Induction of Host Defence through Biocontrol agents in *Sorghum bicolor* Moench against *Gloeocercospora sorghi* Bain & Edg.

Thesis

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G.B. PANT UNIVERSITY OF AGRICULTURE & TECHNOLOGY,
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By

GAURAV VERMA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

(PLANT PATHOLOGY)

AUGUST, 2014

CERTIFICATE

This is to certify that the thesis entitled **"Induction of Host Defence through Biocontrol agents in *Sorghum bicolor* Moench against *Gloeocercospora sorghi* Bain & Edg."** submitted in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** with major in **PLANT PATHOLOGY** and minor in **ENTOMOLOGY** of the College of Post Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bona-fide* research carried out by **Mr. Gaurav Verma, Id. No. 40932**, under my guidelines, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been duly acknowledged.

Pantnagar
August, 2014

(Yogendra Singh)
Chairman
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CERTIFICATE

We, the undersigned, members of the advisory committee of **Mr. Gaurav Verma**, **Id. No. 40932**, a candidate for the degree of **DOCTOR OF PHILOSOPHY** with major in **PLANT PATHOLOGY** and minor in **ENTOMOLOGY** agree that the thesis entitled **"Induction of Host Defence through Biocontrol agents in *Sorghum bicolor* Moench against *Gloeocercospora sorghi* Bain & Edg."** may be submitted in partial fulfillment of the requirements for the degree.

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% = per cent,

°C = degree Celsius,

µ = micro,

µl = microliter,

µM = micromole

AM = arbuscular mycorrhiza,

bp = base pair,

cDNA = complementary deoxyribonucleic acid,

CHI = chitinase,

CHS = chalcone synthase,

cm = centimeter,

CRD = complete randomized design,

Ct = crossing threshold,

cv. = cultivars,

DAS = days after sowing,

GIDR = Gloeocercospora induced defence response,

gm = gram,

ha = hectare,

HPI= hours post inoculation,

HPT = hours post treatment,

hr = hour,

ISR = induced systemic resistance,

kg = kilogram,

L = liter,

m = meter,

mg = milligram,

ml = milliliter,

mm = millimeter,

N = nitrogen,

P = phosphorus,

PAL = phenylalanine ammonia-lyase,

PC-4 = Pant chari-4,

PC-5 = Pant chari-5,

Pf = *Pseudomonas fluorescens,*

pH = *potenz* of hydrogen ion,

psi = pounds per square inch,

RBD = randomized block design,

RH = relative humidity,

RNA = ribonucleic acid,

rpm = revolution per minute,

RT-PCR = real time polymerase chain reaction,

s = second,

SAR = systemic acquired resistance,

TH = *Trichoderma harzianum,*

VI = vigour index,

INTRODUCTION

CHAPTER 1 INTRODUCTION

Sorghum (*Sorghum bicolor* Moench), a grain crop originating from Africa is grown worldwide for both food and forage **(Doggett 1988)**. Sorghums have also long been cultivated in China, with records possibly dating back to the 3rd century A.D., and over time many landraces and varieties developed or were bred **(Qingshan and Dahlberg, 2001)**.

The genus Sorghum belongs to the tribe Andropogoneae of the family Poaceae and includes both wild species and species cultivated for their grain, fodder, syrup and other commercial purposes. There are several types of sorghum including grain sorghum, grass sorghum (for pasture and hay), sweet sorghum (for syrup) and broom corn. Among known species the genus, *Sorghum bicolor* (L) Moench is important. Common names of sorghum in different countries are sorghum (United States, Australia), Durra (Africa), Jowar (India) and Bachanta (Ethiopia). They are sensitive to day length or photoperiod and need consistent day length of up to 12 hours to trigger an internal mechanism to initiate reproductive growth, first flowers and later seeds. Sorghum is a self-pollinated diploid (2n=2x=20) C4 grass with a high photosynthetic efficiency. Its small genome size (730 Mbp, about 25% the size of maize or sugarcane) is fully sequenced and makes sorghum an attractive model for functional genomics of C4 grasses. Sorghum is one among the few resilient crops that can adapt well to future climate change conditions, particularly the increasing drought, soil salinity and high temperatures **(ICRISAT, 2014)**.

Sorghum is cultivated widely throughout the arid, semi arid tropics and temperate regions within the latitude of 45^{o} N to 45^{o}S. Its peculiar quality of withstanding drought makes it a potential alternative in dryland and rainfed conditions in semi-arid tropics (SAT) **(Ross and Webster, 1970)**. India ranks first in acreage among the sorghum growing countries and second in production with an area of about 9.49 mha, production of 7.78 mt and productivity of about 981 kg/ha **(Rao *et al.*, 2008)**. The sorghum area in India was more than 16 million ha in 1981, but has gradually decreased to 7.8 million ha in 2007-08 (still 20% of the world's sorghum area). In India, it is grown successfully in areas having average rainfall between 500 and 1000 mm with the temperature requirement of 25-30^{o}C. It is grown as *Kharif* crop in North India while in western and southern part of the country it is also grown as *Rabi* crop. More than 60% of the total sorghum is raised as rainfed crop in *Kharif* season (June to Oct.) in *Rabi* (Oct. to Feb.) and summer season,

depending upon the weather conditions. Of this, 3.5 million ha was grown in the rainy (*kharif*) season and 4.3 million ha in the postrainy (*rabi*) season. Production increased from 9 million tons in the early 1970s to 12 million tons in the early 1980s and maintained this level for over a decade until the early 1990s, followed by a steep decline to 7.3 million tons. Despite the decrease in area over the years, production has been sustained at 7.3 million tons (2009) due mainly to adoption of improved varieties and hybrids **(ICRISAT, 2014)**. The major states in the country where this cereal grain is produced are Maharashtra, Karnataka, Gujarat, Madhya Pradesh, Andhra Pradesh, Rajasthan and Uttar Pradesh. Maharashtra produces the maximum sorghum in India.

Sorghum grain contains 11.3% protein, 3.3% fat and 56–73% starch. It is relatively rich in iron, zinc, phosphorus and B-complex vitamins. Tannins, found particularly in red-grained types, contain antioxidants that protect against cell damage, a major cause of diseases and aging. Sorghum starch is gluten-free, making sorghum a good alternative to wheat flour for individuals suffering from celiac disease. Sorghum is also used for production of ethanol, starch, adhesives and paper other than being used as food and feed. In developed countries sorghum is mainly used for animal feed. Recent work has shown that sorghum and millet (*Pennisetum glaucum* (L.) R. Br) are rich in antioxidants and gluten-free, which make them an attractive alternative for wheat allergy sufferers **(Dahlbert *et al*., 2004).**

Recently interest in utilization of sweet sorghum for ethanol production has increased in India due to its four times less growing period (4 months) and water requirement (8000 m^3 over two crops) and three times less cost of cultivation than those of sugarcane **(Soltani and Almodares, 1994; Dayakar Rao *et al.,* 2004)**. Also the sweet sorghum is best suited for ethanol as its juice has higher total reducing sugar content compared to sugarcane juice with 90 per cent fermentation efficiency **(Ratnavathi *et al.,* 2004; Huligol *et al.,* 2004)**. The more important is that the ethanol production from sweet sorghum and its use as fuel is environment friendly, as it is of clean burning nature, rating high octane compared to compressed natural gas **(Arbatti, 2001)**.Sorghum plants are known to contain cyanogenic glucoside dhurrin, a group of nitrogenous secondary compounds, which during enzymatic hydrolysis release hydrocyanic acid (HCN), glucose and p-hydroxy benzaldehyde. Cyanogenic glucoside in young sorghum plants accumulate mainly in leaves and at maturity is fairly evenly distributed between leaves and stem

(Morton, 1981; Kim and Voigtlaender, 1985). It was found that HCN content at initial stages of the plant growth may play an important role in the resistant reaction in leaf blight diseases of sorghum **(Mohan and Lakshman, 1987).**

Being cultivated in a range of environments, sorghum is constantly challenged by range of plant pathogens, especially the foliar pathogens. Numerous diseases have been reported in sorghum such as charcoal rot, fusarium root and stalk rot, rough leaf spot, downy mildew, sorghum red stripe and anthracnose **(Tarr, 1962)**. Zonate leaf spot caused by *Gloeocercospora sorghi* is emerging as one of the destructive foliar disease in India **(Bain and Edgerton, 1943).** It is one of the most important disease of crop in humid tropical areas especially in Tarai region of the Uttarakhand, which has been identified as a hot spot for this disease. The disease was first reported from Louisiana State, USA by **Bain (1941)** and was described in detail by **Bain and Edgerton (1943)**. It occurs everywhere in the world where sorghum is grown and the various cultivars presently grown in our country show varying degree of susceptibility to the disease. The rate of infection of this disease is directly correlated with plant density, more the density, more is the infection.

It is very difficult to eliminate any pathogen completely from its host, but time to time many management strategies have been worked out. Sometimes and in certain areas the damages caused by this disease is so serious that it requires fungicidal protection to get considerable yields. Pesticides are necessary at present, but are not the long term solution to crop health. Besides their non- target effects and hazardous nature, many of them are now losing their effectiveness because of development of resistant strains. Moreover, the application of chemicals to manage diseases of sorghum, which is an important fodder crop and is fed to the cattle, is to be avoided. Breeding for resistance, which continues to be the most practical and feasible method to control plant diseases is not able to keep pace with the development of more virulent pathogens. Considering the hazardous nature and development of resistance to chemicals, the increased emphasis on use of biological means for the management of plant diseases is justified.

Biological control and its abbreviated synonym “biocontrol” can be achieved either through introduction of biocontrol agent (BCA) directly or by adopting practices which favour build–up of BCA(s) under natural conditions. Several fungal and bacterial biocontrol agents have been used for achieving disease control of various plant species. Biological control can result from many different types of interactions between organisms,

researchers have focused on characterizing the mechanisms operating in different experimental situations. In all cases, pathogens are antagonized by the presence and activities of other organisms that they encounter. Direct antagonism results from physical contact and/or a high-degree of selectivity for the pathogen by the mechanism(s) expressed by the BCA(s). In such a scheme, hyperparasitism by obligate parasites of a plant pathogen would be considered the most direct type of antagonism because the activities of no other organism would be required to exert a suppressive effect. In contrast, indirect antagonisms result from activities that do not involve sensing or targeting a pathogen by the BCA(s). Stimulation of plant host defense pathways by non-pathogenic BCAs is the most indirect form of antagonism.

Plants actively respond to a variety of environmental stimuli, including gravity, light, temperature, physical stress, water and nutrient availability. Plants also respond to a variety of chemical stimuli produced by soil- and plant-associated microbes. Such stimuli can either induce or condition plant host defenses through biochemical changes that enhance resistance against subsequent infection by a variety of pathogens. Induction of host defenses can be local and/or systemic in nature, depending on the type, source, and amount of stimuli. Recently, phytopathologists have begun to characterize the determinants and pathways of induced resistance stimulated by biological control agents and other non-pathogenic microbes. The first of these pathways, termed systemic acquired resistance (SAR), is mediated by salicylic acid (SA), a compound which is frequently produced following pathogen infection and typically leads to the expression of pathogenesis-related (PR) proteins. These PR proteins include a variety of enzymes some of which may act directly to lyse invading cells, reinforce cell wall boundaries to resist infections, or induce localized cell death. A second phenotype, first referred to as induced systemic resistance (ISR), is mediated by jasmonic acid (JA) and/or ethylene, which are produced following applications of some nonpathogenic rhizobacteria. Interestingly, the SA- and JA- dependent defense pathways can be mutually antagonistic, and some bacterial pathogens take advantage of this to overcome the SAR. For example, pathogenic strains of *Pseudomonas syringae* produce coronatine, which is similar to JA, to overcome the SA-mediated pathway **(He *et al.,* 2004)**. Because the various host-resistance pathways can be activated to varying degrees by different microbes and insect feeding, it is plausible that multiple stimuli are constantly being received and processed by the plant. Thus, the magnitude and duration of host defense induction will likely vary over time. Only if

induction can be controlled, i.e. by overwhelming or synergistically interacting with endogenous signals, will host resistance be increased.

A number of strains of root-colonizing microbes have been identified as potential elicitors of plant host defenses. Some biocontrol strains of *Pseudomonas* sp. and *Trichoderma* sp. are known to strongly induce plant host defenses **(Haas and Defago 2005, Harman 2004)**. In several instances, inoculations with plant-growth-promoting rhizobacteria (PGPR) were effective in controlling multiple diseases caused by different pathogens, including anthracnose (*Colletotrichum lagenarium*), angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*) and bacterial wilt (*Erwinia tracheiphila*). A number of chemical elicitors of SAR and ISR may be produced by the PGPR strains upon inoculation, including salicylic acid, siderophore, lipopolysaccharides, and 2,3-butanediol, and other volatile substances **(Van Loon *et al.,* 1998; Ryu *et al.,* 2004)**. Some events similar to those found in plant–pathogen interactions have been also found in the plant interaction with AMF. These events include signal perception, signal transduction and defence gene activation. Salzer and Boller suggest that AM fungi secrete chitin elicitors, which could induce a defence response **(Salzer and Boller, 2000)**. For instance, the elicitor derived from an extract of extraradical mycelium of *Glomus intraradices* was able to induce phytoalexin synthesis in soybean cotyledons **(Lambais, 2000)**. Interestingly, recent results show that *G. intraradices* induces the expression of a chalcone synthase, the first enzyme in the metabolism of flavonoid compound, such as phytoalexin, in *Medicago truncatula* **(Bonanomi *et al*., 2001)**.

Considering the importance of the crop in the national economy as important grain and fodder crop, the destructive nature of this disease and lack of information on biocontrol of the pathogen *Gloeocercospora sorghi,* it is imperative to work out the suitable management strategies against the disease.

Therefore, the present study is aimed at following objectives:

1. Isolation, purification and pathogenecity test of *Gloeocercospora sorghi.*
2. Screening of *Glomus intraradices* and isolates of *Trichoderma harzianum* and *Pseudomonas fluorescens* against *G. sorghi* under *in vitro* and glasshouse conditions.
3. Evaluation of the effect of *Glomus intraradices* and selected isolates of *Trichoderma harzianum* and *Pseudomonas fluorescens* on growth promotion,

disease reduction, chlorophyll content and green fodder yield of *Sorghum bicolor* against *G. sorghi* under field condition.

4. Determination of the expression of defense response genes during priming and boosting (post inoculation) through *Glomus intraradices*, *Trichoderma harzianum* and *Pseudomonas fluorescens* using real time-RT-PCR.

REVIEW OF LITERATURE

A perusal of literature on diseases of sorghum showed that zonate leaf spot is one of the major constraints in the Tarai region of Uttarakhand. The following account summarizes present knowledge on the disease about symptoms, pathogens, their interactions, the effect of bioagents and their mechanisms in disease suppression as well as plant growth promotion.

2.1 Historical background and nomenclature

A leaf spot was noted on the leaves of several varieties of sweet sorghum (*Sorghum vulgare* Pers.) growing on the experimental station of Louisiana State University, USA in the year 1940 **(Bain, 1941 a,b)**. Entire leaves of most of the plants were covered with large and somewhat zonate spots, and in these spots were numerous black sclerotia like bodies. It resembled in many ways a fungus that Miura described from Manchuria in year 1921 under the name *Ramulispora andropogonis* Miura. Later in 1932, the name was changed to *Titaeospora andropogonis* (Miura) by **Tai (1932)**. The genus *Titaeospora*, however is characterized by the presence of branched conidia. Although, the conidia of sorghum fungus are definitely not branched, there was a possibility that the branching of the spore was not a fixed character and occurred only under certain conditions. Because of the apparent similarity of other characters, the sorghum fungus was then tentatively considered the same as Manchuria fungus **(Bain 1941 a,b)**. This was however proved to be wrong later **(Bain and Edgerton, 1942)**.

In the summer of 1941, a fungus, definitely determined as *Andropogonis titaeospora,* was collected on Johnson grass [*Sorghum halepense* (L.) Pers.]. This fungus bore the typical branched spores, as described for *Titaeospora*, and was shown to be different from the sorghum fungus. Thus it seemed clearly that the sorghum fungus was not only a new species but also did not fit satisfactorily in any form of the commonly known genera of the fungi imperfecti.

In the fall of year 1941, the culture of the fungus was sent to C.L. Shear for identification. He tentatively gave the name *Gloeocercospora heterospora* new genera and species. Based on the study of conidia from single spore cultures and from the host, it was evident that the difference in the conidia is only variations of one form. Since the term *heterospora* suggests more than one distinct type of conidium and since the organism

occurs on the species of sorghum, the name *Gloeocercospora* and the specific name *Gloeocercospora sorghi* and *Gloeocercospora inconspicua* was given by **Deighton (1971)**.

2.2 Geographical distribution

Zonate leaf spot disease is one of the most important diseases of sorghum. It occurs in almost all the areas where the crop is grown including many countries in North and South America (Argentina, Venezuela), Central America (Salvador, Panama, Nicaragua), West Indies, India, Pakistan, Korea and Japan. It is also present in several African countries including Sudan, Ghana, Nyasaland, Tanzania, Nigeria and Uganda.

2.3 Economic importance

Zonate leaf spot occurs worldwide wherever sorghum is grown, especially in warm wet areas of world. This disease can kill or check the growth of young plants to a considerable extent if congenial conditions are available but with advent of dry weather generally, recovery has been observed and in such cases, little damage occurs. **Saccas (1954)** mentioned very severe infections in the North of Oubangui (N. Africa) and on the mid Chari (West Africa) leading in some cases to complete destruction of plants. **Zummo (1971)** found that *G. sorghi* may cause sweet sorghum plants to die, when there is a high incident of the disease at seedling stage. Severe infection at a later stage may result in premature defoliation and a reduction of the yield of the stalks and the sugar content of the juice. **Malaguti and Tovar (1972)** observed 90 percent incidence of *G. sorghi* on sorghum in Venezuela. According to **Odvody *et al.*, (1974)** the incidence of zonate leaf spot in Nebraska was 50 percent, with 5 percent to 10 percent area completely destroyed. On sorghum, damage varies as per varieties. The grain yield of different varieties of sorghum is chiefly reduced by zonate leaf spot as reported by **Sharma and Jain (1975)**. The damage also varies with climatic conditions, growth stage of host plant at infection time. **Chiranjeevi and Tripathi (1976)** reported decreased content of chlorophyll and carotenoid content of sorghum leaves and thus the decreased yield of sorghum grain. **Agnihotri and Pandey (1977)** reported that *G. sorghi* can damage up to 85 percent of the photosynthetic leaf area under humid and cloudy weather conditions. **Ahmad and Gupta (1978)** found in 16 forage cultivars that with increase of the intensity of *G. sorghi* attack, the leaf weight decreased and leaf dry matter content increased. **Kumar (1980)** observed the occurrence and incidence of two major diseases, anthracnose (*Colletotrichum*

graminicola) and zonate leaf spot (*G. sorghi*) on forage sorghum in semi arid regions of the India. **Kalappanavar and Hiremath (2000)** reported that total sugar content significantly decreased with the age of the plants, while the amount of phenols and O-dihydroxyphenols significantly increased while evaluating the relationship between biochemical traits and multiple disease resistance to sooty stripe (*Ramulispora sorghi*), Zonate leaf spot (*G. sorghi*), anthracnose (*C. graminicola*) and rust diseases.

2.1.1. Symptoms and signs

The lesions appear as small, reddish or brownish, water soaked spots sometimes have a narrow, pale-green halo. Later, as the spots enlarge they become dark colored , except in certain varieties where they are light brown and become somewhat elongate and parallel to the veins .They are finally formed (possibly by coalescence) into large ,semi-circular or irregular lesions several centimeters in diameter **(Bain and Edgerton, 1943).** A smaller lesion usually has a light brown centre surrounded by light to dark –red border but frequently in large lesions there may be an alternation of dark and light zones. These leaf lesions may occur along the margin or towards the mid-rib, or they may cover the entire leaf when infection is heavy. Often the younger red lesions are so numerous as to form red irregular patches. Because of this characteristic type of spotting, the name "zonate leaf spot" has been assigned to it.

A few weeks after infection occurs, minute spherical to lenticular sclerotia appear in the necrotic areas of infected leaves and sheaths. Leaves and sheaths are the only part of the plant on which symptoms have been observed. However the fungus has also been isolated from surface sterilized seeds and glumes, which indicate that these also become infected **(Bain and Edgerton, 1943).**

In South Texas, **Odvody and Madden (1984)** also found that *G. sorghi* caused the leaf sheath blight on grain sorghum in fields and nurseries. It infected basal and underlying leaf sheaths on susceptible cultivars under the saturated soil and hot, humid conditions and sclerotia being produced in large quantities to give a grey appearance. Sheath infection by fungus either preceded or occurred simultaneously with leaf blade infection. **Puranik and Suryanarayan (1966)** found *G. sorghi* on khas (*Vetiveria zizanioides)* and noted small diffused brown spots with irregular margin on leaves .The isolate readily infected seedlings of some sorghum varieties. Pinkish gelatinous spore masses have been recorded

on natural substrata in wet conditions by **Ciccarone (1949); Sprague (1950) and Tarr (1962)**. But conidial production doesn't always occur in gelatinous, masses under dry conditions especially **(Rawla, 1973)**. **Rawla (1973)** gave detailed description based on Indian material on *Sorghum vulgare* Pers. Leaf spots are elliptical, oblong or orbicular, upto 8 × 5 cm., zonate with alternate paler and darker brown zones; appearing elongated reddish brown to purple stripes sometimes of dimension 15-50 × 2-3 mm.

