Ga3 producing fusarium and its impact on growth

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Isolation and characterization of Gibberellic acid 3 producing *Fusarium* sp. from Belgaum agriculture land and its impact on green pea and rice growth promotion

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 acid 3 (GA_3) . This research mainly involves the production of GA_3 from fungal species and to apply it on crop plants. *Fusarium* species were isolated from Belgaum agriculture soil and screened for GA_3 production under submerged fermentation. Strain showing maximum GA_3 yield (strain M104) was taken 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 – 40 °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 6. 56-increase in GA_3 production was observed. The GA_3 production was confirmed by thin layer chromatography. The GA_3 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 GA_3 treated crops showed uniform growth and they were

taller than non-treated plants, suggesting its application in increasing the crop plant harvests.

Key words: *Fusarium* sp, isolation, gibberellic acid, optimization, submerged fermentation, crop plants.

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 $C_{19}H_{22}O_6$. 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, act as secondary metabolite (Bruckner and Blecschmidt, 1991; Karakoc and Aksoz, 2006). Till now, 136 gibberellins were isolated from various plants, and among that gibberellic acid 3 shows maximum biological activity (Rodrigues *et al* ., 2011). The use of GA_3 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) (Rodrigues *et al* ., 2011).

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 (Bilkay *et al* ., 2010). Three routes to obtain GA_3 have

been reported, viz. extraction from plants, chemical synthesis and microbial fermentation. Among this the third method is the most common method to produce GA_3 (Rios-Iribe *et al* ., 2011). GA3 is industrially produced by *Gibberella fujikuroi* / *Fusarium moniliforme* under submerged (Santos *et al* ., 2003; Karakoc and Aksoz, 2006). 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 *Azospirillu* species (Rademacher, 1994). All above species produced very low yield of GA3 except *Fusarium* species in which most of the strains show the highest yield of GA_3 than any other microbes (Rangaswamy, 2012). The search for new fungal species like *Fusarium* species capable of producing an important amount of GA_3 is a continuous exercise. The aim of the present study was therefore to isolate and characterize a GA_3 producing *Fusarium* sp. from soil, optimize the culture conditions for maximum GA_3 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 taken from Belgaum agriculture area (Karnataka state, India). This 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 (Castellá *et al* ., 1977). The resulted various colonies were picked up and further inoculated in a potato dextrose agar (PDA) plate and incubated for a week for secondary pigmentation. The colony with different morphology and colour pigmentation were sub cultured on PDA slants and labelled (Avinash *et al* ., 2003). The lactophenol cotton blue technique was used to study the characteristics of the fungal isolate (William and Cross, 1971).

Screening of the isolates for GA_3 production under submerged fermentation

The Czapack 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 (Kahlon *et al* ., 1986; Karakoc *et al* ., 2006; Rangaswamy, 2012).

GA_3 pre-treatment, extraction and estimation

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. GA_3 was extracted trice using equal amount of ethyl acetate/$NaHCO_3$ (Cho *et al* ., 1979). The ethyl extract was kept on hot air oven at 50 °C overnight to

remove ethyl acetate and obtain crystals of GA_3 (Kahlon *et al* ., 1986; Karakoc and Aksoz, 2006; Karakoc *et al* ., 2006; Bilkay *et al* ., 2010; Rangaswamy, 2012). It was estimated by Berrios *et al* . (2004) spectrophotometric method and absorption was read at 254 nm in UV-VIS spectrophotometer (Elico, SL-159 model, India).

Confirmation of GA_3 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. GA_3 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 $FeCl_3$ and heated in oven at 80 °C for 10 min. The GA3 appeared as greenish black/spot fluorescence under UV light (Cavell *et al* ., 1967; Srivastava *et al* ., 2003).

Optimization of culture conditions for maximum GA3 production by *Fusarium* sp. (isolate M-104).

The incubation time for GA_3 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 GA_3 production. The effect of pH on GA_3 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 GA_3 production was investigated by inoculating the fungal spores in CD broth of pH 7. 0 and by incubating at three different temperatures 20, 30, 40, and 50 °C with other conditions remained same. The effect of carbon sources on GA3 secretion 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 GA_3 secretion was analysed by replacing the sodium nitrate in the CD broth of pH 5. 5 by glycine, ammonium chloride and ammonium sulfate at 30 °C for 8 days.

