Isolation and Characterization of

Gibberella fujikuroi from Different Biospheres

Bhuvaneshwari, R

(12PBT004)

A Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women

Coimbatore – 641 043

In Partial Fulfilment of the Requirement of the

Degree of Master of Science in Biotechnology

March, 2014

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Signature of Supervisor

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Bhuvaneshwari, R

CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
	LIST OF TABLES	
	LIST OF FIGURES	
	LIST OF PLATES	
1.0	**INTRODUCTION**	
2.0	**REVIEW OF LITERATURE**	
3.0	**MATERIALS AND METHODOLOGY**	
4.0	**RESULTS AND DISCUSSION**	
5.0	**SUMMARY AND CONCLUSION**	

6.0	**BIBLIOGRAPHY**	
	APPENDICES	

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
1.	**NAMES OF THE PLACES WHERE THE SAMPLES ARE COLLECTED**	
2.	**NAMES OF THE STRAINS COLLECTED FROM DIFFERENT PLACES**	
3.	**MORPHOLOGY OF THE ISOLATED STRAINS IN PDA**	
4.	**COLONY MORPHOLOGY OF *Gibberella fujikuroi* IN RBA**	
5.	**COLONY MORPHOLOGY OF *Gibberella fujikuroi* STRAINS IN CDA**	

6.	**COLONY MORPHOLOGY OF THE *Gibberella fujikuroi* ON SDA**	
7.	**SPORULATION ON DIFFERENT MEDIA**	
8.	**WET WEIGHT AND DRY WEIGHT OF THE MYCELIA**	
9.	**CONCENTRATION OF GIBBERELLIC ACID IN STRAINS OF *Gibberella fujikuroi***	

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
1.	**CONCENTRATION OF GIBBERELLIC ACID IN STRAINS OF *Gibberella fujikuroi***	

LIST OF PLATES

PLATE NO.	TITLE	PAGE NO.
1.	DISEASE AFFECTED PLANT	
2.	EMPTY PANICLE PRODUCED IN DISEASE AFFECTED PLANT AND NORMAL SEEDLINGS	
3.	MORPHOLOGY OF THE STRAINS IN PDA	
4.	MORPHOLOGY OF THE STRAINS IN RBA	
5.	MORPHOLOGY OF THE STRAINS IN CDA	
6.	MORPHOLOGY OF THE STRAINS IN SDA	

7.	**SHAPE OF MACRO AND MICROSPORES PRODUCED BY *Gibberella fujikuroi***	
8.	**MICRO CONIDIA FORMED IN FALSE HEADS**	
9.	**QUALITATIVE ANALYSIS OF GIBBERELLIC ACID IN TLC PLATE UNDER WHITE LIGHT**	
10.	**QUALITATIVE ANALYSIS OF GIBBERELLIC ACID IN TLC UNDER UV LIGHT**	

LIST OF APPENDICES

APPENDIX NO.	TITLE	PAGE NO.
1.	**ISOLATION AND MAINTENANCE OF *Gibberella fujikuroi* FROM RICE (*Oryza sativa)* (Karov *et al.*, 2009)**	
2.	**MORPHOLOGICAL IDENTIFICATION OF *Gibberella fujikuroi* (Rajhoo *et al.*, 2008)**	
3.	**EXTRACTION OF GIBBERELLIC ACID**	
4.	**QUALITATIVE ANALYSIS OF GIBBERELLIC ACID BY THIN LAYER CHROMATOGRAPHY (Zainudin *et al.*, 2008)**	
5.	**QUANTITATIVE ESTIMATION OF GIBBERELLIC ACID (Berrios *et al.*, 2004)**	

INTRODUCTION

1.0 INTRODUCTION

Food legumes play an important and diverse role in the farming systems and in the diets of poor people around the world. These are ideal crops for simultaneously achieving four developmental goals in targeted population reducing poverty, improving human health, nutrition, and enhancing ecosystem resilience (Shahir 2014).

Present agricultural scenario includes soil borne and seed borne diseases. Some diseases can affect the production of crop and thus can also contaminate food. These include many fungi, virus, bacteria and insects. These can also affect socioeconomics of the crop production (Elwakil *et al.,* 2009).

India is an agricultural based country where in agriculture continues to play a dominant role and it contributes about 26% Gross Domestic Product (GDP). Yield of any crop depends on the quality of seeds. Seed is the most important input for increased crop production. Pathogen free healthy seed is needed for desired plant populations and good harvest because several phytopathogens are seed-borne which can cause enormous crop losses. In this connection seed health, the productivity of the crop, management practices and supplementary inputs exert profound influences (Islam *et al.,* 2009)

Many plant diseases are caused by pathogens, which are difficult to see or recognize without magnification. Fungi, bacteria, viruses, nematodes, and even plants can be pathogenic on garden plants. Contamination of Rice (*Oryza sativa* L.), sorghum (*Sorghum vulgare* Pers.) and maize (*Zea mays* L.) grains during storage by fungi is a major concern (Reddy *et al.,* 2008).

Collectively, fungi and fungal-like organisms (FLOs) cause more plant diseases than any other group of plant. The impact that fungi have with regards to plant health, food loss and human nutrition (Masum *et al.,* 2009).

Fungi are not only involved in biodeterioration of grains but some are also known to produce mycotoxins which cause obvious reduction in crop, animal live stock production and diseases in humans. However, the serious problem with storage of seeds is that, it is highly susceptible to many fungi associated with grain mould disease such as species of

Fusarium, Aspergillus, Drechslera, Rhizopus, Pencillium, Alternaria, Curvularia and *Cladosporium*. *Fusarium verticillioides* (Sacc) Nirenberg is a fungal pathogen with a wide range of plant hosts such as maize, sorghum, rice etc., along with plant diseases. *F. verticillioides* infection also results in food safety problems for humans and animals as the fungus produces mycotoxins which cannot easily be removed or detoxified from grains (Maheswar *et al.,* 2009). Currently, fumonisins are considered as the most agriculturally significant environmental toxins produced by *F. verticillioides* and other *Fusarium* species that grow in grains during storage (Prabhashankar *et al.,* 2013).

In addition to being agents of preharvest and postharvest diseases and rots, fungi produce highly toxic, hallucinogenic, and carcinogenic chemicals. Aflatoxin, a chemical produced by several *Aspergillus* species. These fungi can grow on corn and fill the seed with the toxin that not only attacks the liver, but is one of the most carcinogenic substances known (Ellis *et al.,* 2008).

Fungi belonging to *Fusarium* section Liseola, primarily *F.moniliforme* J. Sheld., the sexual stage (Teleomorph) is *Gibberella fujikuroi* (Sawada). It can infect numerous crops worldwide including: asparagus, figs, mango, nectarines, plums, pine, pine apple, rice, sugarcane, sorghum, corn, and pearl millet (Maheshwar *et al.,* 2009).

Gibberella fujikuroi (Teleomorph) or *Fusarium verticilloids* (Sacc.) or *Fusarium moniliforme* is wide spread pathogen of many plants include maize, rice, barley, sugarcane, pine, mango, pine apple, pearl millet, and sunflower (Bomke *et al.,* 2008). They are not only affecting the plant kingdom but also affecting animals as well as humans (Izzati *et al.,* 2011).

The species *Gibberella fujikuroi* can produce significant amounts of fumonisins. Fumonisins are analog of sphingamine can competitively inhibit sphingo lipid biosynthesis, and especially the isoform B1 (FB1). FB1 presents a great mycotoxicological concern because of its abundance in maize grains. FB1 causes leucoencephalomalacia in horses, pulmonary edema in swine, poor performance in poultry, altered hepatic and immune function in cattle (Faria *et al.,* 2012 and Rahjoo *et al.,* 2011).

Fumonisin produced by these fungi have been linked to human mycotoxicosis. It has been associated with human esophageal cancer. And it also causes exogenous endophthalmitis in humans. *Gibberella fujikuroi* can cause mycosis in acute lymphocytic leukemia patients (Faria *et al.,* 2012; Rahjoo *et al.,* 2011 and Strange *et al.,* 2005). Portals of the entry may be the respiratory tract, disrupted skin barrier, nail, or corneal injury. *Fusarium verticilloids* (Sacc.) Nirenberg was reported as the agent of keratitis, cutaneous infections and mycotoma (Donat *et al.,* 2011).

Fusarium species may also produce secondary metabolites that involve Mycotoxins and Phytotoxins (Studl *et al.,* 2012). The secondary metabolites produced by this filamentous fungi can be classified into distinct chemical groups as polyketides, non ribosomal peptide moiety, terpenes and alkaloids (Schwerdt *et al.,* 2009). They produce Gibberellins, Carotenoids, Bikaverin and Fusaric acid (Hoffmeister *et al.,* 2007).

Among all the secondary metabolites produced by this pathogen gibberellic acid is the most considerable secondary metabolite. It was the one first isolated secondary metabolite from the phytotoxic metabolites of this phytopathogenic fungus, *Gibberella fujikuroi* (Sondergard *et al.,* 2011). Gibberellic acid is produced by submerged fermentation or solid substrate cultures of the fungus *Gibberlla fujikuroi.* It is able to give higher yields of gibberellic acid on a variety of media (Syalputra *et al.,* 2013).

Gibberellic acid (GA3), is the most important gibberellin, is a class of diterpenoid that functions as plant growth regulator. It affects stem elongation, elimination of dormancy, flowering, sex expression, enzyme induction, leaf and fruit senescence. GA3 is a high valued industrially important biochemical (Karakoc *et al.,* 2006).

GA3 is presently produced largely by submerged fermentation techniques using *Fusarium moniliforme* or *Gibberella fujikuroi*. Other bacteria that belong to the genus *Azoto-bacter* and *Azospirillum* also synthesize GA3. Recently, a *Pseudomonas* sp. isolated from wastes of processed olive has also been shown to produce GA3 (285 mg/L). The factors that account for high cost of GA3 in present market scenario are the low yield of GA3 produced and its presence in dilute form in submerged fermentation; leading to higher costs of downstream processing and disposal of waste water. GA3 can also be produced by

the solid-state fermentation (SSF), which has got a tremendous potential for production of secondary metabolites (Vidhya, 2012).

These characteristics that is producing some useful secondary metabolites especially the gibberellic acid, a plant growth promoting hormone make *Gibberella fujikuroi* species good candidates for use as good producers of gibberellic acid in large scale (Vidhya, 2012).

Eventhough the bakanae disease (Foolish seedling) is not a big problem during the rice cultivation in South India especially in Tamilnadu and Kerala, it is very important to characterize these *Gibberella fujikuroi* species, and also to find out the elite gibberellic acid producing strain. This will be helpful in the commercial production of gibberellic acid.