2.1.2. The causal organism

2.1.2.1. Systemic Classification

Kingdom	:	Mycota
Division	:	Eumycota
Sub-division	:	Deuteromycotina
Class	:	Hyphomycetes
Order	:	Moniliales
Family	:	Tuberculariaceae
Genus	:	*Gloeocercospora*
Species	:	*sorghi*

(Ainsworth *et al.*, 1973)

Generally the pathogen development was restricted to subcuticular hyphae or stroma in the primary leaf epidermis until 36 to 48 hours after inoculation, but on advancement intercellular colonization became prevalent. In contrast ingress into 3-5 weeks old leaves of 8 to 10 weeks old plants respectively occurred most frequently through leaf trichomes. After penetration pathogen development and lesion appearance in these older leaves of older plants, was similar to that in seedlings. However the lesions appear more slowly and did not coalesce as rapidly on older leaves of older plants **(Myers and Fry, 1978).**

Hyaline septate hyphae emerge through the stomata and branch to form a sporodochial column, which at maturity is more or less definitely stalked. From this arises the sporodochium, a superficial fructification situated above the stomatal aperture. The sporodochia are characteristically salmon pink in color, easily visible to naked eyes and

occur in clusters often tending to be arranged in lines more or less parallel to the veins . Under dry conditions, they may be sparse or absent. In culture the clusters of conidiophores may become so dense as to form bouquet like aggregates, resembling the sporodochia that are found on the leaves **(Bain and Edgerton, 1943).**

Rawla (1973) reported that scanty aerial growth, sporodochia and sclerotia were formed independent of each other. Sporodochia are pink, spherical to sub-spherical, raised upto 0.5 mm diameter, scattered or gregarious often confluent and forming irregular patches, composed of radial hyphae with short conidigenous cells. Conidiophores are hyaline branched or unbranched, septate, short (5-10µ) and aggregated together in dense clusters. In culture they are reported to arise pleurogenously from hyphae as densely clustered short branches **(Bain and Edgerton, 1943).**

A conidium is attached somewhat to the side of the slightly swollen apex of each conidiophore or its branch. The conidia are born in a pinkish to salmon slimy matrix. They are either straight or curved, tapering from base to apex, few to many septate, hyaline and elongated to filiform. The length varies from 20 to 195 µ and slightly over 3 µ in width. In culture the conidia, which develop in pink, bead like slimy mass, do not differ materially from those on leaf lesions. These masses often coalesce to form larger ones (**Bain and Edgerton, 1943**). **Luttrel (1950)** gave the dimension of conidia 1.4-3.2×20-195 µ and mean dimension as about 2.8-46 µ. **Saccas (1954)** gave its dimension as 2.3 × 87 µ in French Equatorial Africa. In Argentina conidia measured 2-3 × 36-176 µ **(Muntanola, 1954).**

The black sclerotia develop within the tissues of the older leaf lesions. They occur at definite intervals and in lines parallel to the veins, which suggest that they are formed under the stomates. In section sclerotia are found to be elliptical. Each sclerotium has a central portion composed of pseudoparenchymatous tissue that is surrounded by a hard layer composed of thick walled cells **(Bain and Edgerton, 1943).** Sclerotia have not been reported under natural conditions in Venezuela, where the leaves decay quickly, possibly before sclerotia are differentiated **(Ciccarone, 1949).**

2.1.3. Survival

Several workers have investigated this aspect and reported that the seasonal carryover no doubt occurs through survival in diseased crop debris, seeds from infected plants and on alternate hosts.

2.1.3.1 Crop debris and soil

Singh and Pavgi (1982) carried out an experiment on the survival of zonate leaf spot pathogen using sterilized and unsterilized field soil. They reported that the mycelium kept in sterilized and unsterilized soil survived for two months and one month respectively. Sclerotia of *G. sorghi* in leaf debris, however, remained viable in the soil from season to season. Sclerotia may remain viable for longer period inside the necrotic tissues of the host **(Bain and Edgerton, 1943).**

Dean (1968) reported that cultures of *G. sorghi* were obtained from the sclerotium bearing leaves over wintered in wire bags on or above the ground, but not from the leaf fragments buried in soil during the same time. It is probable that *G. sorghi* over winters as sclerotia and primary spring infection occurs by rain splashes carrying conidia produced by germinating sclerotia on the soil surface.

2.1.3.2 Seeds

Bain (1950) detected *G. sorghi* on the seeds of *Sorghum vulgare* Pers. In India **Mishra *et al.*, (1969)** reported that the pathogen was found on the seeds of *Sorghum vulgare* under the different storage conditions. They also reported that the pathogen found to cause the seed-rot as well as seedling blight. *G. sorghi* was recovered from 68 percent sorghum seeds in a severely diseased field in Ebina. The emergence of these seeds in autoclaved soil was as low as 20 percent and 80 percent of the emerged seedlings had leaf lesions. The fungus was recovered from stem near the ground from 2 out of 39 seedlings originating from pathogen free seeds but grown in infested soil collected from Ebina **(Watanabe *et al.*, 1978).**

Dhanraj (1979) reported the seedling-blight in varietal trials. A report from Taiwan **(Wu, 1983)** characterized the disease from its seed born nature. *G. sorghi* was frequently transmitted through the seeds to seedlings.

2.2 Biological Control

Plant diseases need to be controlled to maintain the quality and abundance of food, feed and fibre produced by growers around the world. A number of different strategies may be used to manage and control plant pathogens. The broad definition of biological control proposed by **Cook and Baker (1983)** is: "the reduction of the amount of inoculum

or disease-production activity of a pathogen accomplished by or through one or more organisms than man". This broad definition includes the use of less virulent pathogen, more resistant cultivars of the host, and microbial antagonists "that interfere with the survival or disease production activity of the pathogen".

2.2.1 The importance of plant growth promoting rhizobacteria (PGPR) and Biocontrol fungi (BCF)

The first clear indication of improved plant growth and biological control of root pathogens due to seed bacterization with rhizobacteria came from the works of **Burr *et al.*, (1978)** and **Kloepper *et al.*, (1980)** who reported the plant growth promoting effects of *Pseudomonas* strains which were antagonistic to a wide range of plant pathogens *in vitro*. These studies also provided the first evidence that the rhizosphere microbiota could be modified significantly with microorganisms introduced with the planting material. **Kloepper *et al.*, (1989)** coined the term plant growth promoting rhizobacteria (PGPR) to include bacteria inhabiting the root and rhizosphere soil which have the ability to increase plant growth.

2.2.1.1 Plant Growth promotion

Rhizobacterial strains were found to increase plant growth after inoculation in seeds and therefore called "Plant growth promoting rhizobacteria" **(Kloepper *et al.*, 1980)**. The mechanisms of growth promotion by these PGPR are complex and appear to comprise both changes in the microbial balance in the rhizosphere and alterations in host plant physiology **(Glick *et al.*, 1999)**.

Plant growth promoting rhizobacteria, including fluorescent pseudomonads are capable of surviving and colonizing the rhizosphere of all field crops. They promote plant growth by secreting auxins gibberellins and cytokinins **(Vidyasekaran, 1998)**. PGPR has a significant impact on plant growth and development in both indirect and direct ways. Indirect promotion of plant growth occurs when bacteria prevent some of the deleterious effects of a phytopathogenic organism by one or more mechanisms. On the other hand, the direct promotion of plant growth by PGPR generally entails providing the plant with

compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment **(Glick, 1995; Glick *et al.,*¸1999)**.

Plant growth benefits due to the addition of PGPR include increase in germination rates, root growth, yield including grain, leaf area, chlorophyll content, magnesium, nitrogen and protein content, hydraulic activity, tolerance to drought and salt stress, shoot and root weights and delayed leaf senescence **(Lucy *et al.*, 2004)**. Seed treatment with PGPR resulted in increased yield and growth in potato under field conditions **(Kloepper *et al.,* 1980)**.

Van Peer and Schippers (1988) documented the increased root and shoot fresh weight of tomato, cucumber, lettuce and potato as a result of bacterization with *Pseudomonas* strains. **Mashooda Begum *et al.,* (2003)** studied the effectiveness of plant growth promoting rhizobacterial isolates against some seed borne fungal diseases. Among them *B. pumilus* (SE-34), *B. pasteurii* (T4), *B. subtilis* (IN 937-6) and *B. subtilis* (GB-03) strains stood first in the improvement of crop, both in greenhouse and field condition. Potential strains increased the biomass of plants, total number of leaves, fruits, length, girth, biomass of the fruit. The colonization of these bacterial strains reduced the incidence of seed mycoflora which indirectly enhanced the per cent seed germination and vigour index of seedlings. **Minakshi *et al.,* (2005)** isolated a total of 113 rhizobacteria from different rhizotic zones of pigeonpea. Seed treatment using four isolates, *viz.* RS29, RS39, RS41 and RP3 resulted in 90 per cent seed germination in contrast with 50 per cent obtained in untreated control after 72 h of incubation and the isolates RS34, ER17, RP7 and RS41 increased shoot height and shoot dry biomass as compared to uninoculated control whereas isolates RS45, RS36, RS37, ER23, RP24 influenced root dry biomass significantly.

2.2.1.2 Rhizobacteria as biocontrol agents

Rhizobacteria are ideal for use as biocontrol agents since they inhabit the rhizosphere that provides the front line defence for roots against attack by pathogens. Pathogens encounter antagonism from rhizobacteria before and during their primary

infection of roots. Rhizobacteria are reported to provide protection against diverse plant pathogens.

2.2.1.2.1 *Pseudomonas* species

The genus *Pseudomonas* comprises the relatively large and important group of gram-negative, non-spore forming, motile rod bacteria (Bergey´s Manual of Systematic Bacteriology, second ed. Vol. 2). They are ubiquitous in nature and are one of the best-studied soil-borne bacterial groups. Some members of the genus are characterized by production of diffusible and/or insoluble pigments.

Pseudomonads are well-known for their ability to degrade compounds, which are difficult to utilize by other organisms **(Khan and Ahmad, 2006)**. Consequently, they are important organisms in bioremediation. They produce wide varieties of antibiotics, which confer a competitive advantage and microbial fitness to survive in most environments **(Haas and Keel, 2003; Paulsen *et al.*, 2005)**. This genus also comprises human, animal and plant pathogens; besides, there are also important beneficial bacteria such as plant growth promoters and biocontrol agents **(Raaijmakers *et al.*, 2002)**.

Sedra and Malouhy (1994) studied six antagonists from 420 samples obtained from conducive and suppressive soils, for their inhibitory activity against *F. oxysporum* f.sp. *albedinis*. These antagonists suppressed the growth of *F. oxysporum* f.sp. *albedinis in vitro* by 24-47 per cent and its sporulation by 70-99 per cent.

Gupta *et al.*, (1999) isolated *P. aeruginosa* from potato rhizosphere that displayed a strong antagonistic activity against important fungal pathogens, *viz. Macrophomina phaseolina* and *Fusarium oxysporum*. In addition, *Pseudomonas* spp. are common rhizosphere organisms and have been shown to be excellent root colonizers **(Lugtenberg *et al.*, 2001; Raaijmakers and Weller, 2001)**.

Tripathi and Johri (2002) studied the biocontrol potential of fluorescent pseudomonas isolated from rhizosphere of pea and wheat *in vitro* and *in vivo* against maize sheath blight caused by *Rhizoctonia solani*. They found some isolates to possess multiple disease control potential, while some others exhibited biocontrol potential against specific pathogens, which indicated that fluorescent pseudomonads are diverse with respect to their biocontrol potential.

Plant growth-promoting rhizobacterial strain belonging to fluorescent pseudomonads were isolated from the rhizosphere of rice and sugarcane. Among 40 strains that were confirmed as fluorescent psueodmonads, 18 exhibited strong antifungal activity against *Fusarium oxysporum* and *Rhizoctonia bataticola*, mainly through production of antifungal metabolites **(Kumar *et al.*, 2002).**

Due to their ability to produce variable metabolites and to utilize several organic compounds most biocontrol pseudomonads are not specific for one pathogen or plant species only, but have a wide host range and suppress several pathogens. For instance, **Siddiqui and Shaukat (2003)** reported the suppression of four root-infecting fungi, *Macrophomina phaseolina, Fusarium oxysporum, Fusarium solani* and *Rhizoctonia solani* by the biocontrol strain, *Pseudomonas aeruginosa* IE-6 both under laboratory and field conditions.

Antagonistic *Pseudomonas* species have been isolated from agricultural soils as well as soils that were naturally suppressive to different plant pathogens, including *Gaeumannomyces graminis* var. *tritici, Fusarium oxysporum*, *Rhizoctonia solani* (**de Souza *et al.*, 2003; Garbeva *et al.*, 2004; Bergsma-Vlami *et al.*, 2005)**. Natural biological suppression to take-all disease caused by the fungus *Gaeumannomyces graminis* var. *tritici* in fields cultivated to wheat was associated with the dominance of indigenous populations of root-colonizing fluorescent pseudomonads producing the antimicrobial metabolite 2,4-diacetylphloroglucinol **(Raaijmakers and Weller, 1998; de Souza *et al.*, 2003)**.

Tiwari and Thrimurthy (2007) reported that twenty-one isolates of *Pseudomonas fluorescens* were isolated from the rhizosphere of rice, maize, wheat, chickpea, mung, urd, soybean and sunflower from Raipur and Bastar regions. Among these seven isolates which showed bright fluorescence under UV light were further tested. The isolates showed positive response of siderophore production and plant growth promoting activity on rice cv. Bamleshwari. Among the isolates PFR 1 and PFR 2 were found significantly superior to control in increasing the shoot length and root length.

In vitro evaluation of the *P. fluorescens* isolates also confirmed their antagonistic ability against both *Pyricularia grisea* and *Rhizoctonia solani* in dual culture tests. Pure culture of *P. aeruginosa* was obtained from the soil and studied for siderophore

production. The antifungal activity of the strain against three phytopathogenic fungi, *viz. F. moniliforma*, *Altenaria solani* and *Helminthosporoum halodes* was assayed by poison food technique. Inhibition of these fungal pathogens appeared to be due to production of antifungal secondary metabolites by *P. aeruginosa* **(Sharma *et al.*, 2007)**.

2.2.2 Fungi as biocontrol agents

Biocontrol fungi (BCF) are beneficial organisms that reduce the negative effects of plant pathogens and promote positive responses in the plant. They do control diseases and in addition have other benefits, including amelioration of intrinsic physiological stresses in seeds and alleviation of abiotic stresses. As a consequence, plants treated with beneficial fungi may be larger and healthier and have greater yields than plants without them. Fungi are by far the most extensively researched group of biocontrol agents and they have been used against aerial, root and soil microbes. These potential biocontrol fungi are mostly saprophytic in nature and proliferate abundantly in various natural soils. Interest in their use for control of aerial plant pathogen has developed more slowly than in case of root pathogens and this probably reflects the relative lack of information on the ecology of microorganisms on the aerial surfaces in comparison with those in the soil **(Mukhopadhyay and Mukherjee, 1998; Singh *et al.*, 2001; Chaube *et al.*, 2002)**

2.2.2.1 *Trichoderma* spp.

The genus *Trichoderma* consists of anamorphic fungi isolated primarily from soil and decomposing organic matter, with teleomorphs, when known, belonging to the ascomycete genus *Hypocrea* (order *Hypocreales*). Fungal species belonging to this genus are worldwide in occurrence and easily isolated from soil, decaying wood and other plant organic matter.

Trichoderma isolates are characterized by a rapid growth rate in culture and by the production of numerous spores (conidia) with varying shades of green. Their lifestyle is generally saprotrophic with minimal nutritional requirements; they are able to grow rapidly on many substrates, can produce metabolites with demonstrable antibiotic activity and may be mycoparasitic against a wide range of pathogens **(Grondona *et al.*, 1997)**. The abundance of *Trichoderma* spp. in various soils, coupled with a wide metabolic versatility, a dynamic colonization of plant rhizosphere and the ability to antagonize and repress a great number of plant pathogens are direct evidence of the role that these fungal species may play in biological control **(Papavizas, 1985; Chet, 1987)**.

A number of isolates of *Trichoderma* have been found to be effective Biocontrol agents of various soil-borne plant pathogenic fungi under greenhouse and field conditions. The knowledge of mechanisms of interaction of *Trichoderma* spp. with plant pathogenic fungi and the plant host is of importance to enhance the practical application of these beneficial microorganisms. They can work against fungal phytopathogens either directly through mechanisms such as mycoparasitism, competing for nutrients and space, modifying environmental conditions and antibiosis or indirectly promoting plant growth and plant defensive mechanisms. In the direct interactions between *Trichoderma* spp. and the plant pathogenic fungi, mycoparasitism is one of the mechanisms observed with the antagonist that coils around the hyphae of the pathogen, develops hook like structures known as appressoria coupled with production of lytic enzymes and then penetrates the pathogen hyphae **(Kubicek *et al.*, 2001, Rocha-Ramirez *et al.,* 2002; Howell 2003)**.

Trichoderma spp. has also been reported to produce a plethora of secondary metabolites showing antimicrobial activity **(Vinale *et al.,* 2008)**. The chemical composition of these compounds depends on the strains and they may be classified as volatile, water-soluble or water-insoluble compounds **(Ghisalberti and Sivasithamparam, 1991)**.

The competition for space, infestation sites and nutrients has also been shown to be possible mechanisms involved in the biocontrol activities of *Trichoderma* spp. (**Dennis and Webster 1971a, b; Chet 1987; Tronsmo and Hjeljord 1998)**.

The first demonstration of induced resistance was reported in 1997 who described the acquisition of resistance of bean plants towards *Botrytis cinerea* and *Colletotrichum lindemuthianum* after inoculation of the root with the strain T- 39 of *Trichoderma harzianum* **(Yedidia *et al.,* 1999)**. Certain *Trichoderma* isolates invade the vascular tissue or epidermal cells of plant root, giving rise to accumulation of signal molecules, salicylic acid (SA) and jasmonic acid (JA). These compounds induce the PR genes function coding pathogenesis-related proteins (PR protein), expressed by plant to defence pathogen infection **(Hurtado, 2004; Wasternack *et al.*, 2006)**. The PR proteins were classified into 17 families: among them the degrading enzymes chitinases and β-1,3-glucanases are capable of lysing the fungal plant pathogen cell wall. Different reports revealed species

diversity of *Trichoderma* spp. in tomato seed production fields and its effectiveness against *Fusarium* wilt **(Saksirirat *et al.*, 2005; Saepaisan, 2006)**.

2.2.2.1.1 *Trichoderma*: endophytism and plant growth promotion

In recent years, *Trichoderma* spp. have been widely used in agriculture as biocontrol agents and inoculants to provide plant growth promotion. They are involved in fundamental activities that ensure the stability and productivity of both agricultural and natural ecosystems. Some *Trichoderma* strains, described as rhizosphere competent and selectively used for commercial development, can cause an asymptomatic infection of roots, where the fungus colonization is limited to the outer cortical regions. These fungi behave as endophytes, colonizing the root epidermis and outer cortical layers and release bioactive molecules. At the same time, the transcriptome and proteome of plants are substantially altered. This intimate interaction with the plant provides a number of benefits only recently recognized for their variety and importance, including increased resistance of the plant to various biotic stresses through induced or acquired systemic resistance and to abiotic stresses such as water deficit/excess, high salinity and extreme temperature; enhanced nitrogen use efficiency by improved mechanisms of nitrogen reduction and assimilation and reduced over expression of stress genes or accumulation of toxic compounds during plant response to pathogen **(Shoresh *et al.*, 2010)**.

An additional benefit to consumer comes from an increased content of antioxidants in the fruit from plants treated by selected *Trichoderma* strains **(Lorito *et al.*, 2010)**. Moreover, it was also observed that the fertility of soils treated with some *Trichoderma* strains could be significantly improved beyond disease control, which increased the attractiveness of these fungi for a general use in crop production. The effect could be particularly strong in terms of root growth promotion, even though it has been not unusual to detect an increase in stem length and thickness, leaf area, chlorophyll content and yield (size and/or number of flowers or fruits) **(Harman *et al.*, 2004)**. The molecular mechanisms supporting this highly desirable beneficial effect of plant growth promotion are not fully clarified and include improvement of nutrient availability and uptake for the plant **(Altomare *et al.*, 1999, Lorito *et al.*, 2010)**.

Maize plants grown from seeds treated with *T. harzianum* T-22, grown using 40% less of nitrogen in the fertilizer, have obtained a maximum of efficiency equal to that of

untreated plants but with a supply of nitrogen optimal **(Harman, 2000)**. Further analysis show a general increase in the absorption of many elements such as Pb, Mn, Zn, Al and the ability to solubilize some nutrients in the soil, such as phosphates, ions Fe3+, Cu2+, Mn4+, many times not easily available for the plant **(Altomare *et al.*, 1999)**. Moreover, the involvement of growth phytohormones from both plant and fungal origin could be involved in the phenomenon of plant growth promotion **(Vinale *et al.,* 2008)**.

In combination with the direct effects on plant pathogens and with the ability to promote plant growth, *Trichoderma* spp. have also been found to stimulate plant defence mechanisms. The presence of *Trichoderma* in plants involves an induction of resistance, often localized or systemic **(Harman *et al.*, 2004)**. This phenomenon, also observed in field, has been attributed to a fungus-root biochemical cross talk involving many bioactive metabolites produced by the biocontrol agents **(Harman *et al.*, 2004; Shoresh *et al*, 2010; Woo *et al.*, 2006)**.

Many *Trichoderma* strains colonize plant roots of dicots and monocots. During this process *Trichoderma* hyphae coil around the roots, form appressoria-like structures, and finally penetrate the root cortex. During the intercellular *Trichoderma* growth in the root epidermis and cortex the surrounding plant cells have been induced to deposit cell wall material and to produce phenolics compounds. This plant reaction limits the *Trichoderma* growth inside the root **(Vinale *et al.*, 2008)**. Effective *Trichoderma* srains are able to induce a stronger response in the plant compared to pathogen triggered immunity by producing a variety of microbe-associated molecular patterns (MAMP) as hydrophobins, expansin-like proteins, secondary metabolites, and enzymes having direct antimicrobial activity such as peroxidase, chitinase and glucanase. In addition, there is an accumulation of antimicrobial compounds and phytoalexins **(Lorito *et al.*, 2010)**.