Effect of GA_3 on pea plant and paddy crops

Seeds of pea plants were soaked in 200 ppm of GA_3 fermented filtrate for 12 h and then sown in autoclaved soil. After a period of 8 days, 100 ppm of GA_3 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 GA_3 solution for about 2 days and sown in soil. They were sprayed with 200 ppm of GA_3 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 (Tiwari *et al* ., 2011; Susilawati *et al* ., 2014).

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.

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 reseptated which is a unique microscopic feature of *Fusarium* species as shown in the figure1b. The isolation medium containing malachite green was chosen since malachite green inhibits the radial colony growth of the saprophytes and allows only growth of *Fusarium* species (Castellá *et al* ., 1997).

Screening for isolates for GA_3 production

GA_3 can be commercially produced by submerged fermentation using different media but the most common synthetic medium is the Czapack Dox medium (CD broth) (Rangaswamy, 2012). The isolated strains M101, M102, M104 and M110 were subjected to submerged fermentation to check their ability for GA_3 production. The different amounts of GA_3 produced are given in the table 1 and Figure 2, and the strain M104 was the highest producer of GA_3 among the four isolates. Similarly, *Aspergillus niger* strains produced different amounts of GA_3 with the highest of 150. 35 mg/l for *A. niger* Fursan (Cihangir and Aksoz, 1993). Likewise, various amounts of GA_3 were produced by *Lentinus tigrinus* and *Laetiporus* (Ozcan, 2001).

Optimization of culture conditions for maximum GA_3 production by *Fusarium* isolate M104

The optimization of cultural parameters like incubation time, temperature, and pH, and nutritional conditions like nitrogen and carbon sources, is necessary to produce GA_3 in a significant amount. Time course for GA_3 production by the isolate M104 was studied. GA_3 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 time was noted for GA_3 production by *Fusarium monilifome* (Rangaswamy, 2012). 9 days was optimal time for GA_3 secretion by *Fusarium* fujikuroi SG2 (Uthandi *et al* ., 2010) and *Fusarium monilifome* (Kobomoje *et al* ., 2013). In contrast, a higher incubation time of 12 days was observed by for Fusarium moniliforme(Kahlon and Malhotra, 1986) and *Aspergillus niger* (Bilkay *et al* ., 2010). The optimum incubation time for GA_3 production by various fungal species depend therefore on the strain used. The short incubation period observed for GA_3 production by fungal isolate M104 makes the fermentation cost-effective.

Among all pH investigated, the pH 5. 5 showed the maximum production of GA_3 which was 1478. 2 mg/L (Table 2). pH 5. 5 was also optimum for GA_3 production by *Fusarium monilifome* (Kahlon and Malhotra, 1986; Kobomoje *et al* ., 2013) and *Fusarium* fujikuroi SG2 (Uthandi *et al* ., 2010). Bilkay *et al* . (2010) reported pH 5. 0 as optimal time for GA_3 production by *Aspergillus niger* , whereas pH 7. 0 was optimum for GA_3 production by *Fusarium monilifome* (Rangaswamy, 2012).

The effect of temperature on GA $_3$ production was analysed, and maximum production was observed at 30 °C (Table 3). The production of GA $_3$ by various fungal species was also seen at an optimum temperature of 30 °C (Bilkay *et al* ., 2010, Uthandi *et al* ., 2010; Rangaswamy, 2012; Kobomoje *et al* ., 2013). 25 °C was also optimum for GA3 production by *Gibberella fujikuroi* (Gelmi *et al* ., 2002). A low GA3 yield at higher temperature was also recorded for GA3 production by *Aspergillus niger* (Bilkay *et al* ., 2010). A low GA $_3$ production was observed at higher temperatures because metabolic activities get stopped due to enzyme denaturation. The decrease in GA $_3$ secretion by microbial species was ascribed to the variation in enzyme activity or thermal denaturation (Karakoc and Aksoz, 2006).