In the present study, strains of *Gibberellin fujikuroi* are isolated from different parts of Tamilnadu and Kerala, and morphologically characterized and evaluated the elite gibberellic acid producing strain.

The present study was aimed with the following objectives:

- Collection of disease affected rice samples from different placesof TamilNadu and Kerala
- Isolation and Maintenance of *Gibberella fujikuroi* from disease affected rice samples
- Morphological identification of *Gibberella fujikuroi*
- Extraction of gibberellic acid from *Gibberella fujikuroi*
- Qualitative determination of gibberellic acid from *Gibberella fujikuroi*
- Screening of elite gibberllic acid producing organism by quantitative determination.

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

The review of literature pertaining to the study on "**Isolation and Characterization of *Gibberella fujikuroi* from different biospheres"** is discussed under the following headings.

2.1. *Gibberella fujikuroi*

2.2. Plant Diseases caused by *Gibberella fujikuroi* species

2.3. Animal Diseases caused by *Gibberella fujikuroi* species

2.4. Human Diseases caused by *Gibberella fujkuroi* species

2.5. Morphological characteristics of *Gibberella fujikuroi*

2.6. Secondary metabolites produced by *Gibberella fujikuroi*

2.7. Production of gibberellic acid in *Gibberella fujikuroi*

2.7.1. History of gibberellic acid

2.7.2. Role of gibberellic acid in plants

2.7.3. Gibberellic acid production in microbes

2.7.4. Structure of gibberellic acid (GA_3)

2.7.5. Pathway of biosynthesis gibberellic acid in *Gibberella fujikuroi*

2.1. *Gibberella fujikuroi*

Gibberella fujikuroi (Teleomorph) or *Fusarium verticilloids* (Sacc.) or *Fusarium moniliforme* is widespread pathogen of many plants which include maize, rice, barley, sugarcane, pine, mango, pine apple, pearlmillet and sunflower (Bomke *et al.,* 2008). They are not only affecting the plant kingdom they are also affecting animals as well as humans (Izzati *et al.*, 2011).

2.1.1. Taxonomy

Kingdom: Fungi

Phylum: Ascomycota

Class: Sordariomycetes

Sub Class: Hypocreomycetidae

Order: Hypocreales

Family: Nectriaceae

Genus: *Gibberella*

Species: *Fujikuroi*

2.1.2. Synonyms:

Fusarium moniliforme, J.Sheld

Fusarium verticilloids (Sacc), Nirenberg

Gibberella moniliformis, Wireland

Lisea fujikuroi, Sawada

2.2. Plant Diseases caused by *Gibberella fujikuroi*

Gibberella fujikuroi can cause disease in the following plants,

2.2.1. Sorghum:

Sorghum is one of the oldest crops and by the area, it is fourth after rice, wheat and corn. Grain sorghum is cultivated because of the seed which is very rich in proteins and starch. It is extremely energetic feed with more digestible proteins than corn seed. In under developed countries like Africa, Asia and Central America, grain sorghum is used as a staple food (Badoci *et al.,* 2013).

Gibberella fujikuroi can cause Stalk Rot disease in sorghum. Infected plants typically wilt, leaves turn dull greyish – green and the lower stalk turns from dark green to straw colored. The internal pith of the lower stem disintegrates and goes soft. When split open, the stalks exhibit a reddish discoloration. Often black perithecia or mycelium can be observed at lower stalk nodes where the plant is infected (http: //ohioline.osu.edu/ac-fact/0033.html., 2007).

2.2.2. Mango

In mango (*Mangifera indica* L.) *Gibberella fujikuroi* can cause Mango Malformation Disease (MMD). It was first reported in India, it is found elsewhere in Asia (Israel, Malaysia, and Pakistan), Africa (Egypt, South Africa, Sudan and Uganda) and America.

MMD causes inflorescences to shorten, thicken and branch, increases flower numbers and size, increases the number of male flowers and causes sterility or abortion in the remaining hermaphroditic flowers. Leaves may also develop with in the inflorescence (Marasas *et al.,* 2006).

2.2.3. Corn

Corn (*Zea mays* L.) is grown in hot and temperate regions around the world and is the second most cultivated crop in Brazil. Since corn grain possess a high nutritive value, it is used for the preparation of diverse food products, and represents a relevant and important socioeconomic factor in many regions of the world (Rocha *et al.,* 2009).

Corn can be affected by *Gibberella fujikuroi* due the production of fumonicin. Occurrence of fumonicin in corn and corn products has been a worldwide problem. The disease was named as stalk rot (Rahjoo *et al.,* 2008). Infection of maize by *F. verticillioides*

can occur via several routes. The most commonly reported method of kernel infection is through airborne conidia that infect the silks. After invasion through the silks, the fungus infects the kernels, but usually only a small percentage of the infected kernels become symptomatic (Summerell *et al.,* 2011).

2.2.4. Pearl millet

Pearl millet is widely cultivated as a staple grain for human consumption throughout the tropical and subtropical regions of the world. Pearl millet (*Pennisetum glaucum*) is the most widely grown type of millet. Because of its tolerance to difficult growing conditions such as drought, low soil fertility and high temperature, it can be grown in areas where other cereal crops, such as maize (*Zea mays*) or wheat (*Triticum aestivum*), would not survive. Pearl millet production is concentrated in the developing countries which account for over 95% of the production and acreage. India continues to be the single largest producer of pearl millet in the world, although the area has been declining in the traditional growing states of Gujarat, Rajasthan and Haryana (Lee *et al.,* 2012).

Pearl millet is usually grown as a dry land dual purpose grain and fodder crop although it is sometimes irrigated in India, particularly the summer crop grown mainly as a forage crop. Pearl millet grain is the staple diet for farm households in the world's poorest countries and among the poorest people. In the Sahelian region of Africa and rural regions of northwestern India, pearl millet is an important cereal for consumption. Pearl millet Stover is a valuable livestock feed in the growing regions in India and Africa. Exports and imports of pearl millet grain are negligible suggesting low demand, and/or unreliable availability of marketable surpluses for this commodity in world markets (Basavaraj *et al.,* 2010).

The top rot or twisted top disease in pearl millet is caused by *Gibberella fujikuroi*. The symptoms of the top rot affected pear millet are the panicle and immature leaves often remain in the whorl, where they become rotted and covered with a mass of white mycelium. Nodes will frequently be discolored (Jurjevic *et al.,* 2005).

2.2.5. Sugarcane

Sugarcane is the main crop in India mostly cultivated in TamilNadu. It can be also be an important crop in world wide. Sugarcane is cultivated in about 4.2 million ha in tropical and subtropical parts of India. Being an important agricultural crop of the country, it is affected by several pathogens such as fungi, bacteria, viruses and phytoplasmas which are responsible for reducing the yield and quality of the crop (Tiwari *et al.,* 2011).

In the beginning of the 19th century, only few diseases were recognized as important ones. After rapid extension of sugarcane cultivation to different agro climatic zones, monoculture of sugarcane, practices of ratooning over large areas and due to unrestricted seed movements many diseases epiphytotic have occurred in different sugarcane regions. Among the fungal diseases red rot (*Colletotrichum falcatum* Went), Wilt (*Fusarium moniliforme*), root rot (*Pythium graminicolum* Subr.), pineapple disease (*Thielaviopsis parodoxa* (de Seynes) C. Moreau), and smut (*Ustilago scitaminea* Syd.) causes severe losses to the sugarcane. (Tiwari *et al.,* 2012 and Tiwari *et al.,* 2010).

Pokkah boeng is one of the most common sugarcane disease. It is caused by *Gibberella fujikuroi*. Pokkah boeng is Javanese term denoting a malformed or distorted top, was originally described by wakker and went. The knife - cut abnormality sometimes associated with Pokka boeng. Pokkah boeng affected young cane growing vigorously under warm, humid conditions. Most cultivars recovered from the disease as the plants mature (Khani *et al.,* 2013).

2.2.6. Rice

India is reowned rice (*Oryza sativa* L.) producing country and stands second with an annual production of 155 million tones. Rice cultivars take place in all states of India but West Bengal, Uttar Pradesh, Madhya Pradesh, Punjab, Orissa, Bihar and TamilNadu are the major rice producing states. There are several constraints in the production of rice of which diseases caused by bacteria, fungi and nematodes are responsible for major economic losses in India. The crop is affected by as many as 36 seed – borne diseases of which 31 were caused by fungi (Archana and Prakash 2013)

Bakanae is a seedling disease caused by *Gibberella fujikuroi*, but it can be observed throughout the growing season. The earliest symptoms of bakanae are infected seedlings that appear to be elongated, thinner and slightly chlorotic when compared with healthy seedlings. The rapid elongation of infected plants is due to the production of gibberellins, a plant growth hormone, by the fungus. Bakanae plants are often visible above healthy rice plants. Symptom development is influenced by the amount of inoculum present, the strain of the pathogen and the relative quantities of gibberellin and fusaric acid (which causes stunting) produced. Infected seedlings often progressively die from the seedling stage through to maturity. If infected plants survive to heading, the panicles they produce are usually sterile. The flag leaf on mature plants is noticeable by its elevated, more horizontal stance (Karov *et al.,* 2009).

The fungus sporulates profurely on the stems of diseased plants near the water level, and after the water is drained, noticeable pink to white fungal growth appears at the base of the plants. This cottony growth produces masses of conidia which which contaminate the outside of healthy seeds during harvest. Symptom development is affected by temperature. Symptoms do not appear below 20˚C and above 35˚C (Karov *et al.,* 2009).

2.3 Animal diseases caused by *Gibberella fujikuroi*

The species *Gibberella fujikuroi* can produce significant amounts of fumonisins. Being fumonisins are analog of sphingamine can competitively inhibits sphingo lipid biosynthesis, and especially the isoform B1 (FB1). FB1 presents a great mycotoxicological concern because of its abundance in maize grains. FB1 causes leucoencephalomalacia in horses, pulmonary edema in swine, poor performance in poultry, and altered hepatic and immune function in cattle (Faria *et al.,* 2012 and Rahjoo *et al.,* 2011)

2.4 Human diseases caused by *Gibberella fujikuroi*

Fumonisin produced by these fungi have been linked to human mycotoxicosis. It has been associated with human esophageal cancer. And it also causes exogenous endophthalmitis in humans. *Gibberella fujikuroi* can cause mycosis in Acute Lymphocytic Leukemia patients. (Faria *et al.,* 2012; Rahjoo et *al.,* 2011 and Strange *et al.,* 2005). Portals

of the entry may be the respiratory tract, disrupted skin barrier, nail, or corneal injury. *Fusarium verticilloids* (Sacc.) Nirenberg was reported as the agent of keratitis, cutaneous infections and mycotoma (Donat *et al.*, 2011).