2.2.2.2 Arbuscular mycorrhizal (AM) fungi

Mycorrhiza has been defined as a symbiotic association between a fungus and a root or other substrate-contacting organ of a living plant, which is primarily responsible for nutrient transfer **(Brundrett, 2004)**. Many mycorrhizal associations are examples of mutualistic symbiosis arising from an intimate connection between mycorrhizal fungi and plant roots through an exceptional hyphal network specialized in the uptake of water and nutrients. This improves the plants' absorption capability for water and nutrients. In return, mycorrhizal fungi take up to 20% of the carbohydrate photosynthate produced by plants.

According to the location of fungi on plant roots, mycorrhizae are morphologically classified into two major categories; ectomycorrhizae (ECM) and endomycorrhizae (VAM) **(Peyronel *et al.,* 1969; Parniske, 2008)**. The ectomycorrhizal associations are common in temperate and boreal forest trees, including gymnosperms and angiosperms with numerous fungi belonging to the Basidiomycota, Ascomycota and Zygomycota phyla. The infection occurs when the ectomycorrhizal fungi are stimulated by the root metabolites. The fungal hyphae aggregrate around the root and penetrate between the root epidermis and the cortex, resulting in a network of hyphae termed a hartig net surrounding the plant cells within the root cortex and a hyphal sheath known as mantle that covers the root tip. Eventually the entire roots are completely surrounded by mantle. The fungal hyphae on the exterior of the roots usually serve as an extension of the roots and stores large amounts of carbohydrates. Ectomycorrhizae are dispersed either by airborne spores or through the transfer of infected plant tissue **(Quilambo, 2003; Harley and Smith, 1983)**.

Unlike the ectomycorrhizae, the infection of endomycorrhizae begins with the penetration of fungal hyphae into the root cells via the intercellular spaces of the epidermal cells. The hyphal elongation occurs longitudinally in both the outer and inner cortex of the root cells followed by hyphal invagination into the surface layer of the cortical cells. As invagination proceeds, the hyphae start to branch repeatedly and form an arbuscule which is a symbiotic organ that specifically plays an important role in nutrient exchange between the host plant and fungus. The final stage is the degeneration of an arbuscule in the host cortical cell **(Matsubara and Harada, 1996; Gadkar *et al.*, 2001)**. Vesicular arbuscular mycorrhizae were named after their unique structures, arbuscules. However, arbuscular mycorrhizae, has recently replaced the previously more common name. Moreover, arbuscules are normally used to define the vesicular arbuscular mycorrhizal associations **(McGonigle *et al.*, 1990; Toth *et al.*, 1990; Brundrett, 2004)**. The active form of arbuscules persists for not longer than 15 days. Thus progression of colonization requires ongoing arbuscule formation as the fungus spreads in the host roots **(Bonfante, 2003)**.

The arbuscular mycorrhizal association is considered to be the most widely distributed mutualistic association in 80% of all terrestrial plants and this includes the major agricultural crop plants **(Quilambo, 2003)**. Interestingly, the arbuscular mycorrhizae are formed only by fungi in the phylum Glomeromycota which belong to the order

Glomales. The order Glomales established by **Morton and Benny (1990)** comprises of two suborders; Glomineae and Gigasporineae. Gigasporineae is capable of producing extraradical auxillary cells whereas Glomineae generally form intraradical vesicles. *Glomus* and *Sclerocystis* two genera in the suborder Glomaceae are characterized by producing chlamydospores either within the host roots or in the soil. Chlamydospores are borne singly or may form an aggregate in clusters. Members of the genera *Acaulospora* and *Entrophospora* are distinguished by chlamydospores produced within a sporiferous saccule. *Gigaspora* spp. and *Scutellospora* spp. that belong to the family Gigasporaceae are capable of producing azygospores on sporogenous cells **(Quilambo, 2003)**.

The arbuscular mycorrhizal (AM) fungi are considered to be symbiotic organisms that exhibit potential as biological control agents of soil borne diseases just as do *Pseudomonas* spp. and *Bacillus* spp. The use of *Glomus* sp. and *Bacillus* spp. inoculants in an attempt to suppress *Verticillium dahliae*, the causal agent of *Verticillium* wilt in strawberry (*Fragaria* x *ananassa* cv. *Selva*) was successfully achieved in a field experiment **(Tahmatsidou *et al.*, 2006)**. The application of a commercial mycorrhizal inoculum *G. mosseae* BEG29 together with *B. subtilis* M3, *Trichoderma harzianum* DB11, *P. fluorescens* C7r12 and *Gliocladium catenulatum* Gliomis ® effectively decreased oospores and crown rot disease of strawberry caused by *P. cactorum* **(Vestberg *et al.*, 2004)**.

Nwaga *et al.*, (2007) evaluated the antagonistic capacity of *Pseudomonas* spp. either alone or in association with *G. deserticola* against *Pythium aphanidermatu*, the causal agent of damping off and stem rot of cowpea (*Vigna unguiculata* L. Walp). The synergistic effect of *G. deserticola* and *Pseudomonas* spp. was established as the results showed a greater reduction of the disease index after co-inoculation rather than as a single inoculum.

Fritz and Jacobson (2006) investigated the effects of *G. intraradices* BEG 87 on foliar susceptibility to *Alternaria solani* in tomato and found that the presence of AM fungi in tomato plants led to a reduced degree of early blight disease severity. Apart from being a potent biological control agent, AM fungi are also able to facilitate nutrient uptake and promote growth of the host plants. There have been many research publications on the importance of arbuscular mycorrhiza (AM) fungi for agricultural crops that have pointed out that the arbuscular mycorrhizal symbiosis confers numerous benefits to the host plants.

Tarafdar and Marschner (1995) reported that the inoculation of bread wheat *Triticum aestivum* L. with *Glomus mosseae* significantly improved biomass production along with the uptake of macronutrients and trace elements. The effect of the same AM fungus on durum wheat (*T. turgitum* var. *durum*) was investigated three years later. A positive effect of *G. mosseae* on durum wheat tillering was achieved as well as on plant growth and yield that both increased more than the non-inoculated control. Moreover, the phosphorus concentration was increased up to 4 fold compared to control plants **(Karagiannidia and Zinoviadi, 1998)**. *G. fasciculatus* is another mycorrhizal fungus that was earlier proven to produce advantages for plants.

2.3 Biocontrol mechanisms of PGPR and BCF

Since biological control is a result of many different types of interactions among microorganisms, scientists have concentrated on characterization of mechanisms occurring in different experimental situations. In all cases, pathogens are antagonized by the presence and activities of other microorganisms that they encounter. Different modes of actions of biocontrol-active microorganisms in controlling fungal plant disease include mycoparasitism, antibiosis, competition for site and nutrient and induced resistance. The most effective biocontrol active microorganisms appear to antagonize plant pathogens employing several models of action **(Cook, 1993)**.

2.3.1 Mycoparasitism

Mycoparasitism, the direct attack of one fungus to another one, is a very complex process that involves sequential events, including recognition, attack and subsequent penetration and killing of the host. The various mechanisms used by fungi to antagonize or parasitize their competitors include antibiotic production, secretion of lytic enzymes, hyphal interference and direct penetration of the host. Any particular fungus-fungus interaction may encompass more than one of these mechanisms either individually or simultaneously **(Jeffries, 1997)**. Mycoparasitism involves morphological changes, such as coiling and formation of appressorium-like structures, which serve to penetrate the host and contain high concentrations of osmotic solutes such as glycerol **(McIntyre *et al.*, 2004)**. Lysis of the host cell wall of the plant pathogenic fungi has been demonstrated to be an important step in the mycoparasitic attack **(Kubicek *et al.*, 2001; Howell, 2003)**.

2.3.2 Antibiosis

In a general definition, antibiotics are microbial toxins that can, at low concentrations, poison or kill other microorganisms. It has been shown that some antibiotics produced by microorganisms are particularly effective against plant pathogens and the disease they cause **(Homma *et al.*, 1989 and Islam *et al.*, 2005)**. In all cases, the antibiotics have been shown to be particularly effective at suppressing growth of the target pathogen *in vitro* and/or *in situ* conditions. Fungi have been demonstrated to produce a wide variety of toxic substances that have activity against a range of prokaryotic and eukaryotic organisms. The ability of a fungus to produce antibiotic may thus be very important in determining its ability to colonize or maintain its presence on a substrate **(Faull, 1988)**.

2.3.3 Competition

Competition occurs when two (or more) organisms require the same resource and the use of this by one reduces the amount available to the other. The nutrient sources in the soil and rhizosphere are frequently not sufficient for microorganisms and starvation is the most common cause of death for microorganisms. For a successful colonization of phyllosphere and rhizosphere a microbe must effectively compete for the available nutrients. There is a general believe that competition between pathogens and non-pathogens for nutrient resources is an important issue in biocontrol. It is also believed that competition is more critical for soil borne pathogens, including *Fusarium* and *Pythium* species that infect through mycelial contact than foliar pathogens that germinate directly on plant surfaces and infect through appressoria and infection pegs **(Elad and Baker 1985; Keel *et al.*, 1989; Loper and Buyer 1991)**. Competition for rare but essential micronutrients, such as iron, has also been shown to be important in biological disease control. Competition is also possible for oxygen, space and, in the case of autotrophs, light.

2.3.4 Induction of resistance

Plants actively respond to a variety of environmental stimulating factors, including gravity, light, temperature, physical stress, water and nutrient availability and chemicals produced by soil and plant associated microorganisms. Such stimuli can either induce or condition plant host defences through biochemical changes that enhance resistance against subsequent infection by a variety of pathogens. Induction of host defences can be local

and/or systemic in nature, depending on the type, source and amount of stimulating agents **(Audenaert *et al.*, 2002).**

2.4 Induced Resistance

2.4.1 History and developments

Induced resistance was first identified during the early 20th century when plants acquired physiological immunity after attack by a pathogen **(Beauverie, 1901; Ray, 1901; Wingard, 1928; Chester, 1933)**. Later, **Ross *et al.*, (1961 and 1966)** and **McIntyre *et al.*, (1981)** showed that inoculating one leaf of tobacco cultivars containing the '*N'* gene with TMV not only induced resistance to TMV in other non-inoculated leaves of the same plant, but also induced resistance to tobacco necrosis virus, *Phytophthora nicotianae* (B. De Haan), and *Pseudomonas tabaci* (Wolf and Foster) Stevens. They later confirmed that necrotizing pathogens other than TMV could also induce resistance in tobacco. However, IR research received little consideration from the early 1960s to the 1980s. In fact, most of the researches were focused on effects on systemic leaves when viruses or other pathogens were inoculated in the bottom leaves. Induced resistance can be activated by either pathogenic or non-pathogenic microorganisms (for example, some rhizobacteria) in some plants, while in other plants, the same kind of defence can be induced by certain groups of chemicals **(Van Loon *et al.*, 1998)**.

In general, plants can develop resistance against pathogens through active or passive means **(Huang, 1998)**. Passive resistance generally involves constitutive expression of a resistance gene and/or presence of physical barriers. Alternatively, active resistance is induced during the pathogen infection process, which triggers several defence related genes to form a broad-spectrum resistance to multiple pathogen groups. This process of resistance induced either by biotic or abiotic agents against a wide range of pathogens is popularly called, 'induced or acquired resistance' **(Van Loon *et al.*, 1998)**. This defence machinery stimulates plant physiological and biochemical pathways to accumulate numerous phytochemicals, which in turn induce natural systemic resistance to a broad spectrum of pathogens. Thus, there is a great possibility that induced resistance can be utilized to manage many pathogens and parasites. However, induced defence mechanisms can only be implemented effectively under field conditions in grower's fields when thoroughly understood.

2.4.2 Molecular mechanisms

Tobacco has been used as one of the main model plant systems since the late 20th century to understand the mechanisms of induced resistance. With the induced resistance mechanism, not only are there prospects to control plant diseases in an environmentally safe manner, but also to study host defence-related genes and the signal transduction processes involved in priming resistance. The phenomenon of acquiring resistance systemically against a range of pathogens arises from changes in a plant's physiology initially assumed to involve several plant hormones. Several potential components of the SA signaling pathway have been identified, including H_2O_2-scavenging catalase/ascorbate peroxidase, SA-binding protein (SABP2), SA-inducible protein kinase (SIPK), non-expressor of PR1 protein (NPR1), and members of the TGA/OBF family of bZIP transcription factors. Notably, the bZIP factors physically interact with NPR1, which binds a SA-responsive element with promoters of several defence genes encoding pathogenesis-related proteins **(Klessig *et al.*, 2000)**. Despite the extensive research performed for more than two decades on IR, many biochemical and molecular aspects still need to be revealed to fulfill the broad potential of IR as a modern plant disease management tool. The fact that certain biotic and abiotic agents stimulate induced resistance within plants is obvious, but the physiological basis, molecular interactions, and mode of action in tobacco in response to different groups of pathogens are not completely understood.

2.4.3 Types of induced resistance

Under natural conditions, necrotizing pathogenic organisms trigger Systemic Acquired Resistance (SAR) and nonpathogenic rhizobacteria activate Induced Systemic Resistance (ISR). Both types of resistance are effective against a wide range of pathogens **(Pieterse and Van Loon, 2004)**. SAR and ISR are phenotypically similar, but mechanistically different. Like SAR, ISR has been demonstrated to act systemically against fungi, bacteria, and viruses in *Arabidopsis*, bean (*Phaseolus vulgaris* L.), carnation (*Dianthus caryophyllus* L.), cucumber (*Cucumis sativus* L.), radish (*Raphanus sativus* L.), tobacco and tomato **(Van Loon *et al.*, 1998)**. Both SAR and ISR pathways are modulated by NPR1, a common key regulatory protein (**Saskia *et al.*, 2000**).

2.4.4 Crosstalk between signaling pathways

Several reports have confirmed the existence of convergence and interactions among compounds signaling various types of induced resistance, that are triggered simultaneously with the functional outcome of either positive or negative, or neutral interactions **(Bostock, 2005)**. It is not completely clear exactly which mechanisms develop in response to stimuli from pathogenic or non-pathogenic microorganisms. In particular, SA-dependent SAR and JA/ET-dependent ISR can interact either synergistically or antagonistically **(Pieterse and Van Loon, 1999)**. When activated simultaneously, SA-dependent SAR has been shown to dominate JA-dependent ISR under several circumstances **(Bostock, 2005)**.

2.4.5 Induced systemic resistance (ISR)

Induced resistance is defined as an enhancement of the plants defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation. The resulting elevated resistance due to an inducing agent upon infection by pathogen is called induced systemic resistance (ISR) or systemic acquired resistance (SAR) **(Hammerschmidt and Kuc, 1995)**. The induction of systemic resistance by rhizobacteria is referred to as ISR, whereas that by other agencies is called SAR **(Van Loon *et al.*, 1998)**. Once resistance is induced, it will afford non-specific protection against pathogenic fungi, bacteria, nematodes and viruses as well as against insect pests.

A large number of defence enzymes that have been associated with ISR include phenylalanine ammonia lyase (PAL), chitinase, b-1,3-glucanase, peroxidase (PO), polyphenol oxidase (PPO), superoxide dismutase (SOD), catalase (CAT), lipoxygenase (LOX), ascorate peroxidase (APX) and proteinase inhibitors **(Van Loon, 1997)**. These enzymes also bring about liberation of molecules that elicit the initial steps in induction of resistance, phytoalexins and phenolic compounds **(Van Loon *et al.*, 1998)**.

Induced systemic resistance by PGPR has been achieved in large number of crops including *Arabidopsis* **(Pieterse *et al.*, 1996)**, cucumber **(Wei *et al.*, 1996)**, tomato **(Duijff *et al.*, 1997)**, potato **(Doke *et al.*, 1987)**, radish **(Leeman *et al.*, 1996)**, carnation **(Van Peer *et al.*, 1991)**, sugarcane **(Viswanathan and Samiyappan, 1999)**, chilli, brinjal **(Ramamoorthy and Samiyappan, 2001; Bharathi *et al.*, 2004)**, rice **(Vidhyasekaran *et al.*, 1997; Nandakumar *et al.*, 2001)** and mango **(Vivekananthan *et al.*, 2004)** against broad spectrum of pathogens including fungi **(Leeman *et al.*, 1995; Doke *et al.*, 1987)**, bacteria **(Liu *et al.*, 1995)**, nematodes**(Paul and Kumar, 2003)** and viruses **(Murhofer *et al.*, 1994; Kandan *et al.*, 2005)**.

Seed treatment and seedling root dipping induced early and enhanced levels of PO in rice plants **(Nayar, 1996)**. Two peroxidase isoforms were induced in the PGPR-treated rice plants inoculated with the sheath blight pathogen, *R. solani* **(Nandakumar *et al.*, 2001)**. High level expression of PO was reported in *P. fluorescens* Pf1 treated chilli plants challenged with *C. capsici* **(Bharathi *et al.*, 2004)**. Similarly, increased activity of PPO was observed in PGPR treated tomato plants challenged with *F. oxysporum* f.sp. *lycopersici* **(Ramamoorthy *et al.*, 2002)**.

Plants treated with *Pseudomonas* strains initially showed higher level of PAL compared to control **(Chen *et al.*, 2000)**. **Radjacommare *et al.*, (2004)** reported that seedling dip with talc based formulation of *P. fluorescens* induced the activity of PAL in finger millet leaves against blast disease. The inoculation of PGPR strains *P. putida* 89B-27 and *Serratia marcescens* 90-166 and the pathogen, *F. oxysporum* f.sp. *cucumerinum* on separate halves of roots of cucumber seedlings exhibited that both PGPR strains induced systemic resistance against the *Fusarium* wilt as expressed by delayed disease symptom development and reduced number of dead plants **(Liu *et al.*, 1995)**. The same PGPR strains also induced systemic resistance in cucumber against bacterial angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans* **(Liu *et al.*, 1995)**.

Maize plants raised from *P. fluorescens* treated seeds showed higher activity of peroxidase, polyphenol oxidase and PAL, when leaf sheaths were inoculated with the pathogen, *R. solani*. The bacterized seeds with *P. fluorescens* lead to accumulation of higher phenolic compounds and higher activity of PO, PPO and PAL that may play a role in defence mechanism in plants against pathogen **(Sivakumar and Sharma, 2003)**. **Kloepper *et al.*, (2004)** also observed, control of nematode diseases in tomato and bell pepper by treatments with PGPR strains through induction of systemic resistance. **Siddiqui and Shaukat (2002)** observed that the application of PGPR strains to one half of the split root system of tomato caused a significant reduction (42%) in nematode penetration in the other half of the split root system and this was attributed to ISR activity of the strain. **Hariprasad and Umesh (2007)** reported that PGPR application was made by seed, root and foliar spray treatments separately in combinations in field. Among the PGPR strains *Bacillus subtilis* strain GB3 was the most effective in providing significant suppression of bacterial spot and was well correlated with increased activity of defence related enzymes,

viz. peroxidase and PAL. PGPR that were effective in greenhouse were also able to induce resistance in tomato against bacterial spot under field conditions.

2.5 Priming is a mechanism of Induced resistance (IR)

For many years, IR in plants has been suggested to be on the basis of the direct activation of defence responses in systemic tissue of pathogen-infected plants. In case of SAR, these directly induced responses in the systemic tissue include the accumulation of pathogenesis-related (PR) protein **(Durrant and Dong, 2004)**. Many PR-proteins display antimicrobial activity presumably through hydrolytic activities on cell walls of potential microbial pathogens and contact toxicity, and maybe also as compounds involved in plant defence signaling **(Van Loon *et al.*, 2006)**. As research on IR had focused primarily on the role of PR-proteins and other directly induced defence-related compounds, it has not been widely appreciated that the enhanced defensive capacity characteristic of IR is also associated with a sensitized state in which the plant responds more rapidly and/or more robustly with the activation of defence responses after exposure to a biotic or abiotic stressor **(Conrath and Go¨ llner, 2008; Conrath *et al.*, 2002, 2006; Kuc´, 1987)**. The state of enhanced capacity to activate stress-induced defence responses has been called the ‘‘primed’’ (or ‘‘sensitized’’) state of the plant. As a matter of fact, as early as the 1980s, **Kuc´ (1987)** had already argued that priming would be an important component of SAR. Yet, although priming could be a unifying mechanism for the different types of IR in plants, the phenomenon did not attract much attention at the time **(Van Loon, 2000)**. In the 1990s, an important role of priming in SAR was supported by the finding that there is close correlation between the capability of various chemicals to activate resistance against tobacco mosaic virus (TMV) in tobacco **(Conrath *et al.*, 1995)** and their capacity to prime for enhanced Phenylalanine Ammonia-Lyase (PAL) gene expression induced by microbe-associated molecular pattern (MAMP) elicitor treatment in cultured parsley cells **(Katz *et al.*, 1998; Thulke and Conrath, 1998)**, or upon infection of Arabidopsis plants with *Pseudomonas syringe* pv. *tabaci* **(Kohler *et al.*, 2002)**.

