optimum for GA₃ production by *Gibberella fujikuroi* [9]. A low GA₃ yield at higher temperature was also recorded for GA₃ production by *Aspergillus niger* [4]. A low

GA₃ production was observed at higher temperatures because metabolic activities get stopped due to enzyme denaturation. The decrease in GA₃ secretion by microbial species was attributed to the variation in enzyme activity or thermal denaturation [11].

The effect of carbon sources on GA₃ production was investigated. Maximum GA₃ production was seen when glucose was used as carbon source (Table 2). Similarly, glucose was best carbon source for GA₃ production by *Fusarium moniliforme* [17, 12]. However, a mixture of glucose and rice flour was necessary to obtain GA₃ production by *Fusarium fujikuroi* SG2 [25]. When the concentration of glucose was increased, a decrease in enzyme production is seen due to catabolite repression [24].

After analysing the effect of nitrogen sources on GA₃ production, a significant yield was observed with ammonium chloride (Table 2). Similarly, an important yield was seen when ammonium chloride was used as nitrogen source for GA₃ production by *Fusarium fujikuroi* SG2 [25]. A low amount was seen when glycine was used as nitrogen source (Table 2). This can be attributed to the fact that glycine is a slowly consumed organic nitrogen source [19]. After exhaustion of nitrogen source, GA₃ secretion starts and an important amount of carbon source is consumed [24, 19].

The submerged fermentation for GA₃ production by the isolate M104 was carried out under shaking conditions (100 rpm) to allow proper mixing of nutrients, favouring oxygen circulation and GA₃ production. A 3-fold increase was recorded for GA₃ production by *Aspergillus niger* when the culture flasks were agitated [4]. Rodrigues et al. (2011) reported that GA₃ production has to be carried with aeration since GA₃ biosynthesis requires various oxidative steps catalysed by different oxygenases. After optimization, a 5.56-enhancement in GA₃ secretion was observed.

Thin Layer Chromatography (TLC)

After GA₃ extraction, crystals of GA₃ were obtained as shown on the Figure 3. After carrying TLC, the value of Resolution factor (R_f) of GA₃ was calculated as follow: $R_f = \text{distance from origin to solvent peak} / \text{distance from origin to sample spot detected} = 7.9 \text{ cm} / 10.8 \text{ cm} = 0.7315$ (Figure 4).



Figure 3: GA₃ extracted from *Fusarium* isolate M104.

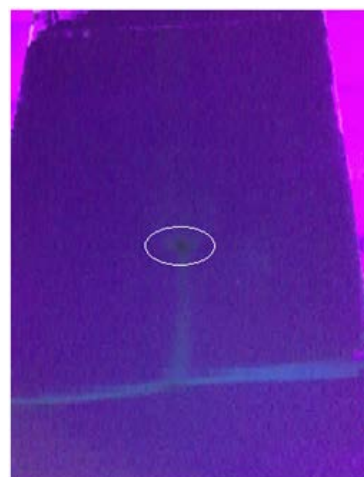


Figure 4: Thin layer chromatography of GA₃ obtained from isolate M104.

The value was closing approximate to the GA₃ standard value. Similarly, an approximate R_f value was recorded for the GA₃ extracted from *Fusarium moniliforme* [17]. The TLC was also used to confirm the GA₃ produced by *Fusarium solani* [3].

Effect of GA₃ on pea plants

It was observed that the pea plants sprayed with GA₃ were 7cm taller than the pea plants without the GA₃ within a period of two weeks (Figure 5). Similarly, size of the lily plants was increased following exogenous GA₃ treatment and this was attributable to the protein synthesis stimulation [13]. Likewise, the hybridized rice plant height was increased after GA₃ extract application [21].

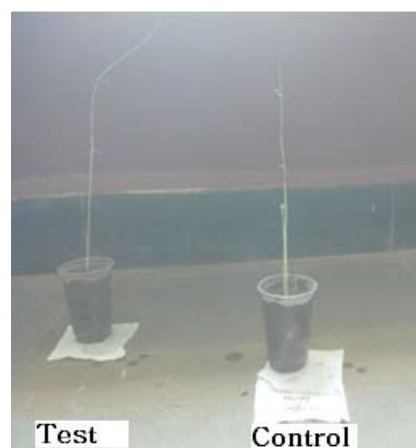


Figure 5: Effect of GA₃ on pea plant. 100 ppm of GA₃ was sprayed on the plant for each alternative day for another 8 days.

The control was soaked in water and sown in autoclaved soil and sprayed with distilled water only.

Effect of GA₃ on paddy crops

All the 10 paddy seeds treated with GA₃ were able to germinate and had uniform growth, colour and height and average height was 9.5 cm within a total period of 25 days. The untreated seeds were able to germinate and had unequal growth and average height was 8.5 cm (Figure 6). Similarly, the shoot and root

heights, and the yield of chana and wheat crops were increased after GA₃ extract application [15]. After GA₃ application, an important harvest was recorded for hybrid rice plant, following a better plant growth and physiological properties [22]. The GA₃ application also led to a significant yield for faba bean, compared to Ca²⁺ ion, and this was attributed to the improvement of growth and photosynthetic activity by the plant hormone [1].

Figure 6: Effect of GA₃ on paddy crops: Uniform growth (left) and non-uniform growth (right). Paddy seeds were soaked in 300 ppm of GA₃ solution for about 2 days and sown in soil. They were sprayed with 200 ppm of GA₃ after growth. The control seeds were soaked in water for the same period and sprayed with only water. The observation was carried out for 25 days.



Conclusion

Four strains of *Fusarium* were screened from Belgaum agriculture land by using a selective medium known as malachite green agar. They were confirmed as belonging to *Fusarium* species by lactophenol cotton blue spore staining method. The GA₃ production depends on nutritional and physicochemical conditions. Strain M104 showed the highest GA₃ production in CD broth. After optimization, a 5.56-fold increase in GA₃ production was achieved. The pea plant sprayed with GA₃ fungal extract was taller than the unsprayed one. The effect of GA₃ on paddy seeds showed uniform and

more growth than control (without GA₃). The isolate M104 can thus be used as a potent fungal species for GA₃ production.

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