2.5 Morphological characteristics of *Gibberella fujikuroi*

2.5.1. Macroscopic Morphology

Macroscopic morphology may vary significantly on different media, rapid growth, colonies initially white becoming tinged with lavender with a colorless to purple reverse in Potato dextrose agar.

2.5.2. Microscopic Morphology

The pathogen sexually produces ascospores that are formed with in a sacc known as ascus. Asci are contained in the fruiting bodies called ascocarps which are referred to as perithecia. They are spherical to oval and somewhat roughened outside. The asci are cylindrical, piston – shaped, flattened above. The spores are one septate. They are occasionally larger.

Hyphae are branched and septated. The fungus has micro and macro conidiospores bearing micro and macroconidia respectively. The micro conidiospores are single, lateral and subulate phialides. They are formed from aerial hyphae. The microconidia are more or less agglutinated in chains.

The macro conidiospores have basal cells with 2 – 3 apical phialids, which produce macroconidia. The macroconidia are delicate, slightly sickle – shaped or almost straight. They are narrow at both the ends and are occasionally somewhat bent into a hook at the apex and distinctly or slightly foot – celled at the base (Karov *et al.*, 2009).

2.6. Secondary metabolites produced by *Gibberella fujikuroi*

Besides being pathogens of plants, *Fusarium* species may also produce secondary metabolites that involve Mycotoxins and Phytotoxins. The secondary metabolites produced by this filamentous fungi can be classified into distinct chemical groups as polyketides, non

ribosomal peptide moiety, terpenes and alkaloids (Studl et al., 2012; Schwerdt *et al.*, 2009 and Hoffmeister *et al.*, 2007). Some of the secondary metabolites produced by this fungi are Gibberellins, Caroteinoids, Bikaverin and Fusaric acid.

2.6.1. Carotenoids

These are the fat soluble pigments produced by photosynthetic organisms and many heterotrophic bacteria and fungi. In plants they function as accessory pigments of the photosynthetic machinery. And in animals they are the source of physiologically important apocaroteinoids. *Gibberella fujikuroi* produces the orange apocaroteinoid, neurosporaxanthin and minor amounts of other caroteinoids including β – caroteinoids and presumably retinol. Caroteinoids are mevalonate derived terpenoids produced from the some precursor geranyl geranyl pyro phosphate, although their synthesis occurs in different cell compartments (Martinez *et al.*, 2012).

2.6.2. Bikaverin

It is red polyketide pigment with antibiotic properties against protozoa and fungi (Limon et al., 2010) where synthesis begins with the condensation of acetate units. The biochemistry and regulation of these biosynthetic pathways has received considerable attention (Martinez *et al.*, 2012).

2.6.3. Fusaric acid

One of the oldest known secondary metabolites of *Fusarium fujikuroi* is fusaric acid (5 – butyl picolinic acid, FA), a mycotoxin with low to high moderate toxicity to animals and humans, but with high phytotoxic properties. Searching for the causing agent of the rice bakanae disease, Japanese scientists first isolated fusaric acid from the culture fluid. Due to wide production of fusaric acid by many *Fusarium* species, this compound was suggested to be used as a marker for *Fusarium* contamination of food and feed. Compared to other fusaric acid producers isolates of *Fusarium fujikuroi* were shown to produce high concentration of fusaric acid (Niehaus *et al.*, 2014)

This metabolite is toxic to various fungi, plant and bacteria but also has pharmacological activities. For example, fusaric acid was found to inhibit dopamine – β – hydroxylase and therefore cause hyposensitive effects in different animals. Interestingly, it was also found to have beneficial health affects, for instance against Acanthomoeba (Boonman *et al.*, 2012) or HIV – 1 dementia (Ramatar *et al.*, 2012)

2.7. Gibberellic acid production in *Gibberella fujikuroi*

Among all the secondary metabolites produced by this pathogen Gibberellic acid most considerable secondary metabolite. It was the one first isolated secondary metabolite from the phytotoxic metabolites of this phytopathogenic fungus, *Gibberella fujikuroi* (Sondergard *et al.*, 2011). Gibberellic acid is produced by submerged fermentation or solid substrate cultures of the fungus *Gibberella fujikuroi* (Syalputra *et al.*, 2013). Gibberellic acid (GA3), the most important gibberellin, is a class of diterpenoid that functions as plant growth regulator. It affects stem elongation, elimination of dormancy, flowering, sex expression, enzyme induction and leaf and fruit senescence. GA3 is a high valued industrially important biologic compound with various applications in agriculture with price ranging around $ 25/g in the international market (Karakoc *et al.*, 2006)

GA3 is presently produced largely by submerged fermentation techniques using *Fusarium moniliforme* or *Gibberella fujikuroi*. Other microorganism that belong to the genus *Azoto-bacter* and *Azospirillum* also synthesize GA3. Recently, a *Pseudomonas* sp. isolated from wastes of processed olive oil has also been shown to produce GA3 (285 mg/L).The factors that account for high cost of GA3 in present market scenario are the low yield of GA3 produced and its presence in dilute form in submerged fermentation leading to higher costs of downstream processing and disposal of waste water. GA3 can also be produced by the solid-state fermentation (SSF), which has got a tremendous potential for production of secondary metabolites (Vidhya 2012).

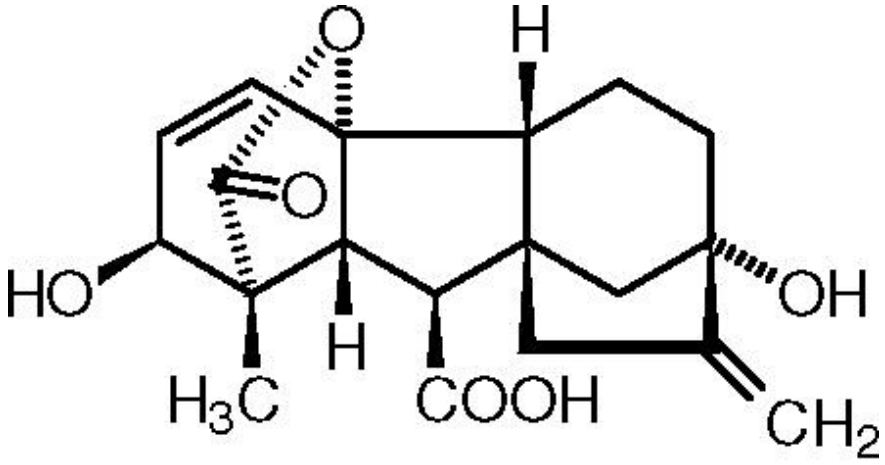
2.7.2. Role of Gibberellic acid in Plants

Gibberellic acid is a diterpenoid compound in a class of gibberellin. It is a plant hormone promoter. Controlling developmental processes such as the induction of hydrolytic

enzyme activity during Seed germination, stem elongation, premature flowering and retards leaves and fruit senescence. These plant regulators can produce by submerged fermentation of these fungi *Gibberella fujikuroi* (Wiemann *et al.,* 2012 and Brock *et al.,* 2011). Gibberellic acid is an important growth promoter in plants, and the worldwide demand for it is steadily increasing (Gokdere *et al.,* 2014).

2.7.3. Structure of Gibberellic acid

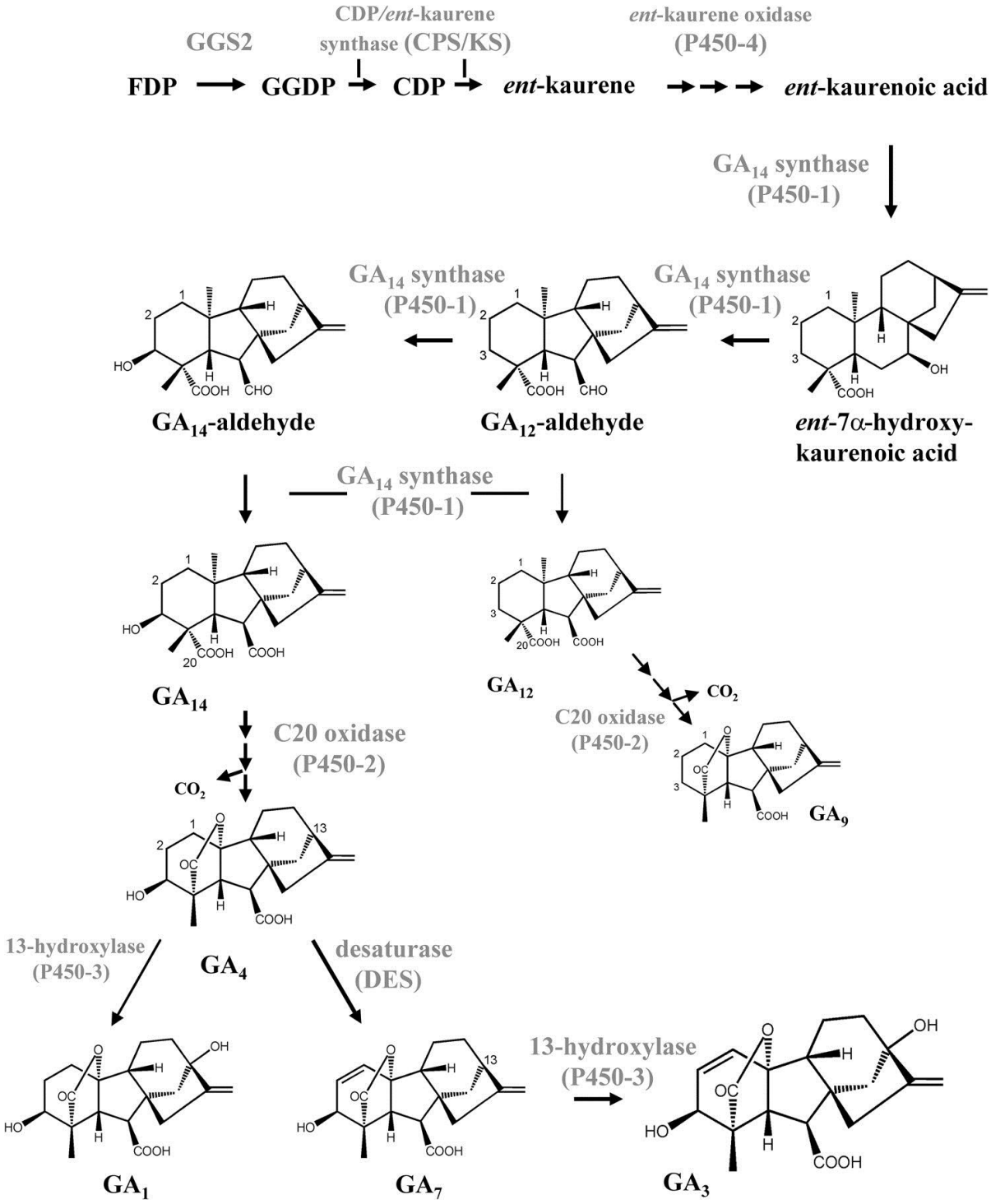
The gibberellic acid is chemically named as 2,4,7 – trihydroxy – 7 – methyl 8 – methylene gib – 3 – ene -1. Chemically this compound is a carboxylic acid (Bombang *et al.,* 2013)



gibberellic acid

2.7.4. Biosynthetic pathway of Gibberellic acid in *Gibberella fujikuroi*

Gibberellic acid biosynthesis in plants is divided into two steps, fungal biosynthesis is divided into two steps. The intermediates in both are same but the final step only different in fungi (Bomke *et al.,* 2008; Blomhoff *et al.,* 2006 and Telfer *et al.,* 2005).