Most of the studies that investigated priming by beneficial microorganisms made use of ISR-eliciting PGPR. The first evidence that priming of plant defence responses is involved in ISR came from experiments with carnation (*Dianthus caryophyllus*). Inoculation with *Fusarium oxysporum* f. sp. *dianth*i of carnation plants displaying ISR led

to a faster rise in phytoalexin levels than in non induced control plants **(Van Peer *et al.*, 1991)**. In a similar manner, *Bacillus pumilus* (strain SE34) induced systemic resistance against the root-rot fungus *F. oxysporum* f. sp. *pisi* in bean **(Benhamou *et al.*, 1996)**. Upon challenge infection with the same fungus, the walls of root cells were rapidly strengthened at sites of attempted fungal penetration through apposition of callose and phenolic material **(Benhamou *et al.*, 1996)**. In Arabidopsis, priming associated with the systemic resistance induced by root-colonizing *Pseudomonas fluorescens* strain WCS417r has been studied at the molecular level. Although WCS417r-elicited ISR is effective against a broad and distinctive spectrum of pathogens, it is not associated with the activation of genes encoding PR-proteins **(Pieterse *et al.*, 1996)**. Analyses of the Arabidopsis transcriptome have shown that locally in the colonized roots, WCS417r bacteria induce the expression of 94 genes **(Le´on-Kloosterziel *et al.*, 2005; Verhagen *et al.*, 2004)**. However, in the systemic leaves, no significant alteration in gene expression was observed. Thus, WCS417r-elicited ISR is not associated with obvious changes in gene expression in distant leaves **(Verhagen *et al.*, 2004)**. In Arabidopsis expressing WCS417r-mediated ISR, 81 genes showed enhanced expression upon infection of the leaves with *Pseudomonas syringae* pv. *tabaci*, indicating that these plants were primed to respond in a faster and/or more robust manner to pathogen attack **(Van Wees *et al.*, 1999; Verhagen *et al.*, 2004)**. Most of the genes with potentiated induction have been described as being regulated by either JA or ET, or both. The findings confirmed earlier results demonstrating that colonization of the roots by WCS417r primed Arabidopsis for augmented induction of the JA- and/or ET-responsive genes Vegetative Storage Protein-2 (VSP2), Plant Defensin-1.2 (PDF1.2), Hevein-Like Protein (HEL), and ACC (1-Aminocyclopropane-1-carboxylic acid) Oxidase (ACO) **(Hase *et al.*, 2003; Van Wees *et al.*, 1999)**. In contrast to gene expression, significant alterations in the production of either JA or ET have not been observed in the plants exhibiting ISR **(Pieterse *et al.*, 2000)**. These observations argue that the state of ISR is based on an enhanced sensitivity to these plant hormones rather than just on an increase in their production **(Pieterse *et al.*, 2000)**. Studies with other PGPR on different plant species generally confirm that ISR is associated with primed expression of defence genes **(Van Wees *et al.*, 2008)**. **Ryu *et al.*,**

(2004) demonstrated that some plant-growth promoting *Bacillus* spp. can prime plants by the release of volatile organic compounds (VOCs).

In addition to SAR and ISR, the primed state is a common feature also of resistance responses that are induced by beneficial microorganisms other than PGPR. For example, colonization of tomato roots by mycorrhizal fungi protected the plant systemically against *Phytophthora parasitica* with no detectable accumulation of PR-proteins before pathogen assault. Only after P. *parasitica* attack, mycorrhizal plants accumulated significantly more PR- 1a and ß-1, 3 glucanase than non-mycorrhizal plants **(Cordier *et al.*, 1998; Pozo *et al.*, 1999, 2002a)**. Ultrastructural studies revealed that plants with established mycorrhizal symbiosis also displayed pectin-rich, callose containing cell wall depositions at the sites of attempted pathogen infection, whereas non-mycorrhizal plants did not **(Cordier *et al.*, 1998; Pozo *et al.*, 1999, 2002a)**. Certain plant growth-promoting fungi can similarly induce priming in plants. Effective *Trichoderma* srains are able to induce a stronger response in the plant compared to pathogen triggered immunity by producing a variety of microbe-associated molecular patterns (MAMP) as hydrophobins, expansin-like proteins, secondary metabolites, and enzymes having direct antimicrobial activity such as peroxidase, chitinase and glucanase. In addition, there is an accumulation of antimicrobial compounds and phytoalexins **(Lorito *et al.*, 2010)**.

2.5.2 PAL

Phenylalanine ammonia-lyase (PAL) is a key enzyme of plant metabolism catalyzing the first reaction in the biosynthesis from L-phenylalanine of a wide variety of natural products based on the phenylpropane skeleton and the synthesis of phenolic compounds **(Cheng *et al.,* 2001).** It is the first enzyme in the phenylpropanoid pathway. In all studies thus far, change in PAL enzymes levels are regulated at the transcription level. PAL transcription is regulated by different stimuli including mechanical wounding, interaction with pathogens and during plant development **(Dixon and Paiva 1995)**. PAL activity has been associated with increases in both lignin deposition **(Whetten and Sederoff 1995)** and production of phytoalexins **(Grahan 1995)**, and transgenic plants with suppressed level of PAL were more sensitive to disease than wild–type plants **(Maher *et al.,* 1994)**. Therefore PAL appears to be important in plant defence against pathogens. In sorghum, the induction of the synthesis of PAL transcripts and the resultant synthesis of

the 3-deoxyanthocyanidin phytoalexins occurs as a response to fungal infection and is probably separated from the induction of PAL and phenolic compound synthesis which occurs as a response to light **(Weiergang *et al.*, 1996)**. **Radjacommare *et al.*, (2004)** reported that seedling dip with talc based formulation of *P. fluorescens* induced the activity of PAL in finger millet leaves against blast disease. The bacterized seeds with *P. fluorescens* lead to accumulation of higher phenolic compounds and higher activity of PO, PPO and PAL that may play a role in defence mechanism in plants against pathogen **(Sivakumar and Sharma, 2003)**.

2.5.3 Chalcone Synthase (CHS)

Phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) have previously been shown to be expressed more quickly or at higher levels during pathogen attack **(Little and Magill 2003)**.

2.5.4 Chitinases

Chitinases have potent antifungal activity against a wide variety of human and plant pathogens. Chitinase induction is often coordinated with the expression of specific ß-1,3-glucanases and other PR protein**s** in response to pathogen attack, as well as in response to treatment with elicitors and abiotic factors. Chitinase genes are differentially regulated in response to development or by colonization of plant tissues by micro-organisms **(Salzer *et al.*, 2000)**. **Pozo *et al.*, (2002b)** used tomato plants and demonstrated hydrolytic enzymes effects using the pathogen *Phytophthora parasitica* and two species of AM fungi (*G. mosseae* and *G. intraradices*). They observed that *G. mosseae* had the ability to reduce infection of *P. parasitica* in tomato roots by inducing the mycorrhiza related hydrolytic enzymes such as chitosanases and ß-1, 3 glucanase that have lytic activity against *Phytophthora* cell walls. Chitinases are enzymes that catalyze the hydrolysis of ß-1, 4-*N*acetylglucosamine linkages present in chitin and chitodextrins. Chitinase is a member of PR-3 proteins. Plant chitinase**s** are classified into six groups based on their primary structure **(Neuhaus, 1999)**. Classes I and IV are characterized by the presence of an N-terminal, cysteine-rich, chitin-binding domain that is also found in protein**s** such as hevein and in non-leguminous plant lectins. Class II chitinases lack the chitin-binding domain but are otherwise similar to class I chitinases. Class III and class V are more distantly related.

They have been isolated from fungi, **(Kang *et al.,* 1998; Mathivanan *et al.,* 1998)** and plants including tobacco **(Melchers *et al.,* 1994)**, cucumber, beans, **(Ye *et al.,* 2000)** peas, and many others plants and bacteria (**Chernin *et al.,* 1997)**. Chitinases have potent antifungal activity against a wide variety of human and plant pathogens including *Trichoderma reesei, Alternaria solani, A. radicina, F. oxysporum, R. solani Guignardia bidwellii, Botrytis cinerea and Coprinus comatus.* Chitinase induction is often coordinated with the expression of specific ß-1,3-glucanases and other PR proteins in response to pathogen attack, as well as in response to treatment with elicitors and abiotic factors. Chitinase genes are differentially regulated in response to development or by colonization of plant tissues by micro-organisms **(Salzer *et al.,* 2000)**.

2.6 Application of induced resistance for plant disease control

Resistance to pathogen infection can be enhanced within plants through exogenous application of biotic and abiotic inducers or elicitors. Biotic elicitors include necrotizing pathogens and non-pathogenic non-necrotizing rhizobacteria or their elicitor fragments. Abiotic elicitors are stress induced by water, heat, pH, nutrition, wounding or chemicals that mimic a component of plant defence signaling pathways. Although induced resistance has significant disease management potential, complete control is often not achieved under natural field conditions. The timing of induction of defence responses is critical to achieve significant control of pathogen infection. The limited information regarding the influence of environmental factors on priming IR restricts the use of elicitors under field conditions. Extensive research on IR over the past two decades has revealed physiological, biochemical, and molecular aspects of the interaction, helping to identify a number of biological and chemical inducers, and making possible the commercialization of several IR-inducing agents. However, more effective commercialization and deployment of IR elicitors will require additional information on compatibility and "best fit" under typical crop protection and production practices **(Vallad and Goodman, 2004; Walters *et al.*, 2005)**.

MATERIALS AND METHODS

3.1 Isolation and identification of pathogen

Leaf samples were collected from zonate leaf spot infected sorghum plants from Livestock Research Centre, Sorghum Pathology block, G.B. Pant University of Agriculture and Technology, Pantnagar and the infected material was brought to laboratory for microscopic examination, isolation and for further studies.

3.1.2 Culture media

The test fungus, *G. sorghi* was grown on oat meal agar medium. Constituents are as follows:

Ingredients	Grams
Oatmeal	60.00
Agar-agar	12.50
Distilled water	1000ml

3.1.3 Preparation

Suspended 72.5 gm OMA powder (HIMEDIA laboratories Pvt. Ltd.) in 1000ml distilled water. It was boiled till completely dissolved. Finally, the medium was sterilized by autoclaving at 15 pounds psi (temperature 121^0C) for 20 minutes.

3.1.4 Glasswares

Cleaned borosil glasswares were used in the experiments. All the petriplates were sterilized in hot air oven at 160^0C for 2 hours.

3.1.5 Isolation of fungus

G. sorghi, the causal organism of zonate leaf spot disease was isolated from infected leaves. The infected leaves were cut with the help of sterilized blade into small pieces of 2-3 mm size having half healthy and half diseased tissues. The small pieces were sterilized with $HgCl_2$ solution (1:1000) for 30 seconds and thoroughly washed in sterilized distilled water for 3 times. Then the pieces were placed between two layers of sterilized blotter paper to remove excess of water. These pieces were then transferred to slants and Petri plates containing OMA medium inside an inoculation chamber under aseptic

conditions, followed by incubation at 28^0C. After 76-96 hours of incubation, the superficial growth was sub-cultured on fresh OMA slants.

3.1.6 Purification and maintenance of the culture

The fungus was purified with hyphal tip method. The pure culture thus obtained was maintained by sub-culturing it every fifteenth day on oat meal agar medium and preserved in refrigerator at 10^0C.

3.1.7 Identification of the fungus

On the basis of cultural and morphological characteristics, the isolated fungus was identified as *G. sorghi*. Slides were prepared in lactophenol and examined under compound microscope to study the morphological characteristics of the pathogen.

3.1.8 Pathogenicity tests and reisolation

The pathogenicity was proved under glass house conditions using healthy seed of susceptible sorghum cultivar Pant Chari-4. Ten seeds were sown in 30 cm plastic pots filled with sterilized soil. As to obtain 30 days old seedlings for inoculation, these pots were kept in glass house and irrigated with water regularly to maintain high moisture conditions. Before inoculation only 5 seedlings per pot were maintained, rest were uprooted. Spore suspension of 15 days old culture of *G. sorghi* was made by adding sterilized distilled water in the culture tube and strained through cheese cloth before spraying on the plants. 30 days old plants were artificially inoculated by spraying the spore suspension (6×10^4 spores /ml) on the sorghum plants and sterilized water was sprayed on control plants. Immediately after inoculation plants were placed in moist chamber for 48-72 hours and then transferred in glass house having a temperature of about 25 ± 1^0C. The symptoms expressed were studied and reisolation was made. The pathogenicity test as above was repeated once more to confirm the result.

3.2 *In vitro* evaluation

3.2.1 Biocontrol agents

Isolates of *T. harzianum* and *P. fluorescens* and an isolate of *G. intraradices* were used throughout the course of investigation. *T. harzianum* and *P. fluorescens* isolates were obtained from Biocontrol Lab. of Deptt. of Plant Pathology, while *G. intraradices* isolate was obtained from Deptt. of Biological Sciences, G. B. Pant University of Agriculture and Technology, Pantnagar.

3.2.2 Dual culture screening

Thirteen *T. harzianum* isolates (viz. TH-R, 6, 10, 15, 18, 25, 28, 31, 32, 36, 38, 39, and 43) and five *P. fluorescens* isolates (viz. Pf-2, 12, 15, 30 and 31) were screened for their antagonistic potential against the pathogen following dual culture technique **(Morton and Stroube, 1955).** Twenty ml of sterilized melted OMA was aseptically poured in a sterilized 90 mm diameter petriplates and allowed to solidify. Five mm of mycelial disc *of G. sorghi* and test biocontrol agents cut with the help of sterilized cork borer from the edge of 4 days old culture plates, were placed on solidified OMA in such a manner that they lie just opposite to each other (approximately 6 cm apart from each other). Inoculated petriplates were incubated at 28 ± 1^{0}C. The process was replicated 3 times and the observations were recorded seven day after incubation. The colony diameter of the test fungus in the treatment in comparison with that of check gave growth inhibition percent by the following formula:

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent inhibition

C = Radial growth in check in mm

T = Radial growth in treated plates in mm

3.2.3 Seed Germination and Seedling vigour

To know the effect of bioagents on the seed germination and vigour of sorghum cv. PC-4 and PC-5, 50 seeds for each treatment were subjected to rolled paper towel method **(ISTA, 1996**) in which the seeds were incubated according to the standard procedures of ISTA. On the 8th day of incubation, seeds were evaluated.

3.2.3.1 Seed germination Assessment

Fifty seeds in three replications for each treatment were assessed and germination tests were conducted using paper towel **(ISTA 1996)**. The number of normal seedlings was counted at two days interval and cumulative germination obtained on the 8th day was recorded. Percent seed germination was then expressed as number of seeds germinated over total number of seeds plated.

3.2.3.2 Seedling vigour index

Ten seedlings in each replication of germination test were collected at random from the test (final count) by paper towel method and the seedling length (root and shoot) was measured. Seedling vigour index was calculated using the following formula (**Baki and Anderson, 1973)**.

Vigour index (VI) = (Mean shoot length + mean root length) x Germination (%)

3.3 Glasshouse screening

3.3.1 Plant growth response (PGR)

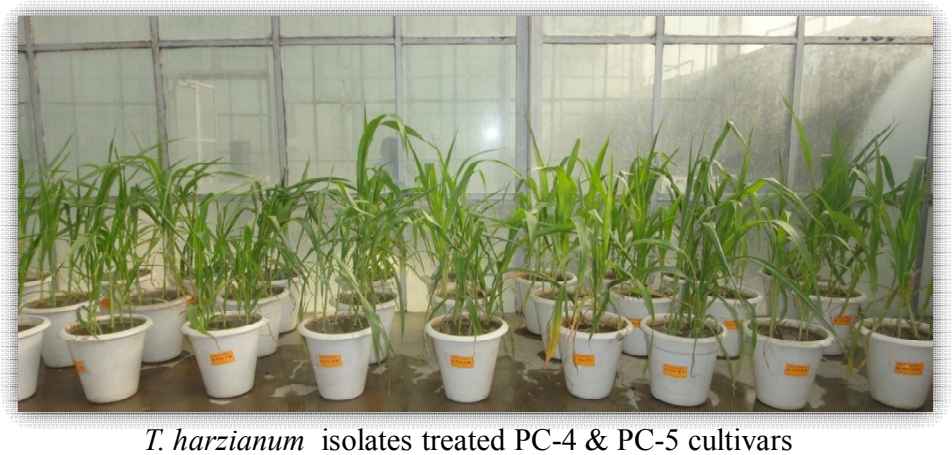
In order to assess the growth promoting/ inhibitory effect of the *P fluorescens* and *T. harzianum* isolates, seed biopriming methods was employed.

3.3.1.1 Seed Biopriming / Seed Bacterization

3.3.1.1.1 *Pseudomonas fluorescens*

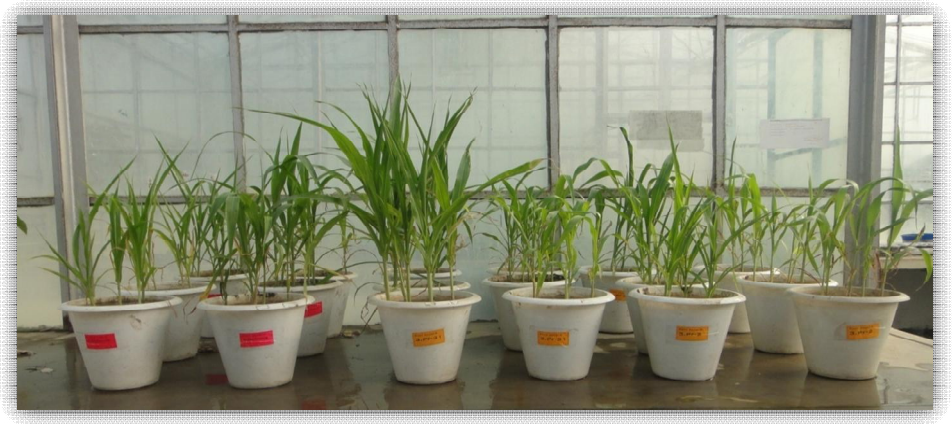
Among several methods developed for application of BCAs, the seed bacterization (treating/ coating seeds with BCAs inoculum) is the most common one **(Chao *et al.*, 1986)**. Sorghum (cv. Pant Chari 4 and Pant Chari 5) seeds were surface disinfected with 1% sodium hypochlorite (NaOCl) for 2 min. and air dried. Bacterial isolates were grown on King's B medium at 28±2°C for 48hrs. Final concentration of 10^8cfu/ml was adjusted and prepared slurry by suspending in 1% (w/v) carboxy methyl cellulose (CMC). A known number of seeds were added to the slurry and treated to obtain uniform coating. These seeds were spread on plastic sheets and air dried for 8-10 hrs. Seeds treated with 1% (w/v) CMC alone served as check. The pots of 30 cm size were filled with sterilized soil and then the coated seeds of susceptible sorghum cultivar Pant Chari-4 and moderately resistant cultivar Pant Chari -5 were sown in different pots filled with sterilized soil. Thirty days old seedlings were artificially inoculated by spraying the spore suspension of the pathogen containing 6×10^4 spores/ml. The observations regarding germination percentage, root, shoot length and stem diameter of plants were taken into account at different time intervals i.e. 15, 30 and 45 DAS and disease severity was recorded at 45 and 65 DAS. Each treatment was replicated thrice. Congenial growth conditions were provided to the experimental plants **(Plate 1)**.

3.3.1.1.2 *Trichoderma harzianum*



T. harzianum isolates treated PC-4 & PC-5 cultivars

PC-4 & PC-5 Control



P. fluorescens and *G. intraradices* isolates treated PC-4 & PC-5 cultivars

Plate 1: Effect of *T. harzianum, P. fluorescens* and *G. intraradices* isolates on growth promotion in PC-4 and PC-5 under glasshouse condition

The pots of 30 cm size were filled with sterilized soil and then the bioprimed seeds of susceptible sorghum cultivar Pant Chari-4 and moderately resistant cultivar Pant Chari - 5 were sown in different pots filled with sterilized soil. *T. harzianum* isolates were used @ 10 g/ kg ($2x10^7$cfu/ml) of sorghum seeds for each treatment. Each isolate was mixed with 1% (w/v) CMC and then seeds were spread on plastic sheets and air dried for 8-10 hrs to ensure full coverage of *T. harzianum* isolates around seeds. The CMC solution acts as a sticking material here and keeps the bioagents glued to the seed surface. Pots were kept inside greenhouse and watered daily to give them high moisture conditions. After 20 days all the other seedlings were uprooted from each pot to keep only 5 seedlings per pot. Thirty days old seedlings were artificially inoculated by spraying the spore suspension of the pathogen containing 6×10^4 spores/ml. The observations regarding germination percentage, root, shoot length and stem diameter of plants were taken into account at different time intervals i.e. 15, 30 and 45 DAS and disease severity was recorded at 45 and 60 DAS. Each treatment was replicated thrice. Congenial growth conditions were provided to the experimental plants. The inoculation of the pots was done when plants were one month old.

3.3.1.2 Soil Drenching

3.3.1.2.1*Glomus intraradices*

Sorghum seeds of both cultivars PC-4 and PC-5 were grown in 30 cm size pots filled with autoclaved sand–soil mixture (9:1 v:v). After one week, soil was drenched carefully upto 3cm from the top and inoculated with 1 gm soil containing *Glomus intraradices* spores. The inoculum used for the pots consisted of chopped roots of pot cultures earlier planted with *Sorghum bicolor* and grown for 6 months in a glasshouse. Fifty grams of AMF inoculum was thoroughly mixed into each pot that received approximately 1000 spores of the AMF species contained at least 20 infective propagules of AMF per gram of chopped root. In non mycorrhizal treatments, each pot was filled with same amount of mycorrhizae free substrate. The observations regarding germination percentage, root, shoot length and stem diameter of plants were taken into account at different time intervals i.e. 15, 30 and 45 DAS and disease severity was recorded at 45 and 60 DAS. Each treatment was replicated thrice. Congenial growth conditions were provided to the experimental plants. The inoculation of the pots was done when plants were one month old.

3.3.2 Plant growth parameters

3.3.2.1 Germination percent

It was recorded after 15 days of sowing with following formula:

$$\text{Germinatio n \%} = \frac{\text{seed germinated}}{\text{Total seeds sown}} \times 100$$

3.3.2.2 Disease severity

The observations on disease severity were recorded in 1-5 scale for each treatment taking random samples. Following formula was used to calculate the percent disease severity:

$$\text{Per cent disease severity (S)} = \frac{\text{Sum of numerical rating}}{\text{Total no. of samples} \times \text{Maximum rating grade}} \times 100$$

3.3.2.3 Shoot length

Plant height was measured from the soil base to the tip of fully expanded leaf and values were recorded in cm.

3.3.2.4 Root length

Root length was measured from the base of the stem to the end of root tip, values were recorded in cm.

3.3.2.5 Stem diameter

For Stem diameter the stem is cut into two and diameter was measured in cm.