The effect of carbon sources on GA $_3$ production was investigated. Maximum GA $_3$ production was seen when glucose was used as carbon source (Table 2). Similarly, glucose was best carbon source for GA $_3$ production by *Fusarium moniliforme* (Rangaswamy, 2012; Kobomoje *et al* ., 2013). However, a mixture of glucose and rice flour was necessary to get GA $_3$ production by *Fusarium* fujikuroi SG2 (Uthandi *et al* ., 2010). When the concentration of glucose was increased, a decrease in enzyme production is observed due to catabolite repression (Tudzynski, 1999).

After analysing the effect of nitrogen sources on GA $_3$ 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 GA $_3$ production by *Fusarium fujikuroi* SG2 (Uthandi *et al* ., 2010). 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 (Rodrigues *et al* ., 2011). After exhaustion of nitrogen source, GA3 secretion starts and an important amount of carbon source is consumed (Tudzynski, 1999; Rodrigues *et al* ., 2011).

The submerged fermentation for GA_3 production by the isolate M104 was carried out under shaking conditions (100 rpm) to allow proper mixing of nutrients, favouring oxygen circulation and GA_3 production. A 3-fold increase was recorded for GA_3 production by *Aspergillus niger* when the culture flasks were agitated (Bilkay *et al* ., 2010). Rodrigues *et al* . (2011) reported that GA_3 production has to be carried with aeration since GA_3 biosynthesis requires various oxidative steps catalysed by different oxygenases. After optimization, a 6. 56-enhancement in GA_3 secretion was observed

Thin layer chromatography (TLC)

After GA_3 extraction, crystals of GA_3 were obtained as shown on the figure 3. After carrying TLC, the value of resolution factor (Rf) of GA_3 was calculated as follow: Rf = distance from origin to solvent peak / distance from origin to sample spot detected = 7. 9 cm / 10. 8 cm = 0. 7315 (Figure 4). The value was closing approximate to the GA_3 standard value. Similarly, an approximate Rf value was recorded for the GA_3 extracted from *Fusarium monilifome* (Rangaswamy, 2012). The TLC was also used to confirm the GA_3 produced by *Fusarium* solani (Bhalla *et al* ., 2010).

Effect of GA_3 on pea plants

It is was observed that the pea plants sprayed with GA_3 was 7 cm taller than the pea plants without the GA_3 within a period of two weeks (Fig. 5). Similarly, size of the lily plants was increased following exogenous GA_3

treatment and this was attributable to the protein synthesis stimulation (Mahmoody and Noori, 2014). Likewise, the hybridized rice plant height was increased after GA_3 extract application (Srivastava *et al* ., 2003).

Effect of GA3 on paddy crops

All the 10 paddy seeds treated with GA_3 were able to germinate and have 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 (Fig. 7). Similarly, the shoot and root heights, and the yield of chana and wheat crops were increased after GA_3 extract application (Pandya and Desai, 2014). After GA_3 application, an important productivity was seen for hybrid rice plant, following a better plant growth and physiological properties (Susilawati *et al* ., 2014). The GA_3 application also led to a significant yield for faba bean, compared to Ca^{2+} ion, and this was attributed to the improvement of growth and photosynthetic activity by the plant hormone (Al-Whaibi *et al* ., 2010).

Figure 7: Effect of GA3 on paddy crops: Uniform growth (left) and non-uniform growth (right). Paddy seeds were soaked in 300 ppm of GA_3 solution for about 2 days and sown in soil. They were sprayed with 200 ppm of GA_3 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 malachite green agar. They were confirmed as belonging to *Fusarium* species by lactophenol cotton blue spore staining

method. The GA_3 production depends on nutritional and physicochemical conditions. Strain M104 showed the highest GA_3 production in CD broth. After optimization, a 5. 56-increase in GA_3 production was achieved. The pea plant sprayed with GA_3 fungal extract was taller than unsprayed one. The effect of GA_3 on paddy seeds showed uniform and more growth than control (without GA_3) . The isolate M104 can thus be used as a potent fungal species for GA_3 production.