GGS2
CDP/ent-kaurene synthase (CPS/KS)
ent-kaurene oxidase (P450-4)
FDP
GGDP
CDP
ent-kaurene
ent-kaurenoic acid
GA14 synthase (P450-1)
GA14-aldehyde
GA12-aldehyde
ent-7α-hydroxy-kaurenoic acid
GA14
GA12
CO2
C20 oxidase (P450-2)
GA9
GA4
13-hydroxylase (P450-3)
desaturase (DES)
GA1
GA7
GA3

EXPERIMENTAL PROCEDURES

3.0. EXPERIMENTAL PROCEDURES

Gibberella fujikuroi (Teleomorph) or *Fusarium verticilloids* (Sacc) or *Fusarium moniliforme* is a wide spread fungus in the large genus f*usarium*, of the liseola section. It is found in humid and sub humid temperate zones, extending into sub-tropical and tropical zones throughout the world. The pathogen is seed borne and seed transmitted. These are the important fungal pathogens of agricultural crops such as rice (*Orzyza sativa*). Besides being pathogens of plants, they also produce plant growth hormone gibberellin acid. It is able to give higher yields of Gibberellic acid on a variety of media.

The methodology pertaining to the study entitled **"Isolation and Characterization of *Gibberella fujikuroi* from different biospheres"** was carried out under the following headings,

3.1 Collection of plant samples from different places.

3.2 Isolation and maintenance of cultures of *Gibberella fujikuroi* from the collected plant samples.

3.3 Morphological Identification of *Gibberella fujikuroi.*

3.3.1 Colony morphology in different media.

3.3.2 Lacto phenol cotton blue staining.

3.3.3 Single spore isolation.

3.3.4 Slide culture technique.

3.4 Extraction of gibberellic acid by liquid – liquid extraction.

3.5 Qualitative analysis of gibberellic acid by thin layer chromatography.

3.5 Quantitative estimation of Gibberellic acid by Spectrophotometric method.

3.1 COLLECTION OF SAMPLES

The Bakanae disease affected plants were collected according to the symptoms described in Karov *et al.,* (2009). The bakanae disease affected plant will produce empty seedlings. This was shown in Plate 2. and height of the plant will be few centimeters taller than the other plants due to the production of gibberellic acid plants which has been showed in Plate 1. The collected plant samples from different places are listed in Table 1.

Table 1. Name of the places where the samples are collected

S.No	Name of the place	Age of the plant	Variety
1.	Vaikkal medu (Sathiyamangalam)	112 days	ADT
2.	Annur	100 days	ASG 16
3.	Mettur (Sathiyamangalam)	120 days	Deluxe ponni
4.	Chandrapuram (Kerala)	117 days	BPT
5.	Palakkad (Kerala)	116 days	AST

Plate 1. Disease affected plant

Plate 1 shows that the height of the disease affected plant is taller than the other plants.

Plate 2: Empty panicle produced in disease affected plant and normal seedlings

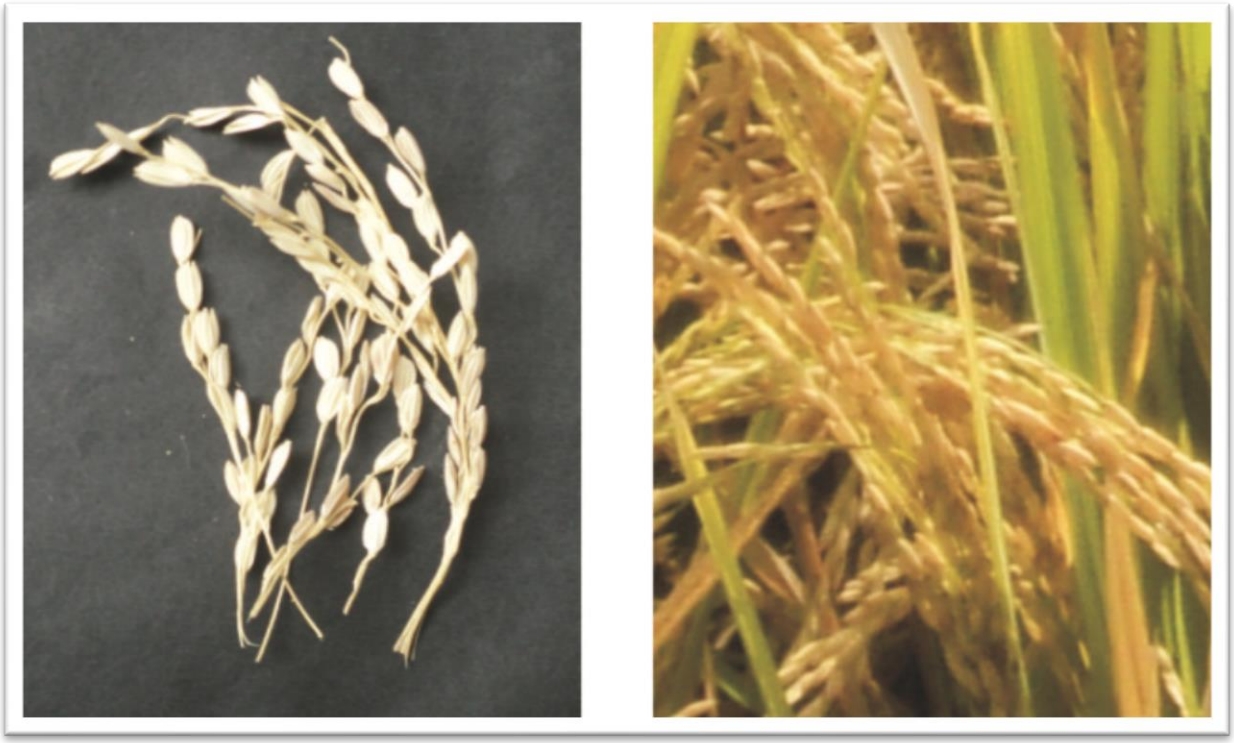


Plate 2 shows that the diffence between disease affected seedlings and normal seeds.

3.2 ISOLATION AND MAINTENANCE OF *Gibberella fujikuroi* FROM RICE (*Oryza sativa)* (Karov *et al.,* 2009)

The stem and leaves of the collected plant sample were cut into small pieces (about $1cm^2$) and surface sterilized for 3 minutes with 1% sodium hypochlorite or 0.1% mercuric chloride. Then rinsed 3 times with double distilled water. After washing inoculate the samples into Potato dextrose agar. Incubate the plates for 5 – 7 days at 24˚C.

3.3 MORPHOLOGICAL IDENTIFICATION OF *Gibberella fujikuroi* (Rajhoo *et al.,* 2008)

3.3.1 Culturing in different media

The culture of the *Gibberella fujikuroi* was inoculated in different media such as Saboraud Dextrose Agar, Potato Dextrose Agar, Czapek Dox Agar and Rose Bengal Agar. Incubated the plates at 23˚C for 7 days.

3.3.2 Single spore isolation Technique

The culture was transferred into Spezieiller Nährstoffarmer Agar or SNA (Composed of (g/L) KH_2PO_4 – 1g, KNO_3 -1g, $MgSO_4.7H_2O$ – 0.5g, KCl – 0.5g Glucose – 0.2g, Sucrose – 0.2g, Agar – 20g.) plates from the PDA plates. The SNA plates were incubated at 23˚C for 10 days. A very small amount of sporulating mycelia was smeared on to 2% water agar plates. The plates were incubated for 16 hours.

3.3.3 Lactophenol cotton blue staining

After isolating single spore the morphological identification was done by observing under the light microscope using lactophenol cotton blue (LPCB) staining.

3.3.4 Slide culture technique

Packs of petri dishes, each containing a cavity slide, cover slip and a filter paper slip were sterilized. Sabouraud Dextrose agar (SDA) was prepared according to manufacturer's guidelines, aliquoted in to small containers and sterilized. An aliquot of the SDA was melted

by heating and pipetted into the cavity of the cavity slide with a sterile pasteur pipette and allowed to set for 5 minutes.

Each fungal isolate to be identified was inoculated onto the cavity slides containing SDA using a sterile straight wire and a sterile cover slip was placed on top of it. The filter paper was moistened with sterile distilled water to humidify the culture environment and the Petri dishes were incubated at room temperature (Wijedasa *et al.*, 2012).

3.4 EXTRACTION OF GIBBERELLIC ACID

3.4.1 Culture media and Growth conditions (Vidya Rangasamy 2012)

Gibberella fujikuroi was grown in 100 ml of CD medium (Composed of (g/L) Sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and $FeSO_4$, 0.01) pH at 7.0 and incubated the culture at 30˚C for 8 days at 120 rpm.

3.4.2 Extraction of gibberellic acid (Berrios *et al.*, 2004)

After the incubation period was completed the culture was centrifuged at 3000 rpm for 10 minutes. The pellet was retained to weigh wet weight and dry weight of the mycelia. The filtrate was then acidified to pH 2.5.Then the filtrate was treated with 20ml of ethyl acetate in separatory funnel, shaked vigorously for 60 seconds. The excess of ethyl acetate (aqueous phase) was discarded from the funnel and allowed to dry by exposing to air.

The extract was retransferred to a separating funnel and retreated with equal volume of ethyl acetate. The solvent fraction (organic phase) was collected and allowed to dry by exposing to air. This process was repeated for 3 times to get a large amount of fine quality of gibberellic acid. The concentrated solution was reevaporated and the residue was dissolved in water containing 0.05% of Tween 20.

3.4.2.1. Wet weight and dry weight of the sample:

After centrifugation the pellet was collected and the wet weight of the samples were taken. After taking wet weight of the sample the pellet was kept in hot air oven and dried. Then the dry weight of the mycelia was taken.