3.4 Field trials

Field experiments were conducted during the *Kharif* season of 2013 at Livestock Research Centre, Sorghum Pathology Block, G.B. Pant University of Agriculture and Technology, Pantnagar to evaluate growth promotion activity, biocontrol potential, cholorophyll content and green fodder yield in response to treatment with selected bioagents against the pathogen in susceptible and moderately resistant cultivars of sorghum i.e. PC-4 and PC-5 respectively. Trials were laid out in Randomized Block Design (RBD) with three replications. The observations regarding germination percentage, root, shoot length and stem diameter of plants were taken into account at different time intervals i.e. 15, 30 and 45 DAS and disease severity was recorded at 45 and 65 DAS. Sample collection for estimation of chlorophyll content in leaves was done at 65 DAS.

3.4.1 Biological control agents

G. intraradices isolate*, T. harzianum* and *P. fluorescens* isolates found effective in glass house experiments were further evaluated in field trials. *T. harzianum* isolates Th-R, 18, 28 , 31 and 32 while *P. flouresence* isolates viz. Pf-2 and 31 were used in field trials through seed biopriming. *G. intraradices* was applied as soil drenching one week after sowing as per procedure described in glasshouse experiment.

3.4.2 Seed Biopriming

Seeds were bioprimed with *T.harzianum* and *P. flouresence* isolates just before sowing as per procedure described in glass house experiments. After properly mixing up biocontrol agents with seeds, sowing was done as per randomization. The observations regarding germination percentage, root, shoot length and stem diameter of plants were taken into account at different time intervals i.e. 15, 30 and 45 DAS and disease severity was recorded at 45 and 65 DAS. Each treatment was replicated thrice.

3.4.3 Preparation of the field

The field was first got ploughed in the first week of June using soil turning plough. The field was then harrowed twice by using disk harrow for better pulverization and leveled with leveler.

3.4.4 Fertilizer schedule

Recommended dose of P_2O_5 (50 Kg/ha) and K_2O (40 Kg/ha) in the form of single superphosphate and Muriate of Potash were broadcasted and mixed thoroughly before sowing. Nitrogen in the form of Urea @ 120 Kg/ha was applied in two split doses. First half was applied along with phosphorus and potash whereas, the second half was top dressed after 40 days of sowing.

3.4.5 Field layout

The experiment was carried out in randomized block design with three replications. Sorghum cultivars viz. Pc-4 and PC-5 were planted in two rows of 6 m length with a sub plot of 2x1m at Sorghum Pathology Block, Livestock Research Centre of the University.

3.4.6 Sowing and post sowing operations

The sowing was done during *Kharif* season, 2013. The spacing dimensions were 45 × 15 cm. The seed was sown @ 15 Kg/ha at the depth of 3-4 cm. Weeding and irrigation

were done from time to time, as and when required. Thinning was done to maintain the distance of 15 cm between plant to plant after 25 DAS.

3.4.7 Artificial inoculation

All the plants in the first row of each cultivar were artificially inoculated by spraying the spore suspension of *G. sorghi* between 6-7 pm after 30 days of sowing as night temperature and humidity are conducive for infection.

3.4.8 Preparation of inoculum on sorghum grains

The test fungus *G. sorghi* isolated and purified from fresh diseased leaves of sorghum was used throughout the investigation. For preparation of inoculum sorghum grains were used soaked in water for 24hrs, after thorough washing in running tap water. The soaked 50gm grains were filled in 250 ml Erlenmeyer flask after removing excess water. Grain filled flask was shaken to prevent formation of grain clumps. The sorghum grain medium was inoculated with actively growing culture of *G. sorghi* in OMA plates. One to two discs cut from the fungus colony were seeded in each flask aseptically and then flasks were incubated at 28 ± 1^{0}C for 10-15 days. During incubation, the grains in flasks were regularly shaken for uniform fungal growth on all grains was then used as inoculums for artificial inoculation in field experiments.

3.4.9 Observation on Plant growth parameter

Seed germination, root length, shoot length and stem diameter was recorded as per formula and method described in glasshouse screening.

3.4.10 Disease observation

Observations on incidence of the disease was recorded in 1 to 5 scale proposed by All India Coordinated Sorghum Improvement Project before boot leaf emergence and one week before harvesting as follows:

1 = Highly resistant (No symptoms)

2 = Resistant (upto 10% intensity)

3 = Moderately resistant (11-25% intensity)

4 = Susceptible (26-50% intensity)

5 = Highly susceptible (above 50% intensity)

Following formula was used to calculate the percent disease severity:

$$\text{Per cent disease severity (S)} = \frac{\textit{Sum}\ \text{of numerical rating}}{\text{Total no. of samples} \times \text{Maximum rating grade}} \times 100$$

3.4.11 Chlorophyll content

Dimethyl sulfoxide solvent (DMSO) was used for chlorophyll extraction from leaf of both cultivars of sorghum in dark according to method described by **Hiscox and Israelstam (1979)**. Absorbance of extracts was read by spectrophotometer at 645 and 663 nm. Chlorophyll *a* (mg/gm fresh weight), Chlorophyll *b* (mg/ gm fresh weight) and total chlorophyll content (mg/ gm fresh weight) were calculated from absorbance at 663 nm and 645 nm according to **Arnon's (1949)** equations:

$$\text{Chlorophyll } a = \frac{\text{(ml solvent) } [(0.0127 \times \text{Absorbance 663}) - (0.00269\ \text{Absorbance 645})]}{\text{Leaf fresh weight (gm)}}$$

$$\text{Chlorophyll } b = \frac{\text{(ml solvent) } [(0.0229 \times \text{Absorbance 645}) - (0.00468\ \text{Absorbance 663})]}{\text{Leaf fresh weight (gm)}}$$

$$\text{Total chlorophyll} = \frac{\text{(ml solvent)}[(0.0202 \times \text{Absorbance 645}) + (0.00802\ \text{Absorbance 663})]}{\text{Leaf fresh weight (gm)}}$$

3.4.12 Green fodder yield

At the time of harvesting of crop, green fooder yield was estimated of both the cultivars of sorghum. Total yield in kilograms was recorded per sub plot (2 x 1m) of each treatment.

3.4.13 Statistical analysis

The data was analyzed statistically at the computer centre of G.B. Pant University of Agriculture and Technology, Pantnagar, using Completely Randomized Design (CRD) and Randomized Block Design (RBD). The treatments were compared by the means of critical differences (CD) at 5% level of significance.

3.5 Expression of defense response genes

3.5.1 Experimental Scope and Design

This research was designed to quantify defence response genes in two cultivars of sorghum during priming which is induced by *Trichoderma harzianum* (Th-32), *Pseudomonas fluorescens* (Pf-31) and *Glomus intraradices* isolates and carry out a comparative study of boosting (post-inoculation with *Gloeocercospora sorghi*) and *Gloeocercospora sorghi* induced defence response (GIDR). This study was divided into 3 parts:

1. Quantification of defence response genes during **priming** induced by Th-32, Pf-31 and *G. intraradices* isolates in PC-4 and PC-5 cv. of sorghum.
2. Quantification of defence response genes during **boosting** induced by Th-32, Pf-31 and *G. intraradices* isolates in PC-4 and PC-5 cv. of sorghum inoculated with *G.sorghi*.
3. Quantification of defence response genes during **GIDR** in PC-4 and PC-5 cv. of sorghum inoculated with *G.sorghi*.

3.5.2 Glasshouse experiment

3.5.2.1 Biological Material

Several biocontrol isolates of *T. harzianum, P. flouresence* and *Glomus intraradices* were screened for their plant growth promotion and biocontrol potential against *G. sorghi* in Sorghum cultivars *viz*. PC-4 and PC-5. Out of which Th-32, Pf-31 and Glomus were used for gene expression studies.

3.5.2.1.4 Pathogen

The pathogen was periodically sub-cultured onto OMA and grown in darkness at 26°C to maintain actively growing colonies used to induce sporulation for this study. Spores of *G. sorghi* were used in boosting and GIDR experiments. Sporulation was induced by growing colonies on OMA at 26°C with backlight illumination for at least 1.5 weeks.

3.5.2.2 Preparation of spore suspension of bioagents and the pathogen

3.5.2.2.1 *T. harzianum* (Th-32)

The antagonist was multiplied on PDA for 5 days under 12 hr dark and 12 hr light regime and the density of conidia was adjusted to $2x10^7$/ml with the help of hemocytometer.

3.5.2.2.2 *P. fluorescens* (Pf-31)

Pseudomonas fluorescens (Pf-31) isolate was grown for 24 h at 28°C on King's medium B agar plates (King et al., 1954). Subsequently, bacteria were collected and resuspended into double distilled water maintained a density of 10^9 cfu/ml using hemocytometer.

3.5.2.2.3*Glomus intraradices*

The inoculum used for the pots consisted of chopped roots of pot cultures earlier planted with *Sorghum bicolor* and grown for 6 months in a glasshouse. Fifty grams of AMF inoculum was thoroughly mixed into each pot that received approximately 1000 spores of the AMF species contained at least 20 infective propagules of AMF per gram of chopped root. In non mycorrhizal treatments, each pot was filled with same amount of mycorrhizae free substrate.

3.5.2.2.4 Sporulation, Conidia harvesting and Counting

Conidia of *G. sorghi* were used for inoculation. To induce conidia formation, samples of fast growing mycelia maintained on OMA were cultured at 26°C with backlight illuminati on for at least 1.5 weeks. *G. sorghi* conidia-laden OMA plate was flooded with 20 ml of sterile double distilled water containing 0.01% Tween 20 and gently scrapped with a sterile plastic brush to free conidia from the sporodochium. The suspension was filtered through a 50 nm nylon filter. The conidia-containing stock filtrate was immediately set on ice until used for infection. Serial dilutions of the stock filtrate were pipetted onto a hemocytometer and used to estimate the conidia count under a light microscope. Inoculum suspension of 6×10^4 conidia/ml was used in all infection experiments.

3.5.2.3 Bioagents and pathogen treatment for priming and boosting study

3.5.2.3.1 *T. harzianum* Th-32 and *P. fluorescens* Pf-31

Pant chari 4 and Pant chari 5 were grown in pots filled with sterilized soil in glasshouse with 27°C daytime temperature, 18°C night-time temperature and 75% humidity. Twenty three days old plants were treated with Th-32 ($2x10^7$/ml) and Pf-31 (2×10^9/ml) by pipetting a 50µl spore suspension directly onto the roots. Control plants were supplemented with an equal volume of sterile water.

3.5.2.3.2 For *G. intraradices*

Seeds of both cultivars PC-4 and PC-5 were grown in 30 cm size pots filled with autoclaved sand–soil mixture (9:1 v:v). AMF inoculum was mixed in pots as per the procedure described earlier.

3.5.2.3.3 Pathogen inoculation

For boosting study, seven days later these and equal number of mock-treated plants were inoculated with *Gloeocercospora sorghi.* Briefly, a 5µl drop of a *G. sorghi* spore suspension (6 x 10^4 conidia/ml) was inoculated onto two leaves per plant.

3.5.2.4 Sample collection for priming and boosting experiments

3.5.2.4.1 For priming

Six different sets of leaves from mock as well as Th-32 and Pf-31 treated PC-4 and PC-5 cultivars were collected upto 8 days after treatment and used for RT-PCR to study gene expression during priming. Whereas in *G. intraradices* prime study was done 15 days after treatment and subsequently the samples were collected following 8 days.

3.5.2.4.2 For boosting

Boosting study was started after 30 days form sowing. Leaves were collected from five of the same sets of mock treated [**Sb**(PC-4 and PC-5)+ ***G.sorghi***] and Th-32, Pf-31 and Glomus [**Th-32**(PC-4 and PC-5), **Pf-31**(PC-4 and PC-5) and **Glomus**(PC-4 and PC-5)] treated plants after additional *G. sorghi* inoculation.

Treatment	Control
Sorghum cv. PC-4 and PC-5 treated with bioagents	Mock treated plants of Sorghum cv. PC-4 and PC-5
Sorghum cv. PC-4 and PC-5 treated with bioagents followed by *G. sorghi* inoculation	Mock treated plants of Sorghum cv. PC-4 and PC-5 followed by *G. sorghi* inoculation
Plants of Sorghum cv. PC-4 and PC-5 followed by *G. sorghi* inoculation	Mock treated plants of Sorghum cv. PC-4 and PC-5

Study involved	Annotation					
	PC-4	PC-5	PC-4	PC-5	PC-4	PC-5
PRIMING	Sb+Th Vs Sb	Sb+Th Vs Sb	Sb+Pf Vs Sb	Sb+Pf Vs Sb	Sb+Gi Vs Sb	Sb+Gi Vs Sb
BOOSTING	Sb+TH+Gs Vs Sb+Gs	Sb+TH+Gs Vs Sb+Gs	Sb+Pf+Gs Vs Sb+Gs	Sb+Pf+Gs Vs Sb+Gs	Sb+Gi+Gs Vs Sb+Gs	Sb+Gi+Gs Vs Sb+Gs
GIDR	Sb+Gs Vs Sb	Sb+Gs Vs Sb	Sb+Gs Vs Sb	Sb+Gs Vs Sb	Sb+Gs Vs Sb	Sb+Gs Vs Sb

3.5.2.5 Disease Assays

Disease symptoms in both cultivars treated or untreated (control) with bioagents were scored by measuring the diameters of the necrotic lesions on various days after *G. sorghi* inoculation. Each day, lesions diameters were measured and the average lesion diameters of treated and untreated plants were compared using a two-sample one-sided Student's *t*-test.

3.5.3 RT-PCR

RT-PCR is a combination of three steps: (i) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (ii) the amplification of the cDNA using the PCR and (iii) the detection and quantification of amplification products in real time (Gibson et al., 1995). Real time PCR is based on the detection and quantification of a fluorescent reporter **(Lee, 1993, Livak, 1995)**. The signal increases in direct proportion to the amount of PCR product in the reaction. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the cycles indicates the detection of accumulated PCR product. A fixed fluorescence threshold is set significantly above the baseline that can be altered. The CT (threshold cycle) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. When a single PCR product is amplified, rather than using a fluorescent probe, it is much more economical to use an SYBR Green, a double-stranded DNA dye in the PCR reaction since it binds to newly synthesized double-stranded DNA and gives fluorescence**.**

3.5.3.1 RNA Extraction and Purification

3.5.3.1.1 RNA Extraction

Hundred milligrams of fresh sample of leaf were collected and homogenized in liquid nitrogen using pre chilled motar and pestle. Total RNA was extracted to obtain pure and intact RNA from the tissues using **MB601 RNA - XPress TM Reagent** RNA isolation kit (Hi-Media) **(Plate 2)**.

Protocol

1. Sample Preparation

Homogenize tissue samples in RNA-XPress Reagent (1 ml for 50-100 mg of tissue) in a Homogenizer with serrated pestle homogenizer.

2. Phase separation

Incubate the homogenized samples for 5 minutes at room temperature (15-25°C) to permit the complete dissociation of nucleoprotein complexes. Add 200 µl of Chloroform per ml of RNA-Xpress reagent used. Cover the sample tightly, shake vigorously for 15 seconds and allow to stand for 10 minutes at room temperature (15-25°C). Centrifuge the resulting mixture at 12,000 x g (≈ 13,000 rpm) for 15 minutes at 4°C. Following centrifugation, mixture separates into lower deep red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase containing RNA.

3. RNA Precipitation

Transfer the aqueous phase containing RNA to a fresh tube and add 500 µl of Isopropyl alcohol. Allow the sample to stand for 5-10 minutes at room temperature (15-25°C). Centrifuge at 12,000 x g (≈ 13,000 rpm) for 10 minutes at 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4. RNA Wash

Remove the supernatant and wash the RNA pellet by adding 1 ml (minimum) of 75% ethanol. Vortex the sample and then centrifuge at 7,500 x g (≈10,500 rpm) for 5 minutes at 4ºC.

5. Redissolving the RNA

Briefly dry the RNA pellet for 5-10 minutes by air-drying. Add an appropriate volume of RNase-Free Water to the RNA pellet. To facilitate dissolution, mix by repeated pipetting with a micropipette. Incubate at 55-60ºC for 10-15 minutes. Storage of the eluate with purified RNA: The eluate contains pure RNA, recommended to be stored at lower temperature (-80°C).

3.5.3.1.2 Quantification of the isolated RNA

Quantification of the RNA content was done by diluting the samples with RNase-free water. Absorbance of the samples was measured at 260 and 280 nm. A260 reading of 1.0 is equivalent to 40 mg/ml of RNA. The purity of the sample was calculated from the A260/A280 ratio. For values higher than 1.6, the RNA purity was considered acceptable.

3.5.3.1.3 Electrophoretic separation of RNA

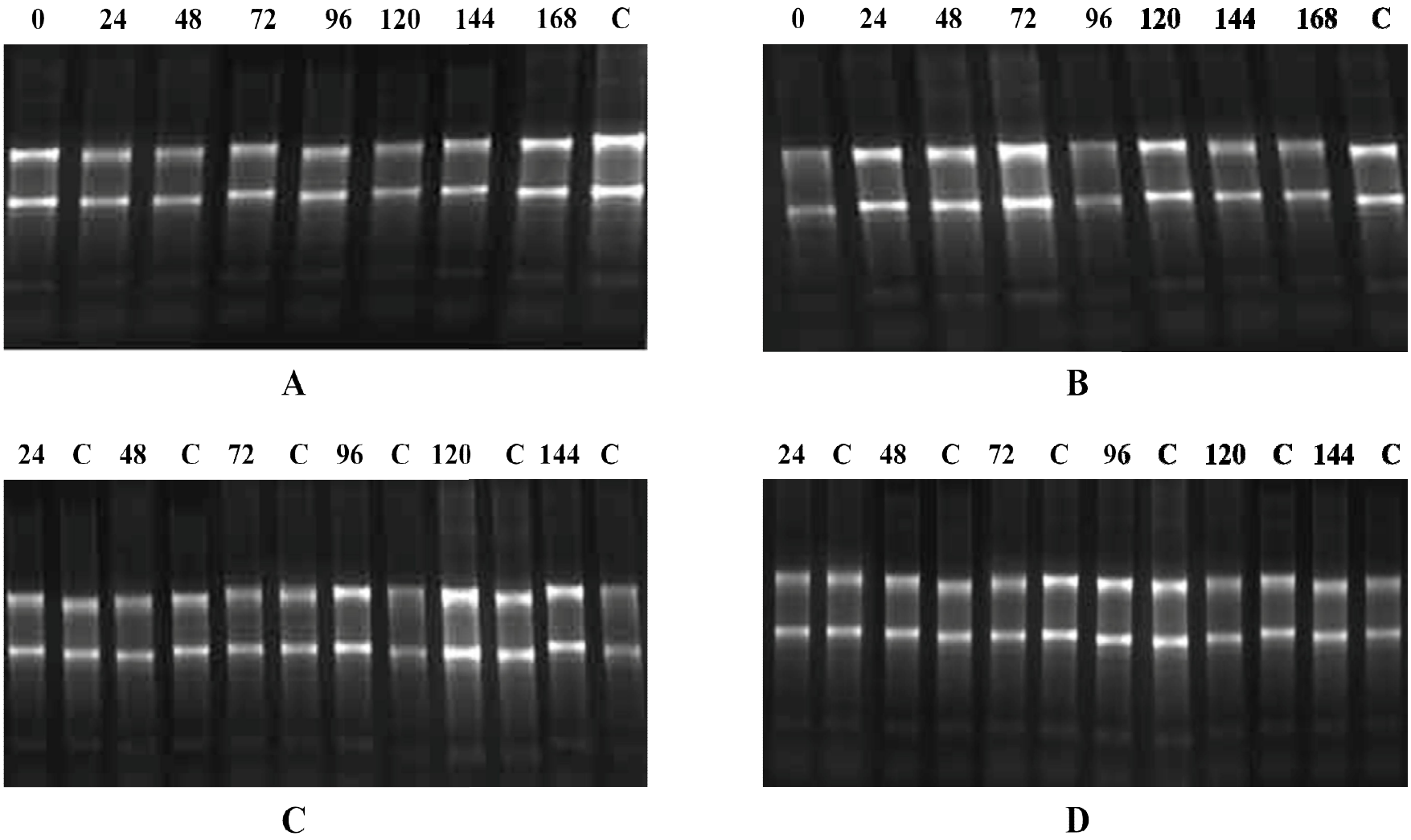


Plate 3: Total RNA isolated of both susceptible (PC-4) and moderately resistant (PC-5) cultivars of *Sorghum bicolor* during priming **(A & B)** and boosting **(C & D)** at different time intervals (HPT & HPI) respectively.

0.8% Agarose was melted in 1X TAE buffer [50X TAE buffer (40 mM Tris acetate, pH: 7.5, and 1 mM ethylenediamine tetraacetic acid) diluted with diethylpyrocarbonate- water]. After cooling down to about 60°C, 0.5 mg/ml ethidium bromide (EtBr) was added. For solidification, the gel medium was poured on horizontal gel trays equipped with combs to form vertical gel loading pockets. The polymerized gels were transferred into a RNase-free gel tank containing 1X TAE buffer. Two microliters of RNA were mixed with 6X loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene glycol, 30% (v/v) glycerol) and loaded in the wells. The RNA was separated on the gels at 100 volts for 1 h.

3.5.3.2 DNase I Treatment

For RT-PCR applications, template RNA must be free of DNA contamination. Prior to cDNA synthesis, RNA is treated with DNase I, RNase-free to remove trace amounts of DNA.

Removal of genomic DNA from RNA preparations

1. Add to an RNase-free tube:

RNA	1 µg
10X reaction buffer with MgCl2	1 µl
DNase I, RNase-free (HI- Media, ML068- 1ml)	1 µl
Water, nuclease-free	to 10 µl

2. Incubate at 37°C for 30 min.

3. 1 µl 50 mM EDTA and incubate at 65°C for 10 min. RNA hydrolyzes during heating with divalent cations in the absence of a chelating agent.

4. This prepared RNA used as a template for reverse transcriptase.

3.5.3.3 cDNA synthesis

cDNA was synthesized from total RNA by **RevertAid First Strand cDNA Synthesis Kit** (Thermo Scientific, K1622). Components of the kit were thawed, mixed and briefly centrifuged, and stored on ice prior to use.

After thawing, mix and briefly centrifuge the components of the kit. Store on ice.

1. Adding the following reagents into a sterile, nuclease-free tube on ice in the indicated order:

Template RNA	Total RNA	1µl
Primer	Oligo $(dT)_{18}$ primer	1µl
Water, nuclease-free	-	to 12 µl

2. Adding the following components in the indicated order:

5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20 µl=l)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV Reverse Transcriptase (20 µ/µl)	2 µl
Total volume	20 µl

3. Mixing gently and centrifuge.

4. Oligo(dT)18 or gene-specific primed cDNA synthesis, incubate for 60 min at 37°C.

5. Terminating the reaction by heating at 70°C for 5 min.

The reverse transcription reaction product is directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended.

3.5.4 Real-time (qRT)-PCR

3.5.4.1 qRT- PCR

Real-time PCR was carried out using **Maxima SYBR Green/ROX qPCR Master Mix (2X)** (Thermo Scientific)

Protocol

Reaction set-up

Gently vortex and briefly centrifuge all solutions after thawing.

1. Prepare a reaction master mix by adding the following components (except template DNA) for each 25 µl reaction to a tube at room temperature:

SYBR Green Master Mix (2X)	12.5 µl
Forward Primer	0.3 µM
Reverse Primer	0.3 µM
Template DNA	≤500 ng
Water, nuclease-free	to 25 µl
Total volume	25 µl

2. Mixing the master mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
3. Adding template DNA to the individual PCR tubes or wells containing the master mix.
4. Gently mixing the reactions without creating bubbles (without doing vortex).
5. Program the thermal cycler according to the recommendations below, place the samples in the cycler and start the program.

Thermal cycling conditions

The PCR parameters were: 10 min at 95˚C, 40 cycles of amplification (10 s at 95˚C, 10 s at 58˚C, 10 s at 72˚C) and a melting curve stage (15 s at 95˚C, 1 min at 60˚C increased to 95˚C with steps of 0.3˚C).

3.5.4.2 Analysed genes

A summary of the primers used, their nucleotide sequence and annealing temperatures, and number of PCR cycles used is shown in **Table 3.1**.

Table 3.1. Summary of primers used for gene expression studies.

Name	Primer Designation	Oligo Sequence	Tm	No. of cycles	Length
Actin	**Forward**	AGGCGCAGTCCAAGA GGGGTA	60	40	21
	Reverse	TGGAAGTTGTGGGGGGCGGTA	60		21
PAL	**Forward**	AAGAAGGTGAACGAGCTGGA	60	40	20
	Reverse	GTCGTTGACGGAGTTGACCT	60		20
CHI	**Forward**	GCTTCGGACTCAGCATTCTC	59	40	20
	Reverse	GCAGCTAATGAATCCCCTGA	59		20
CHS	**Forward**	GTCATCACCATGCACCTCAC	59	40	20
	Reverse	CCAGAACAGGTCGTTCCATT	59		20

3.5.4.3 Calculations using Comparative C_T Method ($\Delta\Delta C_T$ Method)

Real time PCR results were expressed as Ct (cycle threshold) values. This value corresponded to the cycle at which the fluorescence of the SYBR Green dye reached above the threshold or background fluorescence value. In quantifying gene expression, the mRNA levels of the gene of interest were divided by the mRNA levels of the housekeeping gene actin. This normalized for variations in concentration and quality of mRNA among the samples. The calculation of normalized relative quantities (conversion of quantification cycle values (Cq) into normalized relative quantities (NRQs) was first reported by **Livak and Schmittgen (2001**) as NRQ = 2ΔΔCt. Pfaffl modified the model by adjusting for differences in PCR efficiency between gene of interest (goi) and reference genes. In the second step, sample and gene names can be easily annotated or modified. In the next step, reference genes were selected and the quality of raw data examined so that negative or aberrant samples would not be used in comparisons. The samples were ordered and selected. The amplification efficiencies were determined where all quantification models transform (logarithm) quantification cycle values into quantities using an

exponential function with the efficiency of the PCR reaction in its base. Evaluation of normalization was the next step. Normalization can be monitored by inspecting the normalization factor for all samples or by calculating reference gene stability parameters.

3.5.4.4 Data analysis

The experiment was done in triplicates, unless otherwise stated. The minimum sample size used in this assay was 30. Data figures were composed from averages from experiment and triplicates. ANOVA and Turkey's test (Post hoc test) were used to determine significance of observed differences. Statistical significance was determined with 95% level of confidence of $P < 0.05$ at respective degree of freedom (df) - P 0.05, IBM SPSS v.16 computer software was used for analysis. Data were presented as values with ±standard deviations.

RESULTS AND DISCUSSION

4.1 Symptomatology

The symptoms of the disease developed especially when the temperature was high and the weather was cloudy. The spread of the disease in cloudy weather was very rapid.Leaf and leaf sheaths of the plants are the sites of attack of the pathogen. The initial symptoms appear in the form of lesions, which are red brown; water soaked and sometimes with a narrow, pale green halo, when enlarged, become dark and elongated parallel to the vein. They are numerous in number as to give the appearance of irregular blotches. The lesions occur along the leaf margin or near the midrib and eventually become large and semicircular or irregular in shape and several inches long. The lesions join to cover most of the leaf surface when attack is severe. These lesions contain reddish purple bands of tissue alternating with tan or straw colored areas to form a characteristic coarsely zonate pattern.In wet warm weather relatively large pinkish gelatinous fructifications easily visible with the naked eyes, are produced in and around the necrotic areas of the lesions. A few weeks after the infection, minute black spherical lenticular sclerotia 0.1 to 0.2 mm in diameter are seen within the dead tissues of the lesions. They are at definite intervals and in lines between and parallel to the veins. The symptoms on the test plants were formed and produced in the manner and accordance to that as described by workers like **Bain and Edgerton, 1943; Tarr, 1962andPuranik and Suryanarayan, 1966.**

4.1.2 Isolation, identification and pathogenicity test

Isolation from the sorghum leaves showing characteristic symptoms of zonate leaf spot yielded the fungal growth on oat meal agar medium. The fungus was identified as *G. sorghi* on the basis of cultural and morphological characteristics. Initially the colonies were thin and loose cottony but turned thick after 7 days and formed a light- pinkish slimy matrix. The fungal mycelium was septate, branched and hyaline. The conidiophores were hyaline, simple or branched, short and septate. They were observed to arise pleurogenously from hyphae as rather densely clustered short branches. After sometime these clusters of conidiophores became dense to form bouquet like aggregates resembling to sporodochia that are formed on leaves.

Conidia developed in pink and beadlike slimy masses. A conidium is attached somewhat on the side of the slightly swelled apex of the conidiophores and by its broadest end. Conidia are either straight or curved, tapering somewhat from the base to apex, few to

many septate, hyaline and elongated to filiform. Average size of the conidia was measured as 2.5 µ × 85µ, but considerable variations in size was observed. Conidium germinated in water in about 4-5 hours. Frequently, the different cells of the conidium produce germ tubes. In one spore 4-5 germ tubes have been observed. The black sclerotia developed in the culture later and they resembled closely with those which developed in the host. These characters agree very closely with those described by **Bain and Edgerton (1943).** For pathogenicity confirmation, after 48-72 hours of incubation of preinoculated plants in the polythene moist chamber, lesions appeared as small, reddish or brownish water soaked spots that have a narrow, pale green halo. Later as the spots enlarge they become darker. They finally form large, semicircular or irregular lesions several centimeters in diameter. A small lesion has a light brown centre surrounded by a light to dark border, but in a larger lesion there may be an alternation of dark and light zones. On re-isolation, the same fungus was obtained in culture.

4.2 Evaluation of antagonists *in vitro*

4.2.1.1 Screening of *T. harzianum* and *P. fluorescens* isolatesagainst *G. sorghi*

Using dual culture method antagonistic potential of 13 isolates of *T. harzianum*and 5 isolates of *P. fluorescens* was evaluated against the pathogen *G. sorghi***(Table 4.1)**. In dual culture test, all the isolates reduced the colony growth of pathogen. Th-32 performed best which gave 85.78 % inhibition of radial growth followed by Pf-31(83.18 %),Th-R (79.35 %), Pf-2 (76.30%), Th-28(75.73%), Th-31 (70.77%) and Th-18 (70.32%)whereas least inhibition of radial growth was observed in, Th-15(40.41), Th-6(41.08%) and Th-43(42.10%).

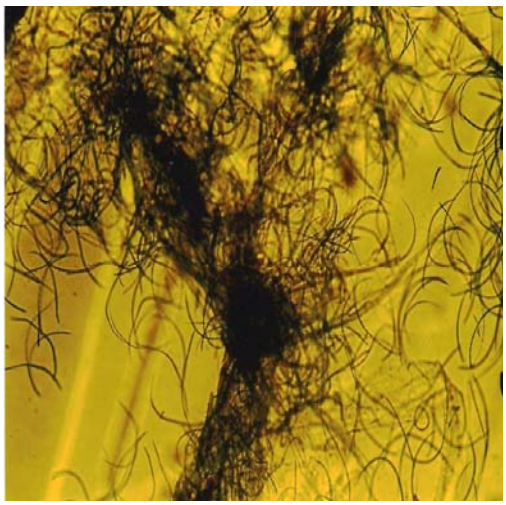
The difference in percent inhibition of radial growth indicates the difference in their antagonistic potential for the test pathogen. These observations are similar to the finding of **Kucuk and Kivance (2004)**. In our results, a clear cut zone of inhibition was observed with all the isolates tested against the pathogen *G. sorghi*. This may be due to mechanism of antibiosis by the antagonists. *Trichoderma* spp. inhibiting the growth of pathogens by the mechanism of antibiosis has been reported by several workers **(Sharma and Doharoo, 1991; Sivan and Chet, 1989; Upadhyay and Mukhopadhyay, 1986; and Howell, 1998).** The results thus obtained indicate a need of*in vitro* testing of more isolates of *T. harzianum*and *P. fluorescens*against the pathogen, which could lead to better ecofriendly management of the disease in future.Similarly, *P. fluorescens* was shown to effectively

A

B

C

D



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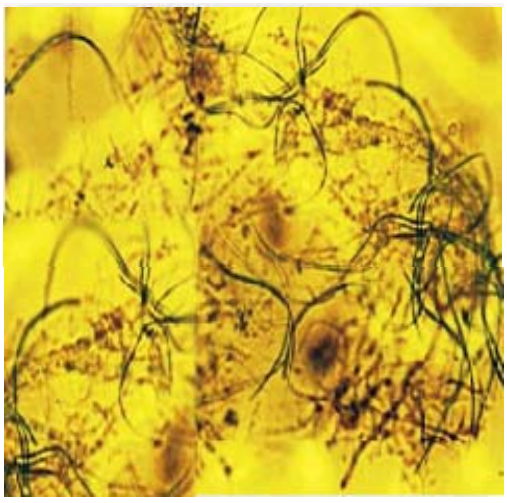


F

Plate 3: Disease symptoms **(A & B)**, mycelial growth of pathogen on OMA **(C & D)** and sporodochium and conidia of formed the pathogen **(E & F)**

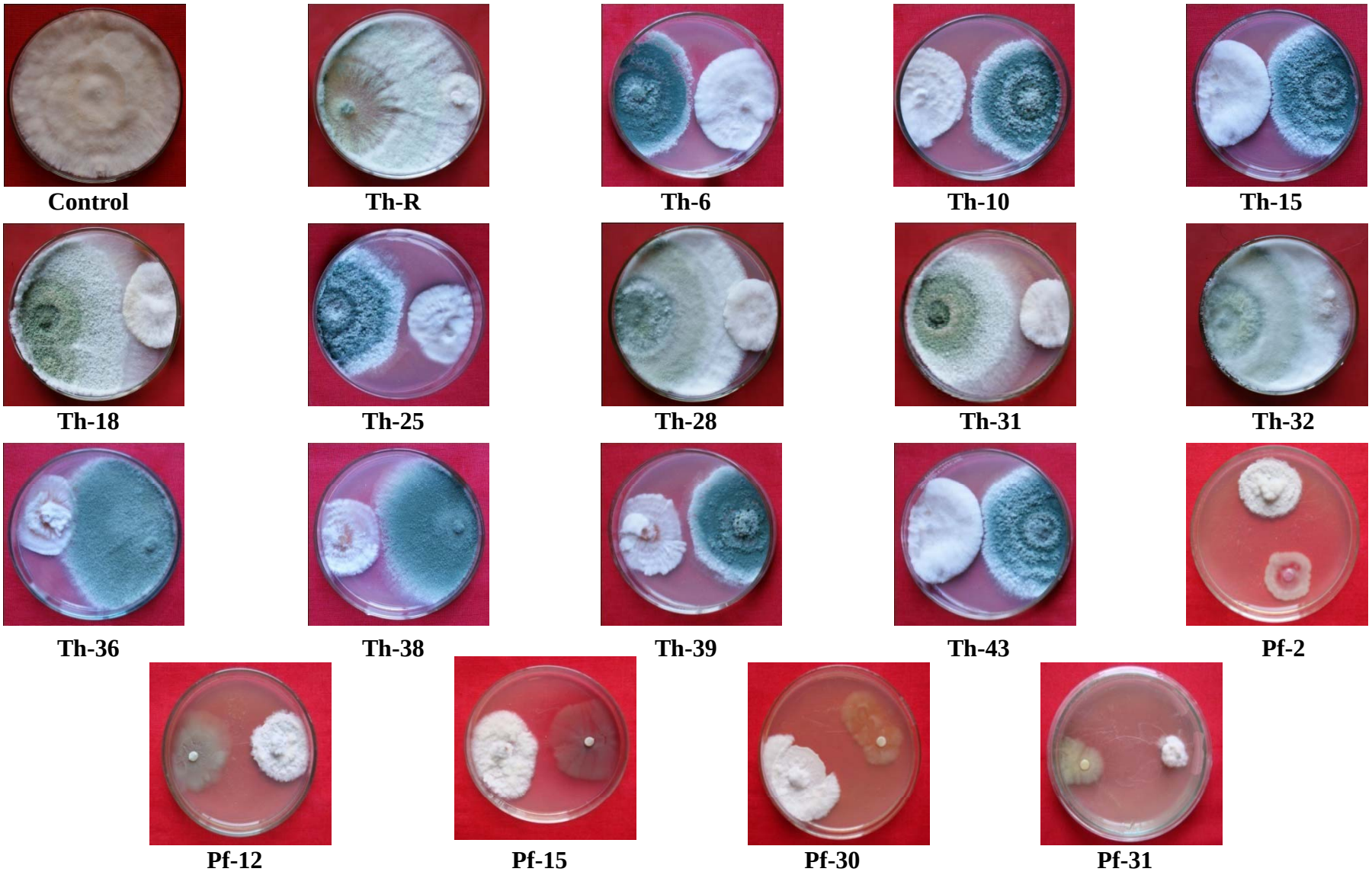


Plate 4. Percent inhibition of radial growth of *G. sorghi* by different isolates of *T. harzianum* and *P. fluorescens*

inhibit R. solani and P. oryzae by agar plate method (**Rosales *et al.*, 1995)**.*In vitro* inhibition by *P. fluorescens* might bedue to the production of Fe- chelating siderophores andhydrogen cyanide which is toxic to pathogenic fungi **(Paulitz and Loper, 1991).** Hence *P. fluorescens* can be explored in agriculture to reduce theincidence of fungal diseases in important crops as reportedpreviously (**Gheorghe*et al.*, 2008).**

Table 4.1: Percent inhibition of radial growth of *G. sorghi* by different isolates of *T. harzianum* and *P. fluorescens*

Treatment No.	Treatment	Radial growth (cm)	Inhibition of radial growth (%)
T1	Th-R	1.83	79.35
T2	Th-6	5.22	41.08
T3	Th-10	4.67	47.29
T4	Th-15	5.28	40.41
T5	Th-18	2.63	70.32
T6	Th-25	4.22	52.37
T7	Th-28	2.15	75.73
T8	Th-31	2.59	70.77
T9	Th-32	1.26	85.78
T10	Th-36	3.58	59.59
T11	Th-38	3.62	59.14
T12	Th-39	4.26	51.92
T13	Th-43	5.13	42.10
T14	Pf-2	2.10	76.30
T15	Pf-12	2.94	66.82
T16	Pf-15	3.85	56.55
T17	Pf-30	4.34	51.02
T18	Pf-31	1.49	83.18
T19	Control	8.86	
	CD at 5%	0.45	-
	CV	7.27-	
	SEM±	0.15	-

4.2.2 Effect of *T. harzianum* and *P. fluorescens* isolates on seedgermination and vigour index.

The collected cultivars of sorghum Pant Chari-4(PC-4)and Pant Chari-5(PC-5)were used for studies on seed germination and vigour index by*T. harzianum* and *P. fluorescens* isolates and data are presented in **Table 4.2 & 4.3** respectively.

4.2.2.1Effect of*T. harzianum*and *P. fluorescens*isolates on seedgermination and vigour index on PC-4

4.2.2.1.1Seed Germination

Highest seed germination was observed in Th-31(88.67%), followed by Pf-31(87.33%), Th-32(86.67%), Th-28(84.67%) and Pf-2(84.67%), whereas least was observed in Th-25(50.33%).

4.2.2.1.2 Shoot length

Th-31(6.27 cm), Th-28(6.17 cm), Pf-31(6.11cm) and Th-32(6.00 cm)resulted in maximum shoot length as compared to other treatments and control(3.70 cm).

4.2.2.1.3Root length

All the treated plants showed significantly higher root length as compared to control. Pf-31(4.77cm) and Pf-2(4.53cm) performed best followed by Th-32 (4.13cm), Th-18(4.07cm) and Th-28(4.03cm).

4.2.2.1.4Plant fresh weight

All the treatments except Th-10 significantly increased the plant fresh weight over control in PC-4cultivar of Sorghum. Maximum fresh weight was recorded withisolates Th-31(4.50gm),Pf-31(4.50gm), Pf-2(4.41gm), Th-32(4.27gm) and Th-28(4.20gm)as compared to control (1.52gm).

4.2.2.1.5Plant dry weight

All the treatments showed significant effect on the plant dry weight in PC-4 variety of Sorghum. Among all the treatments, Pf-2(0.893gm), Pf-31(0.881gm), Th-31(0.870gm), Th-32(0.860gm) and Th-28(0.843gm) isolates were observed increased dry weight as compared to control whereas isolates viz. Th-10(0.290gm), Th-6(0.337gm), Pf-30(0.363gm), Pf-12(0.370gm), Pf-15(0.373gm) and Th-15(0.503gm) were exhibited least dry weight as compared to control (0.243gm)**Table 4.2**.

4.2.2.1.6 Vigour Index (VI)

Treatment with Pf-31 resulted in highest vigour index (950.7 VI) followed by Th-31(910.3 VI), Pf-2(883.3 VI), Th-32(878.2 VI) and Th-28(863.6 VI) whereas Th-25(364.0 VI) was found least effective as compared to control i.e. 267.1VI**Table 4.2**.

Table 4.2:Effect of*T. harzianum* and *P. fluorescens* isolates on seed germination and vigour index of PC-4

Treatment	Germination %	Root length(cm)	Shoot length(cm)	Fresh weight(gm)	Dry weight(gm)	Vigour index
Th-R	71.00	3.97	4.87	2.70	0.593	627.17
Th-6	59.67	3.07	4.03	2.27	0.337	423.63
Th-10	57.33	3.17	3.97	1.93	0.290	408.98
Th-15	59.67	3.57	4.43	2.43	0.503	477.33
Th-18	75.33	4.07	6.00	4.13	0.840	758.36
Th-25	50.33	3.03	4.20	2.30	0.320	364.08
Th-28	84.67	4.03	6.17	4.20	0.843	863.60
Th-31	88.67	4.00	6.27	4.50	0.870	910.31
Th-32	86.67	4.13	6.00	4.27	0.860	878.22
Th-36	58.67	3.17	4.40	2.80	0.528	443.91
Th-38	53.67	3.70	4.07	2.73	0.530	416.81
Th-39	59.00	3.83	4.97	2.93	0.533	519.20
Th-43	57.67	3.53	4.43	2.80	0.503	459.41
Pf-2	84.67	4.53	5.90	4.41	0.893	883.36
Pf-12	58.67	3.76	4.67	3.72	0.370	494.17
Pf-15	53.67	3.89	4.72	3.79	0.373	462.25
Pf-30	59.67	3.67	4.55	3.70	0.363	490.06
Pf-31	87.33	4.77	6.11	4.50	0.881	950.77
Control	46.33	2.07	3.70	1.52	0.243	267.19
CD at 5%	3.32	0.40	0.39	0.47	0.08	-
CV	3.04	6.70	4.77	8.73	8.61	-
SEM±	1.15	0.14	0.14	0.16	0.02	-

4.2.2.2Effect of*T. harzianum*and *P. fluorescens*isolates on seedgermination and vigour index on PC-5

4.2.2.2.1 Seed Germination

Highest germination was observed in Th-32(89.33%), followed by Th-31(88.00%), Pf-31(86.67%), Pf-2(85.33%) and Th-28(85.33%) while in control it was 49.33%.

4.2.2.2.2 Shoot length

Th-28(6.17 cm), Pf-31(6.22cm), Pf-2(6.17cm), Th-32(6.13 cm), Th-18 (6.13 cm) and Th-32(6.03cm) was found most effective in increasing the shoot length.

4.2.2.2.3Root length

All the treated plants showed significantly higher root length as compared to control. Pf-31(5.37cm) and Pf-2(5.17cm) treated plants showed maximum root length followed by Th-32 (4.27cm), Th-18(4.21cm), Th-28(4.20cm) and Th-31(4.08cm) as compared to other treatments and control (2.20cm).