3.5 QUALITATIVE ANALYSIS OF GIBBERELLIC ACID BY THIN LAYER CHROMATOGRAPHY (Zainudin *et al.*, 2008)

TLC plates were prepared. The chromatogram was developed and spotted 10µl of samples were run up to 4 hours in the solvent system composed of Isopropanol:Ammonia:Water at 10:1:1 ratio. Plates were removed and dried at room temperature and sprayed with spraying reagent (Ethanol: Concentrated Sulphuric acid (95:5)). The plates were heated at 80˚C for 10 minutes in hot air oven. Then the plates were allowed to cool and examine under UV – Light (275 nm) for floresecence greenish spots.

3.6 QUANTITATIVE ESTIMATION OF GIBBERELLIC ACID (Berrios *et al.*, 2004)

A sample of 1 ml and 1 ml absolute ethanol were placed in a test tube. 3.75 M Hydrochloric acid was added to the flask up to make 10 ml and then vigorously mixed for 10 s. the tubes were kept one hour at room temperature. After incubation period the absorbancy of the resulting solution was measured at 254 nm and recorded. Temperature was kept at 20 ± 0.5˚C during the process. Standard GA3 solutions prepared by dissolving 0.04 g of pure GA3 in absolute alcohol and diluted to 100 ml in a volumetric flask with absolute alcohol. Regression value was obtained with the help of standard values to calculate the concentration of the gibberellic acid present in the sample.

RESULT AND DISCUSSION

4.0 RESULTS AND DISCUSSION

Gibberella fujikuroi (Teleomorph) or *Fusarium verticilloids* (Sacc) or *Fusarium moniliforme* is a wide spread fungus in the large genus F*usarium*, of the liseola section. It is found in humid and sub humid temperate zones, extending into sub-tropical and tropical zones throughout the world. The pathogen is seed borne and seed transmitted (Lee *et al*., 2012)

The top rot or twisted top disease in pearl millet is caused by *Gibberella fujikuroi* (Badoci *et al.,* 2013). Pokkah boeng is one of the most common sugarcane disease (Marasas, *et al*., 2006). It is caused by *Gibberella fujikuroi. Gibberella fujikuroi* can cause Stalk Rot disease in Sorghum (Summerell *et al*., 2011). In mango (*Mangifera indica* L.) *Gibberella fujikuroi* can cause Mango Malformation Disease (MMD) (Tiwari *et al*., 2011). Bakanae is a seedling disease caused by *Gibberella fujikuroi (*Karov *et al.,* 2009).

Besides being pathogens of plants, they also produce plant growth hormone gibberellic acid. It is able to give higher yields of gibberellic acid on a variety of media. Gibberellic acid (GA3), the most important gibberellin, is a class of diterpenoid that functions as plant growth regulator. It affects stem elongation, elimination of dormancy, flowering, sex expression, enzyme induction and leaf and fruit senescence. GA3 is a high valued industrially important biochemical with various applications in agriculture (Karakoc *et al*.,2006).

GA3 is presently produced largely by submerged fermentation techniques using *Fusarium moniliforme* or *Gibberella fujikuroi*. Other bacteria that belong to the genus *Azoto-bacter* and *Azospirillum* also synthesize GA3. Recently, a *Pseudomonas* sp. isolated from wastes of processed olive has also been shown to produce GA3 (285 mg/L). The factors that account for high cost of GA3 in present market scenario are the low yield of GA3 produced and its presence in dilute form in submerged fermentation; leading to higher costs of downstream processing and disposal of waste water. GA3 can also be produced by the solid-state fermentation (SSF), which has got a tremendous potential for production of secondary metabolites (Vidhya, 2012).

Gibberellic acid (GA3) is an important member of the gibberellins family and acts as a natural plant growth hormone, controlling many development processes, which is gaining great attention all over the world due to its effective use in agriculture, nurseries, tissue culture, tea gardens, etc. The cost of GA3 has restricted its use to preclude application for plant growth promotion, except for certain high value plants. Reduction in its production costs could lead to wider applications for a variety of crops (Rodrigues *et al.*, 2009**)**

The results obtained from the present study was discussed as follows,

4.1 Collection of plant samples from different places TamilNadu and Kerala.

4.2 Isolation and maintenance of cultures of *Gibberella fujikuroi* from the collected plant samples.

4.3 Morphological Identification of *Gibberella fujikuroi.*

4.3.1 Colony morphology in different media.

4.3.2 Single spore isolation.

4.3.3 Lacto phenol cotton blue staining.

4.3.4 Slide culture technique.

4.4 Extraction of gibberellic acid by liquid – liquid extraction.

4.5 Qualitative analysis of gibberellic acid by thin layer chromatography.

4.6 Quantitative estimation of gibberellic acid by Spectrophotometric method.

4.1 COLLECTION OF SAMPLES

The Bakanae disease affected plants were collected according to the symptoms described by Karov *et al.*, (2009). The collected plant samples from different places were listed in Table 1.

4.2 ISOLATION AND MAINTENANCE OF *Gibberella fujikuroi* FROM RICE

Five strains of *Gibberella fujikuroi* were isolated from bakanae disease affected rice plants from five different places of TamilNadu, India using Potato Dextrose Agar medium and named as GAB1 to GAB5 (Table 2). Uthandi *et al.*, (2010) isolated *Gibberella fujikuroi* strain such as *Gibberella fujikuroi* SG2, Pradeep *et al* (2013) isolated *F. moniliforme* strain from the rhizosphere soil of paddy field and named as KUMBF1201 and Rahjoo *et al.*, (2008) isolated *Gibberella fujikuroi* strains such as NRRL22172 and FvMM2-4.

The places from which the *Gibberella fujikuroi* strains isolated were listed in the Table 2. Karov *et al.,* (2009) described the method for the isolation of *Gibberella fujikuroi*. Briefly, the plant samples were cut into small pieces, surface sterilized and inoculated into the Potato Dextrose Agar. The isolated strains are maintained on the Potato Dextrose agar slants at 4˚C.

Table 2: Name of the strains isolated from different places

S.No	Strain	Name of the place
1.	GAB 1	Vaikkal medu (Sathyamangalam)
2.	GAB 2	Periyur (Sathyamangalam)
3.	GAB 3	Annur
4.	GAB 4	Chandrapuram (Kerala)
5.	GAB 5	Palakkad (Kerala)

4.3 MORPHOLOGICAL IDENTIFICATION OF *Gibberella fujikuroi*

4.3.1 Culturing in different media:

In this study, four solid media such as Potato Dextrose Agar (PDA), Rose Bengal Agar (RBA), Czapex's Dox Agar (CDA) and Sabouraud's Dextrose Agar (SDA) were used. All the media were prepared according to the manufacturer's instructions (Hi Media, India). Each Petri dish was poured with 20 ml of sterilized medium and allowed for solidification. After inoculation, petri plate was incubated at 23±2°C for 7 days. After incubation period morphology of the each strain was observed and photographed. The fungi grow on artificial medium forming colonies of the different shape, size and color. Size of colonies enhances day by day attaining the full growth at definite period.

3.3.1.1. Morphology of the strains in Potato Dextrose Agar (PDA)

The colony morphology of the strains GAB 1 was observed as pink cottony growth having irregular margin. Pink cottony growth was observed in the strains GAB 2 and GAB 3 as shown in the Plate 3.

The pale white color of the media was changed into orange color due to the production of pigments. GAB 4 and GAB 5 grew as white cottony irregular margin and purple cottony with irregular margin respectively. Purple colony of the strain GAB 5 indicated the production of pigment. The colony morphology of the *Gibberella fujikuroi* strains grown on PDA was presented in Table 3.

Plate 3: Morphology of the strains in PDA

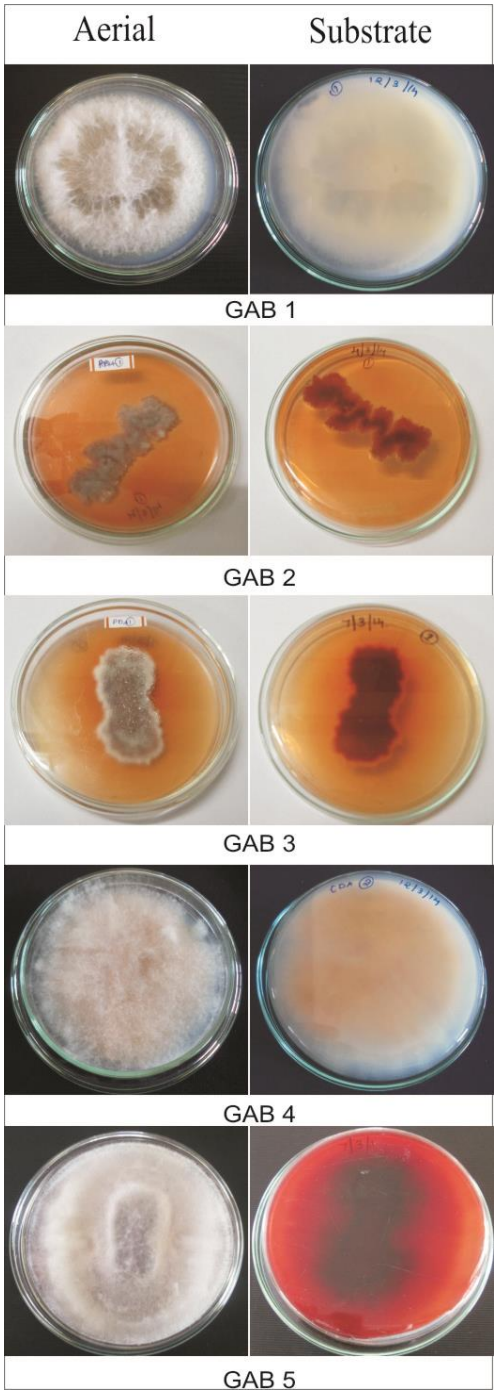


Table 3: Morphology of the isolated strains in Potato dextrose agar

S.No	Strain	Colony morphology
1.	GAB 1	Pink cottony growth with irregular margin was observed
2.	GAB 2	Pink cottony growth was observed and color of the media was changed into Orange due to the pigment production
3.	GAB 3	Pink cottony growth was observed and color of the media was changed into Orange due to the pigment production
4.	GAB 4	White cottony growth with irregular margin was observed
5.	GAB 5	Purple cottony growth with irregular margin was observed

4.3.1.2. Morphology of the strains in Rose Bengal Agar (RBA)

The morphology of the strain GAB 1, GAB 2, GAB 3 and GAB 5 was observed as white cottony with regular margin. Strain GAB 4 has white cottony growth with irregular margin. The colony morphology of the *Gibberella fujikuroi* was shown in Plate 4.