4.2.2.2.4 Plant fresh weight:

All the treatments significantly increased the plant fresh weight over control. However maximum fresh weight was recorded with Pf-31(5.00gm) followed by Pf-2(4.79gm), Th-32(4.40gm), Th-31(4.33gm), as compared to other treatments and control (1.53gm).

4.2.2.2.5 Plant dry weight

All the treatments showed significant effect on the plant dry weight in PC-5. Among all the treatments isolates, Pf-31(0.893gm) followed by Pf-2(0.873gm), Th-32(0.877gm), Th-31(0.863gm), Th-18(0.860gm) and Th-28(0.803gm) were observed increased dry weight as compared to control(0.220gm) whereas isolates viz. Th-10(0.257gm) and Th-25(0.327gm) were exhibited least dry weight as compared to control (0.220gm) **Table 4.3**.

4.2.2.2.6 Vigour Index (VI)

In PC-5 maximum vigour index was recorded with Pf-31(1004.1 VI) followed by Pf-2(967.4 VI), Th-32(929.0 VI), Th-31(890.2 VI) and Th-28(884.6 VI) whereas least vigour index was recorded with Th-25(380.8 VI)**Table 4.3**.

It has been found that bioagent impact on germination by paper towel method is multifold.The increase in seedling vigour index was due to increasedgermination percentage, root length and shoot length of seedlings.Previousstudies have shown that *Pseudomonas fluorescens*spp. enhanced germination and seedlingvigor of different crop plants **(Amruthesh *et al.*, 2003)**.The difference in percent seed germination, shoot length, root length, plant fresh weight and plant dry weightamong the varieties may be due to the inherent genotypic differences. The vigour index (VI) was assessed through mean root, shoot length and percent germination according to **Abdul-baki and Anderson, (1973)**.

Table 4.3:Effect of*T. harzianum* and *P. fluorescens* isolates on seed germination and vigour index of PC-5

Treatment	Germination %	Root length(cm)	Shoot length(cm)	Fresh weight(gm)	Dry weight(gm)	Vigour index
Th-R	73.33	4.04	5.80	3.91	0.797	721.36
Th-6	61.00	3.15	4.10	2.37	0.367	442.25
Th-10	58.00	3.20	4.07	2.07	0.257	421.47
Th-15	60.67	3.64	4.53	2.44	0.397	495.85
Th-18	74.67	4.21	6.13	4.19	0.860	772.55
Th-25	51.00	3.20	4.27	2.37	0.327	380.80
Th-28	85.33	4.20	6.17	4.22	0.803	884.62
Th-31	88.00	4.08	6.03	4.33	0.863	890.27
Th-32	89.33	4.27	6.13	4.40	0.877	929.07
Th-36	59.33	3.40	4.53	2.47	0.538	470.71
Th-38	53.33	3.87	4.17	2.23	0.470	428.44
Th-39	60.67	3.93	4.93	3.00	0.450	537.91
Th-43	58.33	3.97	4.47	2.30	0.423	491.94
Pf-2	85.33	5.17	6.17	4.79	0.873	967.40
Pf-12	58.67	3.90	4.73	3.85	0.490	506.49
Pf-15	53.67	4.13	4.80	3.93	0.525	479.42
Pf-30	59.67	3.81	4.69	3.79	0.480	507.37
Pf-31	86.67	5.37	6.22	5.00	0.893	1004.18
Control	49.33	2.20	3.97	1.53	0.220	304.22
CD at 5%	2.36	0.28	0.27	0.15	0.03	-
CV	2.15	4.45	3.30	2.78	3.36	-
SEM±	0.82	0.99	0.96	0.53	0.01	-

Plant growth promoting bacteria including*Pseudomonas* spp. have been reported to stimulate thedevelopment of healthy root system **(Germinda and Walley, 1996)**and rapid root colonization by beneficial bacteria **(Bolton *et al.*, 1990)**. The present study revealed that the sorghum seeds treated with the antagonists increased the seed germination and induced the plant growth promotion *in vitro*. The shoot length, root length, plant fresh weight, dry weight and vigour index of sorghum seedlings was significantly increased. The growth promotion exerted by *Trichoderma* might be due to the production of secondary metabolites **(Vey*et.al,* 2001; Vinale *et.al,* 2006).**Similar beneficial effects on seed germination and seedling vigour observed with inoculation of *Trichoderma* spp. has been previously reported (**Jadhav and Ambadkar, 2007; Neelamegam, 2004).**It can be concluded that seed dressing with bioagents may be beneficial in enhancing seed germination percentage and vigour of sorghum seeds.

4.2.3 Glasshouse experiments

Glasshouse experiments were conducted using *T. harzianum* and *P. fluorescens* isolates used *in vitro* and one isolate of *G. intraradices* studying their effect on plant growth promotion and disease reduction.

4.2.3.1.1 Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-4 (15 DAS)

4.2.3.1.1.1 Seed Germination

As shown in **Table 4.4** highest seed germination was observed in case of Th-25(85.67%), Th-32(84.00%) and Pf-2(84.00%) were statistically at par followed by Pf-31(80.00%), Th-18(80.33%) and Th-28(80.00%).

4.2.3.1.1.2 Root Length

15 Days after sowing (DAS), there was a significant increase in plant root length in treated pots as compared to untreated control. Maximum root length was observed in Pf-31(5.24cm) treated plants followed by Th-32(5.23cm), Pf-2(4.78cm), Th-31(4.74cm), Th-18(4.48cm), Th-28(4.41cm) and Glomus(4.03cm) whereas isolate Th-6(3.55cm) was found least effective in plant root growth promotion activity.

4.2.3.1.1.3Shoot Length

As shown in **Table 4.4**, the plant shoot lengthof the 15-day old sorghum plants increased significantly as compared to control.Plant shoot length was observed to be maximum in Pf-31(16.30cm) and Th-32(16.30cm) followed by Th-31(13.03cm), Pf-2(13.03cm), Th-18(12.29cm), Th-28(12.00cm), Glomus(10.90 cm) and Th-R(10.34cm) treatments. While least shoot length was found in Pf-15(9.29cm).

4.2.3.1.1.4 Stem Diameter

It is evident from the data presented in **Table 4.4** that maximum stem diameter was recorded with Th-32(0.65cm), Pf-31(0.65cm) followed by Pf-2(0.62cm), Th-28(0.62cm), Glomus(0.61cm), Th-31(0.60cm) and Th-18(0.60cm) while isolates viz. Th-10(0.56cm) and Th-15(0.56cm) exhibited least effect on stem diameter as compared to control(0.49cm).

4.2.3.1.2 Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-5 (15 DAS)

4.2.3.1.2.1 Seed Germination

Table 4.4: Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-4 (15 DAS)

Treatment No.	Treatment	Germination %	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	70.67	3.90	10.34	0.58
T2	Th-6	75.67	3.55	10.12	0.58
T3	Th-10	75.67	3.76	9.93	0.56
T4	Th-15	78.00	3.81	9.91	0.56
T5	Th-18	80.33	4.48	12.29	0.60
T6	Th-25	85.67	4.13	9.60	0.58
T7	Th-28	80.00	4.41	12.00	0.62
T8	Th-31	78.67	4.74	13.03	0.60
T9	Th-32	84.00	5.23	16.30	0.65
T10	Th-36	70.67	3.59	9.80	0.58
T11	Th-38	78.67	3.88	10.19	0.59
T12	Th-39	75.33	3.72	9.29	0.58
T13	Th-43	78.33	3.77	9.88	0.58
T14	Pf-2	84.00	4.78	13.03	0.62
T15	Pf-12	75.67	3.72	10.19	0.59
T16	Pf-15	70.67	3.77	9.29	0.58
T17	Pf-30	78.33	3.78	9.88	0.58
T18	Pf-31	80.00	5.24	16.30	0.65
T19	Glomus	74.00	4.03	10.90	0.61
T20	Control	70.00	2.88	7.98	0.49
	CD at 5%	3.60	0.33	0.72	0.02
	CV	2.84	4.88	4.23	7.44
	SEM±	1.26	0.11	0.27	0.02

As shown in **Table 4.5** highest seed germination was observed in Th-32(89.67%), Pf-31(89.67%) followed by Pf-2(88.00%), Th-31(88.00%), Th-28(88.00%), Th-18(88.00%), Glomus(85.67%) and Th-25(84.00%), whereas least was observed in Th-10(75.67%) as compared to control (72.67%).

4.2.3.1.2.2 Root Length

15 Days after sowing (DAS), there was a significant increase in plant root length in treated pots as compared to untreated control. Maximum root length was observed in Th-32(6.12cm) and Pf-31(6.12cm) treated plants followed by Pf-2(5.83cm), Th-28(5.83cm), Glomus(5.86 cm) Th-18(5.78cm), Th-31(5.49cm) and Th-25(4.89cm) exhibited increased root length as compared to the control (3.42cm) whereas isolates Th-6(3.94cm)wasfound least effective in plant root growth promotion activity.

4.2.3.1.2.3 Shoot Length

As shown in **Table 4.5**, the plant shoot lengthof the 15-day old sorghum plants increased significant as compared to control. Plant shoot length was observed to be maximum in Th-32(18.11cm) and Pf-31(18.11cm) followed by Th-31(17.44cm), Pf-2(16.29cm), Th-28(16.29cm), Th-18(15.55cm) and Glomus(15.60cm) while least shoot length was found in Pf-15(14.20cm.

4.2.3.1.2.4 Stem Diameter

It is evident from the data presented in **Table 4.5** that maximum stem diameter was recorded with Th-32(0.72cm), Pf-31(0.72cm) followed by Th-28(0.68cm), Th-31(0.66cm), Pf-2(0.66cm), Th-18(0.62cm), Glomus(0.62cm) and Th-25(0.62cm) while isolates viz. Th-10(0.59cm), Pf-15(0.59cm) and Th-R(0.59cm) exhibited least effect on stem diameter as compared to control(0.56cm).

4.2.3.2.1 Effect of *T. harzianum*,*P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-4 (30 DAS)

4.2.3.2.1.1 Root Length

Thirty days after sowing (DAS), root length was maximum in Th-28(15.40cm). However Pf-31(15.39cm), Th-32(15.38cm), Th-18(15.30cm), Th-31(15.24cm) and Pf-2(15.07cm) were statistically at par Th-28. Whereas isolates Pf-12(10.54cm), Th-10(10.64cm), Th-15(10.82cm), Pf-30(10.83cm), Th-43(10.86cm) were did not differ significantly than control (9.86cm)**Table 4.6**.

4.2.3.2.1.2Shoot Length

Maximum shoot length was found with isolate Pf-31(55.73cm) followed by Pf-2(54.31cm), Pf-30(51.11cm), Pf-15(48.88cm), Th-32(48.07cm), Pf-12(47.62cm), Th-31(46.83cm),Th-28(45.48cm), Th-18(43.07cm), Th-25(41.83cm) and Glomus(40.13cm) while, isolates Th-6(30.00cm), Th-15(32.49cm) and Th-39(32.11cm) did not differ significantly than control (27.72cm).

4.2.3.2.1.3Stem Diameter

It is evident from the data presented in **Table 4.6** that maximum stem diameter was recorded with Pf-31(2.11cm) followed by Pf-2(1.76cm), Th-32(1.60cm), Th-31(1.57cm), Th-28(1.44cm) and Th-18(1.44cm) while isolate Th-6(0.83cm) did not differ significantly as compared to control(0.66cm).

Table 4.5:Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates ongrowth promotion in PC-5 (15 DAS)

Treatment No.	Treatment	Germination %	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	80.67	4.55	14.26	0.59
T2	Th-6	80.98	3.94	13.75	0.60
T3	Th-10	75.67	4.02	13.97	0.59
T4	Th-15	80.00	4.24	14.07	0.61
T5	Th-18	88.00	5.78	15.55	0.62
T6	Th-25	84.00	4.89	14.58	0.62
T7	Th-28	88.00	5.83	16.29	0.68
T8	Th-31	88.00	5.49	17.44	0.66
T9	Th-32	89.67	6.12	18.11	0.72
T10	Th-36	77.33	4.14	14.24	0.61
T11	Th-38	82.67	4.31	14.20	0.60
T12	Th-39	78.00	4.23	14.77	0.62
T13	Th-43	80.00	4.30	13.94	0.63
T14	Pf-2	88.00	5.83	16.29	0.66
T15	Pf-12	77.33	4.31	14.24	0.60
T16	Pf-15	82.67	4.23	14.20	0.59
T17	Pf-30	78.00	4.30	14.77	0.61
T18	Pf-31	89.67	6.12	18.11	0.72
T19	Glomus	85.67	5.86	15.60	0.62
T20	Control	72.67	3.42	10.22	0.56
	CD at 5%	3.87	0.26	0.37	0.05
	CV	2.84	3.28	1.49	4.89
	SEM±	1.35	0.91	0.12	0.01

4.2.3.2.2 Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-5 (30 DAS)

4.2.3.2.2.1 Root Length

Thirty days after sowing (DAS), root length was maximum in Th-31(17.13cm). However Th-28(16.89cm), Th-18(16.32cm),Pf-31(16.15cm) and Th-32(16.08cm) were statistically at par Th-31. Whereas isolates Th-15(10.92cm) was observed least effective in plant root growth promotion activity **Table 4.7**.

4.2.3.2.2.2Shoot Length

Maximum shoot length was found with isolate Pf-31(68.52cm) followed by Pf-2(65.52cm), Th-32(62.97cm), Th-31(60.17cm), Th-28(58.65cm), Th-18(55.82cm) and

Table 4.6:Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-4 (30 DAS)

Treatment No.	Treatment	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	14.14	36.13	0.93
T2	Th-6	12.12	30.00	0.83
T3	Th-10	10.64	33.91	1.23
T4	Th-15	10.82	32.49	1.15
T5	Th-18	15.30	43.07	1.44
T6	Th-25	11.15	41.83	1.34
T7	Th-28	15.40	45.48	1.44
T8	Th-31	15.24	46.83	1.57
T9	Th-32	15.38	48.07	1.60
T10	Th-36	11.60	39.07	0.99
T11	Th-38	11.12	39.71	1.37
T12	Th-39	11.68	32.11	1.57
T13	Th-43	10.86	38.23	1.23
T14	Pf-2	15.07	54.31	1.76
T15	Pf-12	10.54	47.62	1.22
T16	Pf-15	11.41	48.88	1.34
T17	Pf-30	10.83	51.11	1.30
T18	Pf-31	15.39	55.73	2.11
T19	Glomus	12.23	40.13	1.07
T20	Control	9.86	27.72	0.66
	CD at 5%	1.20	5.54	0.20
	CV	5.79	8.07	9.61
	SEM±	0.41	1.94	0.73

Glomus(55.05cm) while isolates Th-6(42.59cm) and Th-15(45.78cm) did not differ significantly than control (27.72cm) **Table 4.7**.

4.2.3.2.2.3 Stem Diameter

It is evident from the data presented in **Table 4.7** that maximum stem diameter was recorded with Pf-31(2.26cm) followed by Pf-2(1.97cm), Th-32(1.84cm), Th-31(1.79cm), Th-28(1.69cm), Pf-15(1.57cm), Pf-30(1.51cm), Th-18(1.54cm), Pf-12(1.46cm), Glomus(1.33cm) and Th-R(1.12cm) while isolates viz. Th-6(1.02cm) and Th-15(1.04cm) did not differ significantly as compared to control (0.94cm).

Table 4.7:Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-5 (30 DAS)

Treatment No.	Treatment	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	15.00	46.38	1.12
T2	Th-6	14.09	42.59	1.02
T3	Th-10	11.47	46.81	1.40
T4	Th-15	10.92	45.78	1.04
T5	Th-18	16.32	55.82	1.54
T6	Th-25	11.52	54.93	1.51
T7	Th-28	16.89	58.65	1.69
T8	Th-31	17.13	60.17	1.79
T9	Th-32	16.08	62.97	1.84
T10	Th-36	11.82	53.19	1.36
T11	Th-38	10.96	56.56	1.56
T12	Th-39	11.71	57.56	1.70
T13	Th-43	12.15	54.92	1.51
T14	Pf-2	15.80	65.52	1.97
T15	Pf-12	11.33	59.19	1.46
T16	Pf-15	12.05	58.56	1.57
T17	Pf-30	12.01	63.59	1.51
T18	Pf-31	16.15	68.52	2.26
T19	Glomus	14.19	55.05	1.33
T20	Control	10.22	41.22	0.94
	CD at 5%	0.77	3.93	0.20
	CV	3.51	4.30	9.52
	SEM±	0.27	1.37	0.73

4.2.3.3.1 Effect of *T. harzianum,P. fluorescens* and *G. intraradices* isolates on growth promotion in PC-4 (45 DAS)

4.2.3.3.1.1 Root Length

Forty five Days after sowing (DAS), there was a significant increase in plant root length in treated pots as compared to untreated control. Out of all isolates, isolates viz. Th-28(18.75cm) followed by Th-32(18.02cm),Pf-31(17.93cm), Pf-2(17.56cm), Th-31(17.45cm), Th-R(16.59cm), Th-18(16.54cm) and Glomus(15.98cm) exhibited increased root length as compared to the control(10.98cm). Whereas isolates viz. Pf-12(12.58cm) followed by Th-10(13.23cm), Pf-15(13.29cm), Pf-30(13.45cm) and Th-43(13.45cm) were observed least in plant root growth promotion activity **Table 4.8**.

4.2.3.3.1.2Shoot Length

Maximum shoot height were found with isolates viz. Th-32(71.71cm) followed by Th-31(70.49cm), Th-28(70.23cm), Th-18(68.97cm), Pf-31(68.23cm), Pf-2(68.08cm), Th-R(66.70cm), Pf-12(66.40cm) and Glomus(66.17cm) while isolates Th-36(59.08cm) and Pf-15(59.50cm) were recorded least as compared to control (56.29cm)**Table 4.8**.

4.2.3.3.1.3Stem Diameter

It is evident from the data presented in **Table 4.8** that maximum stem diameter was recorded with Pf-31(2.27cm) followed by Pf-2(1.84cm), Th-32(1.76cm), Th-31(1.73cm), Th-28(1.57cm), Th-18(1.54cm) and while isolates viz. Glomus(1.27cm) followed by Th-R(1.12cm), Th-15(1.26cm), Pf-12(1.31cm), Th-38(1.35cm), Pf-30(1.36cm) and Pf-15(1.46cm) did not differ significantly as compared to control(0.79cm).

Table 4.8 Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-4 (45 DAS)

Treatment No.	Treatment	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	16.59	66.70	1.03
T2	Th-6	13.89	63.27	0.96
T3	Th-10	13.23	65.10	1.38
T4	Th-15	13.94	61.23	1.26
T5	Th-18	16.54	68.97	1.54
T6	Th-25	14.12	60.16	1.37
T7	Th-28	18.75	70.23	1.57
T8	Th-31	17.45	70.49	1.73
T9	Th-32	18.02	71.71	1.76
T10	Th-36	13.98	59.08	1.09
T11	Th-38	13.78	62.74	1.50
T12	Th-39	14.35	65.05	1.60
T13	Th-43	13.45	69.48	1.38
T14	Pf-2	17.56	68.08	1.84
T15	Pf-12	12.58	66.40	1.31
T16	Pf-15	13.29	59.50	1.46
T17	Pf-30	13.45	61.77	1.36
T18	Pf-31	17.93	68.23	2.27
T19	Glomus	15.98	66.17	1.27
T20	Control	10.98	56.29	0.79
	CD at 5%	0.97	4.14	0.26
	CV	3.94	3.86	11.17
	SEM±	0.34	1.45	0.91

4.2.3.3.3 Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-5 (45 DAS)

4.2.3.3.3.1 Root Length

45 Days after sowing (DAS), there was a significant increase in plant root length in treated pots as compared to untreated control. Out of all isolates, isolates viz. Th-32(18.99cm), Th-R(18.78cm), Th-31(18.71cm), Th-28(18.68cm), Th-18(18.66cm), Pf-2(18.25cm) and Pf-31(18.04cm) exhibited increased root length as compared to the control(12.07cm). Whereas isolates viz. Pf-12(12.30cm) followed by Pf-15(13.33cm), Pf-30(13.93cm), Th-38(13.97cm) and Th-36(14.12cm) were observed least in plant root growth promotion activity **Table 4.9**.

4.2.3.3.3.2Shoot Length

As shown in **Table 4.9**, the plant shoot lengthof the 45-day old sorghum plants was increased significantly as compared to control. Isolates viz. Th-31(82.82cm), Th-32(82.08cm), Pf-31(80.22cm), Pf-2(79.04cm), Th-28(79.04cm) and Th-R(78.16cm) found with maximum plant shoot length as compared to control(60.23cm) while Th-15(67.67cm) followed by Th-10(68.91cm), Th-6(68.97cm), Th-43(69.20cm) were recorded least as compared to control (60.23cm).

4.2.3.3.3.3Stem Diameter

It is evident from the data presented in **Table 4.9** that maximum stem diameter was recorded with Pf-31(2.31cm) followed by Pf-2(1.90cm), Th-32(1.96cm), Th-31(1.79cm), Th-28(1.74cm), Th-R(1.67cm), Th-18(1.64cm) and Glomus(1.30cm)while isolates viz. Th-38(1.03cm) and Th-36(1.10cm) exhibited least effect on stem diameter as compared to control(1.01cm).