Plate 4: Colony morphology of the isolated strains in RBA

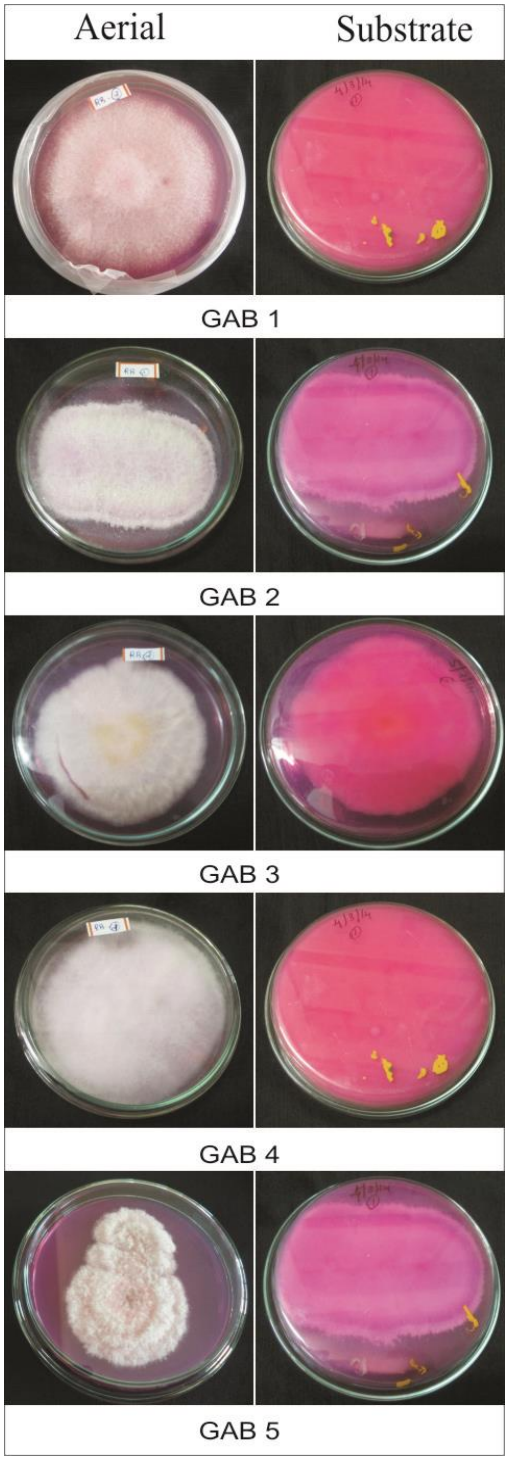


Table 4: Colony morphology of *Gibberella fujikuroi* in RBA

S.No	Strain	Colony morphology
1.	GAB 1	White cottony growth was observed
2.	GAB 2	White cottony growth was observed
3.	GAB 3	White cottony growth was observed
4.	GAB 4	White cottony growth with irregular growth was observed
5.	GAB 5	White cottony growth was observed

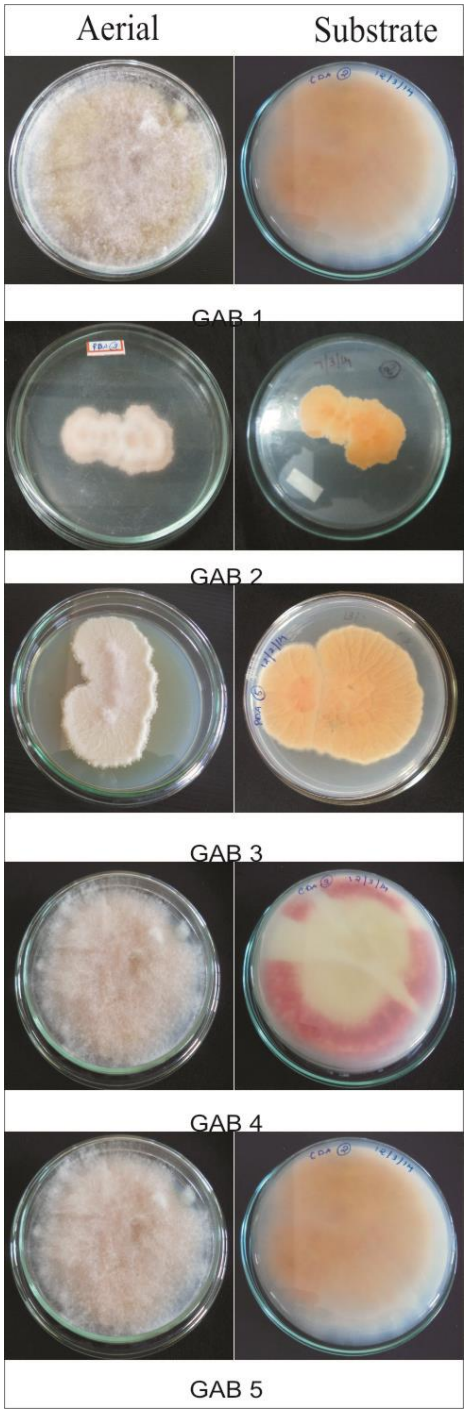
4.3.1.3. Morphology of the strains in Czapek Dox Agar (CDA).

White cottony growth with irregular shape was observed in the strains GAB 1, GAB2 and GAB 5. The colony morphology of the strains GAB 3 and GAB 4 was observed with white cottony growth. With this morphology the isolated strains are identified as *Gibberella fujikuroi*

Table 5: Colony morphology of Gibberella fujikuroi strains in CDA

S.No	Strain	Colony morphology
1.	GAB 1	White cottony growth with irregular shape was observed
2.	GAB 2	White cottony growth with irregular shape was observed
3.	GAB 3	White cottony growth was observed
4.	GAB 4	White cottony growth was observed
5.	GAB 5	White cottony growth with irregular shape was observed

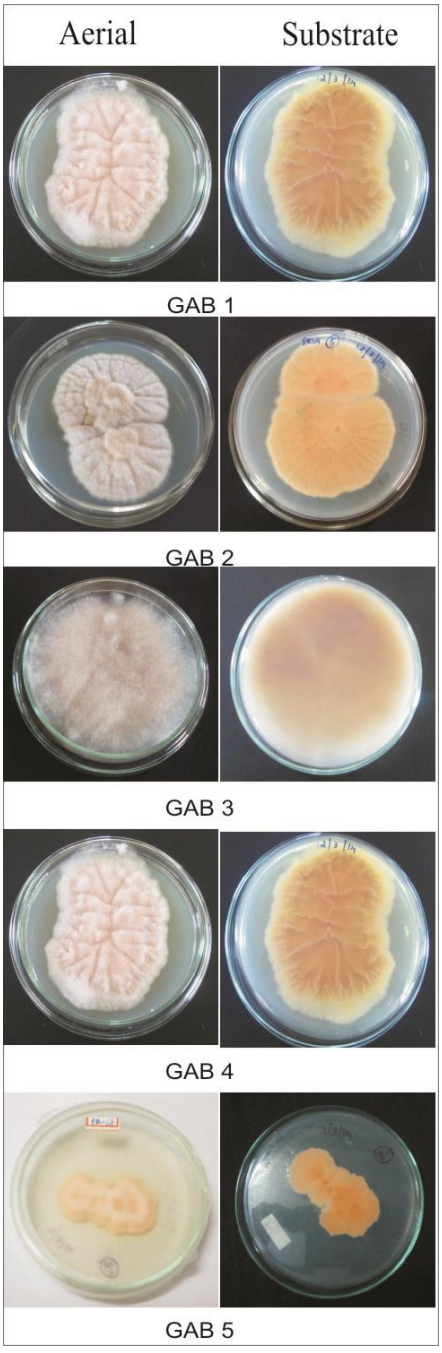
Plate 5: Colony morphology of the *Gibberella fujikuroi* on CDA



4.3.1.4. Morphology of the strains in Sabouraud Dextrose Agar (SDA)

The morphology of the *Gibberella fujikuroi* strains was showed in Plate 6

Plate 6: Colony morphology of *the Gibberella fujikuroi* on SDA



The strains GAB 1, GAB 2, GAB 4 and GAB 5 have pink cottony growth with regular margin but the strain GAB 3 has pink cottony growth with irregular margin. The colony morphology of the *Gibberella fujikuroi* was presented in table 6

Table 6: Colony morphology of the *Gibberella fujikuroi* on SDA

S.No	Strain	Colony morphology
1.	GAB 1	Pink cottony growth, regular margin was observed
2.	GAB 2	Pink cottony growth, regular margin was observed
3.	GAB 3	Pink cottony growth, irregular margin was observed
4.	GAB 4	Pink cottony growth, regular margin was observed
5.	GAB 5	Pink cottony growth, regular margin was observed

Pradeep *et al.,* (2013) reported the colony morphology of *Gibberella fujikuroi* on variety of media (Potato Dextrose Agar, Rose Bengal Agar, Czapek dox agar, Sabouraud Dextrose Agar, Malt Extract Agar, Yeast Malt Agar and Nutrient agar). Gupta *et al.,* (2010) studied the growth characteristics of *Gibberella fujikuroi* in different synthetic media such as Potato Dextrose agar, Czapek Dox Agar and Oat Meal Agar. Our results are agree with Pradeep *et al.,* (2013) and Gupta *et al.,* (2010). With these suggestions we identified the isolated strains are belongs to *Gibberella fujikuroi* species.

Sporulation on different solid media

Sporulation of the isolated strains are also done using lactophenol cotton blue staining observed under light microscope with 40X magnification. Sporulation of the strains on the different media was included in the Table 7.

Table 7: Sporulation on different media

S.No	Strain	Sporulation			
		PDA	RBA	CDA	SDA
1.	GAB 1	++++	+++	++++	++++
2.	GAB 2	+++	+++	++++	++++
3.	GAB 3	+++	++++	+++	++++
4.	GAB 4	++++	++++	+++	+++
5.	GAB 5	++++	+++	++++	++++

+++ - Below 100 spores, ++++ - Above 100 Spores

Strains GAB 1, GAB 2 GAB 3 and GAB 5 produced more than 100 spores, and GAB 3 produced less than 100 spores in Potato Dextrose Agar. In Rose Bengal agar GAB 3 and GAB 4 produce more than 100 spores. Other strains GAB 1, GAB 2 and GAB 5 produces less than 100 spores. The spores produced in Czapek Dox Agar by the strains GAB 1, GAB 2 and GAB 4 are more than 100 where as the strains GAB 3 and GAB 5 produce less spores. All isolates produce more than 100 spores except GAB 4 in Sabouraud Dextrose Agar. Pradeep *et al* (2013) reported the sporulation of *Gibberella fujikuroi* on different media.

4.3.2 Single spore isolation technique

The culture was transferred into Spezieiller Nährstoffarmer Agar (SNA). The SNA plates were incubated at 23˚C for 10 days. A very small amount of sporulating mycelia was smeared on to 2% water agar plates. The plates were incubated for 16 hours (Nasr *et al.*, 2014)

4.3.3 Lactophenol cotton blue staining

After isolating single spore the morphological identification was done by observing under the light microscope using lactophenol cotton blue (LPCB) staining.

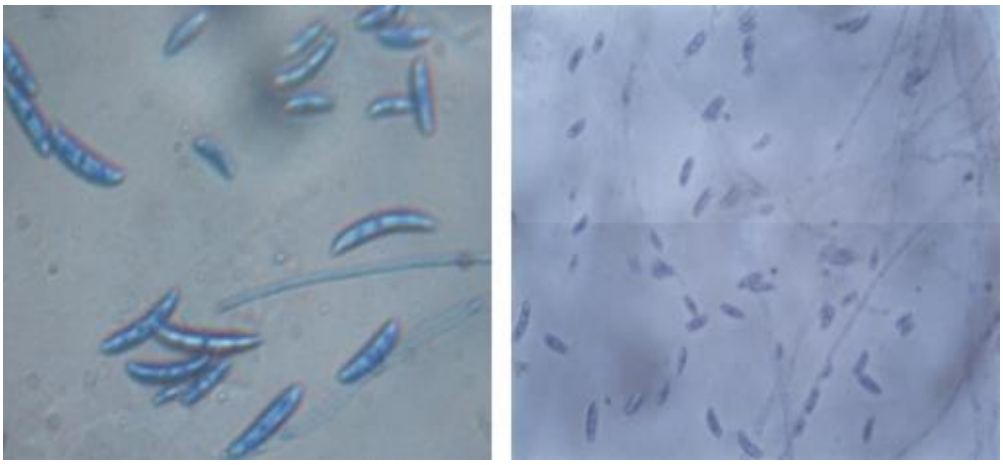
4.3.4 Cavity slide culture technique

The conventional agar block slide culture method does not allow staining to visualize structures in the agar block using lactophenol cotton blue. Only the imprinted material on the cover slide could be stained and visualized. The cavity slide cultures allowed the visualization of structure within the agar block after staining.

This cavity slide culture technique was conducted by the method described by Wijedasa *et al.*, (2012). Briefly, an aliquot of the Potato Dextrose agar was prepared and sterilized and pipetted into the cavity of the cavity slide with a sterile pasteur pipette and allowed to set then each fungal isolate to be identified was inoculated onto the SNA. The filter paper was moistened with sterile distilled water to humidify the culture environment and the Petri dishes were incubated at room temperature (Wijedasa *et al.*, 2012).

Using lactophenol cotton blue staining by single spore isolation technique in cavity slides under light microscope reveals the morphology of *Gibberella fujikuroi*.The fungus has micro and macro conidiophores bearing micro and macro conidia, respectively Microconidiophores are single, lateral and formed from hyphae. Microconidia are one celled, ovoid or oblong, borne singly in false head on laterally borne conidiophores. Some conidia are intermediate, with two or three cells, oblong or slightly curved.

Plate 7: **Shape of macro and microspores produced by *Gibberella fujikuroi***



Macroconidiophores consist of a basal cell bearing 2—3 apical phialides which produce macroconidia. Macroconidia are multi - celled (3 to 5septate). The macroconidia are delicate, slightly sickle shaped or almost straight. They are narrow at both the ends and are occasionally somewhat bent into a hook at the apex and distinctly or slightly foot celled at the base. The microconidia formed in false heads in *Gibberella fujikuroi* was showed in the Plate 8

Plate 8 : Microconidia formed in false heads

Plate 8 shows that the formation micro conidia in false heads

Summerell *et al.,* (2003), suggested the structure of *Gibberella fujikuroi* and Karov *et al* (2009) reported the structure of *Gibberella fujikuroi* isolated from rice. Nasr *et al.*,

(2014) described the method of cavity slide culture technique and studied the micro and macro conidia of the *Gibberella fujikuroi.* According to Summerell *et al* (2003), Karov *et al.,* (2009) and Nasr *et al.,* (2014) with the morphology of the strains described above we identified the isolated strains are *Gibberella fujikuroi*

4.4 EXTRACTION OF GIBBERELLIC ACID

4.4.1 Culture media and Growth conditions

Gibberella fujikuroi was grown in 100 ml of Czapez Dox Broth (CDB) medium pH at 7.0 and incubated the culture for 8 days at 30˚C at 120 rpm (Vidya Ranagasamy 2012).

4.4.2 Extraction of gibberellic acid

After the incubation period was completed the culture was centrifuged at 3000 rpm for 10 minutes. The pellet was stored to measure the wet weight and dry weight of the mycelia. Then the filtrate was acidified to pH 2.5. Gibberellic acid was extracted using liquid – liquid extraction by adding 20ml of ethyl acetate. The aqeous phase was discarded and the organic phase was collected and evaporated. The residue was dissolved in water containing 0.05% of Tween 20 (Berrios *et al.,* 2004).

4.4.2.1. Wet weight and dry weight of the sample:

After centrifugation the pellet was collected and the wet weight of the samples were taken. After taking wet weight of the sample the pellet was kept in hot air oven and dried. Then the dry weight of the mycelia was taken. The wet weight and the dry weight of the 7 strains were included in Table 8.

Table 8: Wet weight and dry weight of the mycelia

S.No	Strain	Wet weight of the mycelia (g/100ml)	Dry weight of the mycelia (g/100ml)
1.	GAB 1	1.8	0.04
2.	GAB 2	11	0.7
3.	GAB 3	14.65	1.2
5.	GAB 5	11	0.8
6.	GAB 6	1.2	0.023

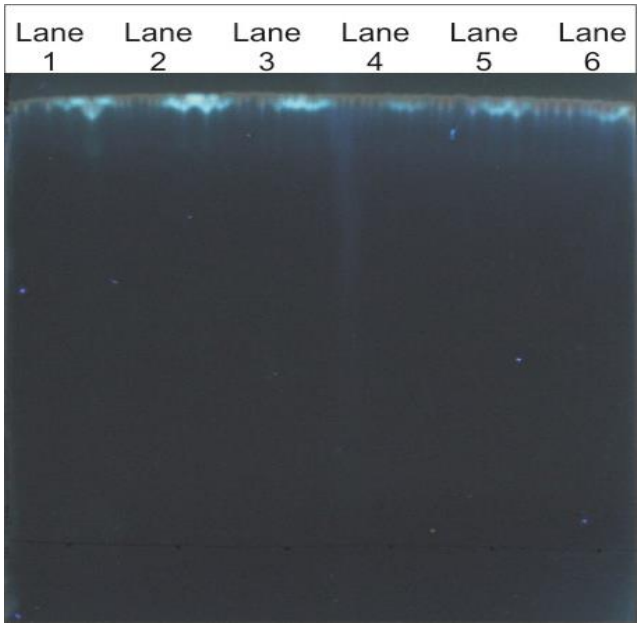
4.5 QUALITATIVE ANALYSIS OF GIBBERELLIC ACID BY THIN LAYER CHROMATOGRAPHY

TLC plates were prepared. Spotted 10µl of samples and chromatogram was developed were run up to 4 hours in isopropanol : Ammonia: Water in the ratio of 10:1:1 as a mobile phase. Then the plates were removed and derivatized with Ethanol : Concentrated sulphuric acid (95:5) (Zainudin *et al.*, 2008) . Then the plates were heated at 80˚C for 10 minutes in hot air oven and allowed to cool. Examine the plates under UV – Light (275 nm) for spots.

Usually the gibberellic acids are invisible under white light (Plate 9). In UV – light the spots are visible as greenish florescence spots revealed that the presence of gibberellic acid in the extract. The greenish florescence spots are indicated in Plate 10

Plate 9: Qualitative analysis of gibberellic acid in TLC plate under white light

Plate 10: Qualitative analysis of gibberellic acid in TLC under UV light



Lane1: Standard gibberellic acid

Lane2: GAB 1

Lane 3: GAB 2

Lane 4: GAB 3

Lane 5: GAB 4

Lane 6: GAB 5

4.6 QUANTITATIVE ESTIMATION OF GIBBERELLIC ACID (Julio Berrios *et al.*, 2004)

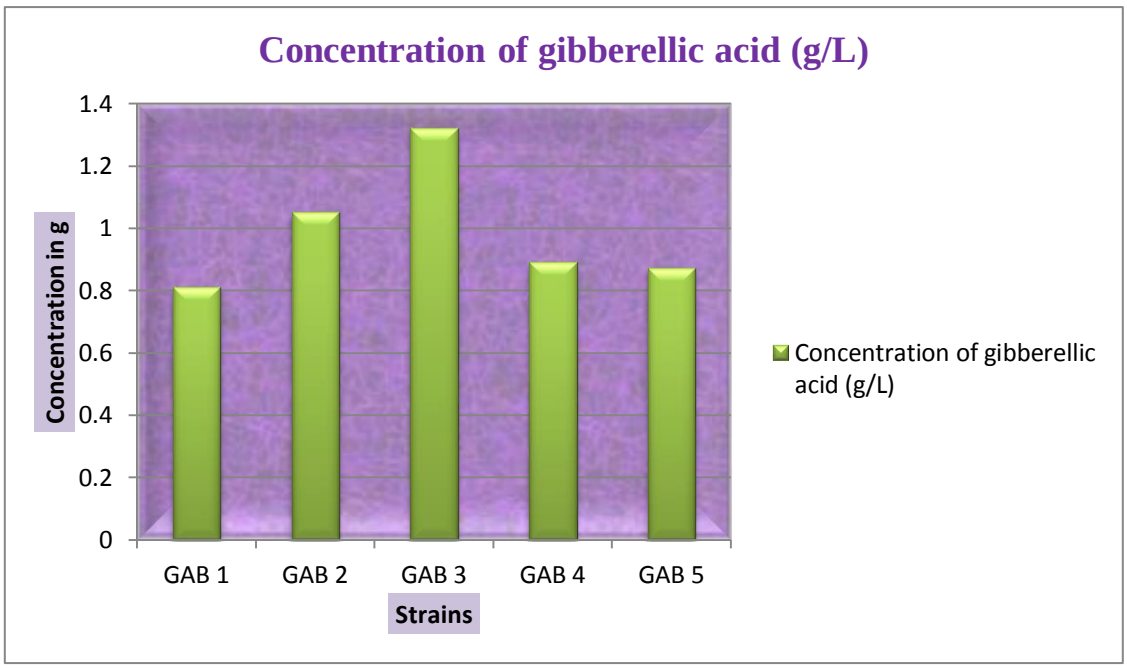
A sample of 1 ml and 1 ml absolute ethanol were placed in a 10 ml volumetric flask. HCl, 3.75 M, was added to the flask up to make 10 ml and then vigorously mixed for 10 s. Incubated the tubes for one hour at room temperature. The absorbancy of the resulting solution was measured at 254 nm and recorded. Temperature was kept at 20 ± 0.5 ◦C during the process.

Out of 5 strains isolated from the rice plant the maximum yield was obtained by strains GAB 2 and GAB 3 concentration of the gibberellic acid present in these strains are 1.05g/L and 1.32g /L respectively. Concentration of gibberellic acid produced by 5 strains of *Gibberella fujikuroi* shown in Table 9

Table 9: Concentration of gibberellic acid in 5 strains of *Gibberella fujikuroi*

S.No	Name of the Strain	Concentration of gibberellic acid (g/L)
1.	GAB 1	0.812
2.	GAB 2	1.05
3.	GAB 3	1.32
5.	GAB 4	0.89
6.	GAB 5	0.873

Figure 1: Concentration of gibberellic acid



Hollmann *et al.*, (1995) reported that 520mg /L of gibberellic acid was produced by *Gibberella fujikuroi.* Escamilla *et al.*, (2000) have reported that 2.862g/L of gibberellic acid was produced by *Gibberella fujikuroi*. Shukla *et al.*, (2005) reported that the amount of gibberellic acid produced by the Gibberella fujikuroi was 1g/L in used glucose and rice meal

as a substrate. Vidhya Rangasamy (2012) reported the production of gibberellic acid in Czapek Dox broth (CDB) by *Gibberella fujikuroi,* 5.8g/L of gibberellic acid was produced using CDB. According to Vidhya Rangasamy the production was less. Our findings revealed that the less production of gibberellic acid by isolated strains of *Gibberella fujikuroi* may be due different climatic conditions, type of soil, type of nutrtion availability.

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCLUSION

Many plant diseases are caused by pathogens, which are difficult to see or recognize without magnification. Fungi, bacteria, viruses, nematodes, and even plants can be pathogenic. Among all the pathogens the fungi and fungi like organisms cause most of the plant diseases. By causing diseases in plants they can affect the crop production this will leads to socioeconomic problems in world wide. *Gibberella* sp. are the seed borne fungi this can cause plant diseases due to the production of some secondary metabolites such as fumonisin, fusaric acid and gibberellic acid. Due to the production of fusaric acid and gibberellic acid this filamentous fungi can cause bakanae disease in rice plant. Eventhough it is a pathogen it can produce the plant hormone gibberellic acid. This plant hormone has a wide range of applications in the fields of agriculture and horticulture.

The present study entitled "**Isolation and characterization of *Gibberella fujikuroi* from different Biospheres**" was conducted with the following objectives.

- Isolation and maintenance of *Gibberella fujikuroi* strains.
- Identification of *Gibberella fujikuroi* using macroscopic and microscopic Morphological Characters
- Extraction of gibberellic acid from *Gibberella fujikuroi*
- Detection of gibberellic acid produced by *Gibberella fujikuroi* by TLC
- Screening of elite gibberellic acid produced strain by spectrophotometric analysis

The results obtained from the present study were summarized as follows:

a. 5 strains of *Gibberella fujikuroi* were isolated from different biospheres and maintained in Potato Dextrose Agar.
b. The structure of macrospore and microspore seen under light microscope suggested the isolated strains as *Gibberella fujikuroi*
c. Gibberellic acid was extracted from the 5 isolated strains.
d. The greenish florescent spots in Thin layer Chromatography Plates under the UV light revealed that presence of gibberelllic acid.

e. Screening of elite gibberellic acid produced strain showed that GAB 2 (1.05g/L) and GAB 3 (1.32g/L) have maximum production of gibberellic acid than the other strains.

From the present study, it can be concluded that the *Gibberella fujikuroi* isolates GAB 2 (1.05g/L) and GAB 3 (1.32g/L) showed higher gibberellic acid producers among 5 isolates. This may be due to climatic condition and type of soil from which they isolated.

Suggestions for future study:

1. Media can be standardized to get high yield of gibberellic acid
2. Commercial production of gibbberellic acid can be done.
3. Media can be standardized for the production of other secondary metabolites such as carotenoids and bikaverin by Gibberella fujikuroi
4. Carotenoids and bikaverin can be commercially produced using Gibberella fujikuroi

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APPENDICES

APPENDIX I

ISOLATION AND MAINTENANCE OF *Gibberella fujikuroi* FROM RICE (*Oryza sativa)* (Karov *et al.*, 2009)

Materials Required:

1. 1% Sodium Hypochlorite
2. Potato dextrose agar
3. 70% alcohol

Procedure:

1. The stem and leaves of the collected plant sample were cut into small pieces (about $1cm^2$)
2. Surface sterilized for 3 minutes with 1% sodium hypochlorite or 0.1% mercuric chloride.
3. Then rinsed 3 times with double distilled water.
4. After washing inoculate the samples into Potato dextrose agar.
5. Incubate the plates for 5 – 7 days at 24˚C.
6. Maintained the isolates on Potato dextrose agar at 4˚C.

APPENDIX II

MORPHOLOGICAL IDENTIFICATION OF *Gibberella fujikuroi* (Rajhoo *et al.*, 2008)

Culturing in different media

Materials required:

1. Potato dextrose agar
2. Saboraud dextrose agar
3. Czapek dox agar
4. Rose Bengal agar
5. Inoculation loop
6. 70% alcohol

Procedure:

1. All the media were prepared and autoclaved
2. A loop full of culture was inoculated in the respective plates containing diiferent media
3. The plates were incubated at 23˚C for 7 days.

Single spore isolation Technique

Materials required:

1. Spezieiller Nährstoffarmer Agar or SNA
   Composed of (g/L) KH_2PO_4 – 1g, KNO_3 -1g, $MgSO_4.7H_2O$ – 0.5g, KCl – 0.5g Glucose – 0.2g, Sucrose – 0.2g, Agar – 20g.
2. Agar
3. 70% Alcohol
4. Inoculation loop
5.

Procedure:

1. SNA agar was prepared and autoclaved.
2. Poured 20ml of medium on to sterilized petri plates.

3. A loop full of culture to be identified was inoculated on petri plates containing SNA medium.
4. Incubated the tubes at 23˚C for 10 days
5. After incubation period the culture was smeared on to 2% water agar plates
6. Incubate the tubes for 16 hours at room temperature.

Lactophenol cotton blue staining

Materials required:

1. Lactophenol cotton blue
2. Sterilized needle
3. Slides
4. Cover slips
5. 70% alcohol

Procedure

1. The glass slides were wiped with 70% alcohol allowed to dry
2. The culture was transferred on to slides with the help sterilized needle
3. Cover the culture cover slip
4. A drop of Lactophenol cotton blue was added to the slides
5. Observed under light microscope

Slide culture technique (Wijedasa *et al.*, 2012).

Materials required:

1. Cavity slides
2. Petridishes
3. Sabouraud dextrose agar
4. Inoculation loop

Procedure

1. Packs of petri dishes, each containing a cavity slide, cover slip and a filter paper slip were sterilized.
2. Sabouraud Dextrose agar (SDA) was prepared according to manufacturer's guidelines, aliquoted in to small containers and sterilized.
3. An aliquot of the SDA was melted by heating and pipetted into the cavity of the cavity slide with a sterile pasteur pipette and allowed to set for 5 minutes.
4. Each fungal isolate to be identified was inoculated onto the cavity slides containing SDA using a sterile straight wire and a sterile cover slip was placed on top of it.
5. The filter paper was moistened with sterile distilled water to humidify the culture environment and the Petri dishes were incubated at room temperature

APPENDIX III

EXTRACTION OF GIBBERELLIC ACID

Culture media and Growth conditions (Vidya Rangasamy 2012)

Materials required:

1. Czapek Dox Broth
   - Composed of (g/L) Sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and $FeSO_4$, 0.01. pH 7.0
2. Inoculation loop
3. 70% alcohol

Procedure:

1. The media was prepared and sterilized
2. A loop full of culture was inoculated on to flasks containing medium.
3. Incubated the culture at 30˚C for 8 days at 120 rpm.

Extraction of gibberellic acid (Berrios *et al.*, 2004)

Materials required:

1. Centifuge tubes
2. Separating funnel
3. Ethyl acetate
4. 10N Sulphuric acid
5. 0.05 Tween 20

Procedure:

1. After the incubation period was completed the culture was centrifuged at 3000 rpm for 10 minutes.
2. The pellet was retained to weigh wet weight and dry weight of the mycelia. The filtrate was then acidified to pH 2.5.
3. Then the filtrate was treated with 20ml of ethyl acetate in separatory funnel, shaked vigorously for 60 seconds.
4. The excess of ethyl acetate (aqueous phase) was discarded from the funnel and allowed to dry by exposing to air.
5. The extract was retransferred to a separating funnel and retreated with equal volume of ethyl acetate.
6. The solvent fraction (organic phase) was collected and allowed to dry by exposing to air.
7. This process was repeated for 3 times to get a large amount of fine quality of gibberellic acid.

8. The concentrated solution was reevaporated and the residue was dissolved in water containing 0.05% of Tween 20.

Wet weight and dry weight of the sample:

Materials required

1. Watch glasses
2. Spatula

Procedure:

1. After centrifugation the pellet was collected and the wet weight of the samples were taken.
2. After taking wet weight of the sample the pellet was kept in hot air oven and dried.
3. Then the dry weight of the mycelia was taken.

APPENDIX IV

QUALITATIVE ANALYSIS OF GIBBERELLIC ACID BY THIN LAYER CHROMATOGRAPHY (Zainudin *et al.*, 2008)

Materials required:

1. Silica coated plate
2. Solvent phase (Isopropanol:Ammonia:Water at 10:1:1 ratio)
3. Spraying reagent (Ethanol: Concentrated Sulphuric acid (95:5))

Procedure:

1. TLC plates were prepared.
2. The chromatogram was developed and spotted 10µl of samples
3. Run up to 4 hours in the solvent system

4. Plates were removed and dried at room temperature
5. Sprayed with spraying reagent.
6. The plates were heated at 80˚C for 10 minutes in hot air oven.
7. Then the plates were allowed to cool and examine under UV – Light (275 nm) for floresecence greenish spots.

APPENDIX V

QUANTITATIVE ESTIMATION OF GIBBERELLIC ACID (Berrios *et al.*, 2004)

Materials required:

1. Ethanol
2. 3.75 M Hydrochloric acid
3. Standard solution
   - 0.04 g of pure GA3 was dissolved 100ml of absolute alcohol

Procedure

1. A sample of 1 ml and 1 ml absolute ethanol were placed in a test tube.
2. 3.75 M Hydrochloric acid was added to the flask up to make 10 ml and then vigorously mixed for 10 s.
3. The tubes were kept one hour at room temperature.
4. After incubation period the absorbancy of the resulting solution was measured at 254 nm and recorded.
5. Temperature was kept at 20 ± 0.5˚C during the process.
6. Regression value was obtained with the help of standard values to calculate the concentration of the gibberellic acid present in the sample.