Studies on the effect of *Glomus intraradices,Trichoderma harzianum* and *Pseudomonas fluorescens*in seed germination, root and shoot growth promotion, increase in stem diameter and there biocontrol potential are important in identifying potential isolates for*G.sorghi* management. In the present study, five isolates of *T. harzianum*(Th-32, Th-31, Th-28, Th-18 and Th-R), two isolates of *P. fluorescens*(Pf-31 and Pf-2) and one isolate of *G. intraradices*were found effective in growth promotion and disease reduction attributes on PC-4 and PC-5 cultivars of sorghum under glasshouse condition. Seed treatment with bacterial antagonists resulted in increased shoot and root lengths of

Table 4.9: Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on growth promotion in PC-5 (45 DAS)

Treatment No.	Treatment	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	18.78	71.16	1.27
T2	Th-6	17.26	68.97	1.10
T3	Th-10	14.29	68.19	1.44
T4	Th-15	15.33	67.67	1.38
T5	Th-18	18.66	73.60	1.64
T6	Th-25	14.82	70.12	1.42
T7	Th-28	18.68	79.04	1.74
T8	Th-31	18.71	82.82	1.79
T9	Th-32	18.99	82.08	1.96
T10	Th-36	14.12	68.56	1.13
T11	Th-38	13.97	69.60	1.53
T12	Th-39	14.28	68.30	1.64
T13	Th-43	14.39	69.20	1.45
T14	Pf-2	18.25	79.04	1.90
T15	Pf-12	12.30	71.70	1.35
T16	Pf-15	13.33	74.86	1.49
T17	Pf-30	13.93	77.56	1.39
T18	Pf-31	18.04	80.22	2.31
T19	Glomus	16.08	72.78	1.30
T20	Control	12.07	60.23	1.01
	CD at 5%	1.02	1.02	4.99
	CV	3.92	3.91	4.16
	SEM±	0.35	0.35	1.74

seedlings**.**The beneficial effects of rhizobacteria (PGPR) on plant growth can be direct or indirect. To exert their beneficial effects, bacteria usually must colonizethe root surface efficiently. Direct plant-growth-promoting rhizobacteriaenhance plant growth in the absence ofpathogens.Some bacteria produce substances that stimulate the growth of plants in the absence ofpathogens. The best understood example isthe hormone auxin. In addition, other hormonesas well as certain volatiles and the cofactorpyrrolquinoline quinone (PQQ) stimulateplant growth Inoculation of seeds with the auxin-generating *P. fluorescens* WCS365 did not result in an increase in the root or shoot weight of cucumber,sweet pepper, or tomato, but led to a significant increase in the root weight of radish **(Kamilova *et al.*, 2006)**. A study was conducted by **Kumar *et al.*, (2012)** in which they observed an improvement in nutrient uptake and growth of sorghum plants on inoculation

with fluorescent *Pseudomonas* sp. P17.Use of diazotrophic bacteria in rice to promote growth and yields in rice is a common practice.

Seed treatment with diazotrophs resulted in growth responses such as seedling emergence, radicle elongation, plumule length, cumulative leaf and root areas, grain and straw yields. Rhizobacterium colonization into a host plant is started when a seed is germinating. At the same time, the rhizobacteria also require adequate nutrition for their growth and development. Several previous studies also showed that the role of*P. fluorescens* as PGPR was correlated to their ability to synthesize plant growth regulator substances, to fix nitrogen or to dissolve phosphate **(Sutariati *et al.*, 2009)**. *P. fluorescens* can produce IAA **(Ashrafuzzaman *et al.,* 2009)**, gibberellins and cytokinins **(Ahmad *et al.*, 2005)**, fix nitrogen **(Mehrab *et al.,* 2010)** and dissolve phosphate **(Park *et al.,* 2009)**.**Nadeem *et al.,* (2010)** added that the main contribution of rhizobacterium *P. fluorescens* associated with host plants was to increase the availability of regulator growth substances, such as, IAA that functions to promote plant growth and increase the availability of plant nutrition such as P that is highly required during the plant growth and development. The utilization of P-dissolving rhizobacteria that can substitute a part or all plant P-requirement results in an increased plant growth and yield. This P-dissolving is brought about by bacteria that produce phosphates that can release bound P from organic substances, and therefore, it can fulfill plant requirement **(Vleesschauwer *et al.,* 2009; Koo & Cho, 2009)**.Increased growth response in lettuce, bean, cucumber, and pepper has been demonstratedfollowing application of *Trichoderma* spp. under greenhouse or field conditions**(Baker, 1989; Kleifeld and Chet 1992; Inbar *et al.*,1994; Ousley *et al.*,1994; Vázquez *et al.,* 2000; Yedidia *et al.*,2001)**

The results presented here also demonstrate a significantincrease in growth of sorghum plants treated*T. harzianum,P. fluorescens* and *G. intraradices* isolates for each of the parameters; seed germination, root length, shoot length and stem diameter under greenhouse condition. It has beensuggested that *T. harzianum* might affect plant growth as a result of its ability to influenceplant hormones and vitamins **(Baker, 1989; Kleifield and Chet 1992; Harman *et al.,* 2004)**. Such substances could influence the early stages of

plant growth with better developmentof plant roots. The enhancement in roots total area and growth rate enablesthe plants to explore a greater volume of soil due to the increase in number of activesite of uptake per unit area. Thus, they might be able to sequester more phosphate andother mineral ions liberated as a result of solublization by microorganisms.In most of the earlier studies *Trichoderma* mediated plant growth promotion hasbeen attributed to indirect mechanisms viz. control of plant pathogens and inducedresistance. Though few of the studies have been focused on the level of minerals andother direct means of growth promotion, they could not establish role of *T. harzianum*isolates for these parameters **(Windham *et al.,* 1986; Baker, 1989; Inbar *et al.,*1994; Ousley *et al.,*1994)**. Based on earlier reports **(Kleifield and Chet 1992; Inbar *et al.,* 1994; Kredics *et al.,* 2001; Yedidia *et al.,* 2001)** and findings presented here we conclude thatplant growth may be improved by inoculation with *T. harzianum* isolates which helpsthe plant to obtain P and other less available minerals from native soil and also leadto early emergence and increased vigor of plants. **Karandashov and Bucher (2005)** reported enhanced N_2- fixing ability in mycorrhizal plants compared with nonmycorrhizalplants.Bacteria may also support the AM symbiosis by increasingbio available phosphate (P) since P will be solubilized byorganic acids produced by plant and bacteria for enhanceduptake by root hairs. Available P concentration is very lowin the non-rhizosphere region because of less microbialactivity. Mycorrhizal fungi can help the plants to scavengethe P beyond their rhizosphere region and make themavailable to the plants. Increase in P content inplants along with modification in root architecture wasobserved in plants co-inoculated with either *P. fluorescens*92 or *P. fluorescens* P190r and *G. mosseae* BEG12 due togreater absorptive surface and enhanced mycelial developmentin *G. mosseae* BEG12 **(Gamalero *et al.*, 2004)**.**Yusran *et al.,* (2009)** observed enhanced uptake of Mn,Zn, and P due to combined inoculation of *Pseudomonas*sp., *Bacillus amyloliquefaciens* FZB42 and AMF. Similarlyincreased N, P, S, Zn, Mn and Cu uptake by *R. leguminosarum*inoculation with mixed inoculum of AMF containing*Gigaspora albida*, *Glomus intraradices* and *Acaulosporascrobiculata* spores was observed in Indian rosewood **(Bisht *et al.*,2009)**.

4.2.3.2Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on reduction of disease severity.

Biocontrol potential of *T. harzianum,P. fluorescens* and *G. intraradices* isolates on disease severity reduction was assessed 45 and 65 DAS in both PC-4 and PC-5 cultivars of Sorghum. It is evident from the data presented on **Table4.10 and 4.11** that there was a significant reduction in disease severity over the control.

4.2.3.2.1.1Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on reduction of disease severity in PC-4.

All the isolates reduced the disease severity significantly over control both 45 and 65 DAS.Isolates namely, Th-32(45.30%) recorded maximum reduction in disease severity followed by Pf-31(44.10%), Th-31(41.80%), Pf-2(39.40%), Th-18(37.07%), Th-R(35.87%) Glomus(35.34%) and Th-28(33.04%) while Th-36(13.90%) was observed to be least effective in disease reduction potential.

Table 4.10 Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on reduction of disease severity in PC-4.

Treatment No.	Treatment	45 DAS	65 DAS	Mean	Decrease in disease Severity (%)
T1	Th-R	24.79	33.92	29.36	35.87
T2	Th-6	31.06	38.08	34.57	24.48
T3	Th-10	32.34	41.34	36.84	19.53
T4	Th-15	33.92	37.49	35.71	22.00
T5	Th-18	25.27	32.34	28.81	37.07
T6	Th-25	29.23	44.09	36.66	19.92
T7	Th-28	24.52	36.78	30.65	33.04
T8	Th-31	24.71	28.56	26.64	41.80
T9	Th-32	23.42	26.66	25.04	45.30
T10	Th-36	32.08	46.76	39.42	13.90
T11	Th-38	32.67	45.01	38.84	15.16
T12	Th-39	33.52	42.49	38.01	17.00
T13	Th-43	32.56	46.14	39.35	14.04
T14	Pf-2	24.41	31.06	27.74	39.40
T15	Pf-12	29.86	39.89	34.88	23.80
T16	Pf-15	28.21	43.19	35.70	22.02
T17	Pf-30	29.12	44.56	36.84	19.53
T18	Pf-31	22.49	28.70	25.59	44.10
T19	Glomus	24.04	35.15	29.60	35.34
T20	Control	35.12	56.45	45.78	
	CD at 5%	2.40	2.52	-	-
	CV	5.07	3.74	-	-
	SEM	0.84	0.85	-	-

4.2.3.2.1.2 Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on reduction of disease severity in PC-4.

All the isolates reduced the disease severity significantly over control both 45 and 65 DAS. Isolates namely, Th-32(43.04%) recorded maximum reduction in disease severity followed by Th-31(40.03%), Pf-2(41.21%), Pf-31(40.73%), Th-18(39.47%), Th-28(35.27%), Th-R(31.61%) and Glomus(29.63%) while isolates viz. Th-38(7.23%) was observed to be least effective in disease reduction potential.

Table 4.11 Effect of *T. harzianum,P. fluorescens*and*G. intraradices* isolates on reduction of disease severity in PC-5.

Treatment No.	Treatment	45 DAS	65 DAS	Mean	Decrease in Disease Severity (%)
T1	Th-R	20.78	25.52	23.15	31.61
T2	Th-6	25.67	27.45	26.56	21.54
T3	Th-10	27.45	29.75	28.60	15.51
T4	Th-15	24.52	26.56	25.54	24.55
T5	Th-18	19.35	21.64	20.49	39.47
T6	Th-25	25.82	27.01	26.41	21.98
T7	Th-28	20.45	23.38	21.91	35.27
T8	Th-31	19.38	21.23	20.30	40.03
T9	Th-32	18.44	20.13	19.28	43.04
T10	Th-36	29.71	31.70	30.71	9.28
T11	Th-38	29.90	32.89	31.40	7.23
T12	Th-39	26.52	27.90	27.21	19.62
T13	Th-43	26.56	28.12	27.34	19.23
T14	Pf-2	18.82	20.97	19.90	41.21
T15	Pf-12	25.87	29.22	27.55	18.61
T16	Pf-15	26.21	32.89	29.55	12.70
T17	Pf-30	25.27	30.04	27.66	18.28
T18	Pf-31	18.49	21.64	20.06	40.73
T19	Glomus	21.75	25.90	23.82	29.63
T20	Control	32.44	35.26	33.85	
	CD at 5%	2.40	2.39	-	-
	CV	6.02	5.38	-	-
	SEM±	0.84	0.84	-	-

In present study biocontrol potential of of *Glomus intraradices*, *Trichoderma harzianum* and *Pseudomonas fluorescens*was screened in glasshouse condition. Several microbes promote plant growth, and many microbial products reducing the disease severity

have been marketed. Several research papers that have appeared in theliterature do reveal the fact that various species and isolates offungal antagonist *Trichoderma* suppress mycelial growth, reduceroot rots, increase plant growth, and induce resistance in variouscrops infected with*Sclerotium rolfsii***(Mukherjee, 1993; Tian *et al.*, 2001; Dutta and Das, 2002; Palomar *et al.*, 2002)**, *Rhizoctonia solani* (**Li *et al.*, 2001; Zapata *et al.*, 2001; Ziedan and Mahmoud, 2002; Gaikwad and Nimbalkar, 2003; Yossen *et al.*, 2003; Fravel and Lewis, 2004)** and *Sclerotinia sclerotiorum* (**Gupta and Agarwal, 1988; Hajlaoui *et al.*, 2001; Singh *et al.*, 2003; Huang and Erickson, 2004).**

4.3 Evaluation of the effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion, disease reduction, chlorophyll content and green fodder yield of Sorghumagainst *G. sorghi* under field condition.

Promising isolates (Th-R, 18, 28, 31, 32, Pf-2, Pf-31 and Glomus) selected on the basis of growth promotion and disease reduction experiments *in vitro* and under glasshouse conditions, were further evaluated forgrowth promotion, disease reduction, chlorophyll content and green fodder yield under field condition.

4.3.1 Plant growth promotion

4.3.1.1 Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-4 (15 DAS)

4.3.1.1.1 Seed Germination

As shown in **Table 4.12**all the treatments except TH-28 significantly increased germination over control. Maximum seed germination was observed in Pf-31(89.67%), followed by Pf-2(88.86%), Th-32(88.56%) and Th-31(88.00%) as compared to control (71.93%).

4.2.3.1.2 Root Length

Maximum root length was observed in case of Pf-31(7.12cm) treated plants, followed by Pf-2(6.78cm), Th-32(6.46cm), Glomus (6.20cm) and Th-31(6.00cm), which was significantly higher as compared to control (4.92cm)**Table 4.12**.

4.2.3.1.3Shoot Length

As shown in **Table 4.12**, the plant shoot lengthof the 15-day old sorghum plants increased significantly in all the treatments as compared to uninoculated control. Plant shoot length was observed to be maximum in Pf-31(25.89cm), followed by Th-32(25.14cm), Th-32(22.12cm) and Pf-2(21.24cm) while least shoot length was found with Th-R(17.44cm)**.**

4.2.3.1.4Stem Diameter

It is evident from the data presented in **Table 4.12** that maximum stem diameter was recorded with Th-32(0.75cm) followed by Pf-31(0.73cm), Th-31(0.71cm), and Pf-2(0.73cm) while least was recorded with Th-R(0.65cm)**.**

Table 4.12: Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-4 (15 DAS)

Treatment No.	Treatment	Germination %	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	80.67	5.27	17.44	0.65
T2	Th-18	87.66	5.13	18.56	0.69
T3	Th-28	78.93	5.78	20.10	0.69
T4	Th-31	88.00	6.00	22.12	0.71
T5	Th-32	88.56	6.46	25.14	0.75
T6	Pf-2	88.86	6.78	21.24	0.69
T7	Pf-31	89.67	7.12	25.89	0.73
T8	Glomus	85.67	6.20	18.45	0.68
T9	Control	71.93	4.92	13.45	0.58
	CD at 5%	8.12	0.45	3.24	0.03
	CV	5.56	4.43	9.26	3.21
	SEM±	1.71	0.15	1.08	0.01

4.3.1.2 Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-5 (15 DAS)

4.3.1.2.1 Seed Germination

As shown in **Table 4.13** all the treatments significantly increased germination over control. Maximum seed germination was observed in Th-32(89.26%), followed by Th-28(88.85%), Th-31(88.56%) and Th-18(88.56%) while least was recorded with Th-R (88.00%).

4.3.1.2.2 Root Length

Maximum root length was observed in case of Pf-31(10.89cm) treated plants, followed by Pf-2(10.45cm) and Th-32(10.12cm) which was significantly higher as compared to control (6.45cm)**Table 4.13**.

4.3.1.2.3 Shoot Length

As shown in **Table 4.13**, the plant shoot lengthof the 15-day old sorghum plants increased significantly in all the treatments except Th-R as compared to other treatments and uninoculated control. Plant shoot length was observed to be maximum in Th-32(28.93cm) followed by Pf-31(27.98cm), Th-31(24.56cm) and Pf-2(24.56cm) while least shoot length was found with Th-R(17.95cm)**.**

4.3.1.2.4Stem Diameter

It is evident from the data presented in **Table 4.13** that maximum stem diameter was recorded with Th-32(0.91cm) followed by Pf-31(0.87cm), Th-28(0.87cm) and Th-31(0.86cm) while least was recorded with Th-R(0.82cm)**.**

Table 4.13: Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-5 (15 DAS)

Treatment No.	Treatment	Germination %	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	88.00	8.91	17.95	0.82
T2	Th-18	88.56	7.89	21.45	0.85
T3	Th-28	88.85	8.99	20.98	0.87
T4	Th-31	88.56	9.64	24.56	0.86
T5	Th-32	89.26	10.12	28.93	0.91
T6	Pf-2	83.82	10.45	24.56	0.83
T7	Pf-31	87.90	10.89	27.98	0.87
T8	Glomus	82.37	9.78	21.89	0.84
T9	Control	75.34	6.45	15.69	0.63
	CD at 5%	3.72	0.46	3.24	0.03
	CV	2.50	2.87	8.28	2.65
	SEM±	1.24	0.15	1.08	0.01

4.3.1.3 Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-4 (30 DAS)

4.3.1.3.1 Root Length

30 Days after sowing (DAS), there was a significant increase in plant root length in treated pots as compared to untreated control. Maximum root length was observed in case of Pf-31(23.89cm), followed by Th-32(22.91cm), Glomus (18.99cm) and Pf-2(17.89cm) while least root length was recorded in case of Th-18(14.95cm)**Table 4.14**.

4.3.1.3.2Shoot Length

As shown in **Table 4.14** after 30 days after sowing, significantincreases in plant shootlength were found with all the isolates except Th-R. Maximum shoot length was observed withPf-31(74.12cm), followed by Th-32(72.98cm) and Pf-2(70.89cm).Least shoot length was recorded in case of Th-R(52.45cm).

4.3.1.3.3Stem Diameter

It is evident from the data presented in **Table 4.14** that maximum stem diameter was recorded with Th-32(1.01cm) followed by Pf-31(0.98cm) and Th-31(0.92cm), while least was recorded in case of Th-28(0.81cm).

Table 4.14: Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-4 (30 DAS)

Treatment No.	Treatment	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	17.45	52.45	0.82
T2	Th-18	14.95	58.98	0.84
T3	Th-28	16.89	62.91	0.81
T4	Th-31	16.12	68.25	0.92
T5	Th-32	22.91	72.98	1.01
T6	Pf-2	17.89	70.89	0.85
T7	Pf-31	23.89	74.12	0.98
T8	Glomus	18.99	56.45	0.82
T9	Control	10.89	44.08	0.69
	CD at 5%	3.71	9.46	0.09
	CV	12.08	8.77	6.53
	SEM	1.24	1.15	0.03

4.3.1.4 Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-5 (30 DAS)

4.3.1.4.1 Root Length

30 Days after sowing (DAS), there was a significant increase in all treatments except Th-18 in plant root length as compared to untreated control. Maximum root length was observed in case of Pf-31(24.98cm), followed by Th-32(24.01cm) and Glomus (19.45cm) while least root length was recorded in case of Th-18(15.56cm)**Table 4.15**.

4.3.1.4.2Shoot Length

As shown in **Table 4.15** after 30 days after sowing, significantincreases in plant shootlength were found with all the isolates. Maximum shoot length was observed with Th-32(86.89cm), followed by Pf-31(86.45cm),Pf-2(84.89cm) and Th-31(83.98cm). Least shoot length was recorded in case of Th-R(66.98cm).

4.3.1.4.3Stem Diameter

It is evident from the data presented in **Table 4.15** that maximum stem diameter was recorded with Th-32(0.99cm) followed by Pf-31(0.97cm), Pf-2(0.95cm) and Th-31(0.95cm), while least was recorded in case of Th-R (0.91cm).

Table 4.15: Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-5 (30 DAS)

Treatment No.	Treatment	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	18.89	66.98	0.91
T2	Th-18	15.56	72.89	0.93
T3	Th-28	17.12	73.45	0.93
T4	Th-31	16.89	83.98	0.95
T5	Th-32	24.01	86.89	0.99
T6	Pf-2	18.98	84.89	0.95
T7	Pf-31	24.98	86.45	0.97
T8	Glomus	19.45	70.18	0.93
T9	Control	12.98	47.98	0.73
	CD at 5%	3.71	9.46	0.09
	CV	11.45	7.30	6.10
	SEM±	1.24	3.15	0.03

4.3.1.5 Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-4 (45 DAS)

4.3.1.5.1 Root Length

45 Days after sowing (DAS), there was a significant increase in all treatments in plant root length as compared to untreated control. Maximum root length was observed in case of Th-32(26.89cm), followed by Pf-31(25.89cm) and Th-R (22.89cm) while least root length was recorded in case of Th-18(19.68cm)**Table 4.16**.

4.3.1.5.2Shoot Length

As shown in **Table 4.16** after 45 days after sowing, significantincreases in plant shootlength were found with all the isolates. Maximum shoot length was observed with Pf-31(172.12cm) followed byTh-32(169.89cm),Pf-2(166.95cm) and Th-31(163.92cm). Least shoot length was recorded in case of Th-R(142.45cm).

4.3.1.5.3Stem Diameter

It is evident from the data presented in **Table 4.16** that maximum stem diameter was recorded with Th-32(1.35cm) followed by Pf-2(1.33cm) and Pf-31(1.31cm) while least was recorded in case of Th-R (1.18cm).

Table 4.16: Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-4 (45 DAS)

Treatment No.	Treatment	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	22.89	142.45	1.18
T2	Th-18	19.69	146.45	1.29
T3	Th-28	21.12	155.45	1.21
T4	Th-31	20.89	163.92	1.28
T5	Th-32	26.89	169.89	1.35
T6	Pf-2	19.89	166.95	1.33
T7	Pf-31	25.89	172.12	1.31
T8	Glomus	20.12	145.89	1.24
T9	Control	11.89	110.21	0.81
	CD at 5%	3.71	9.46	0.09
	CV	10.21	3.58	4.60
	SEM±	1.24	2.15	0.03

4.3.1.6 Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-5 (45 DAS)

4.3.1.6.1 Root Length

45 Days after sowing (DAS), there was a significant increase in all treatments in plant root length as compared to untreated control. Maximum root length was observed in case of Th-32(27.12cm) and Pf-31(27.10cm) while least root length was recorded in case of Th-18(21.12cm)**Table 4.17**.

4.3.1.6.2Shoot Length

As shown in **Table 4.17** after 45 days after sowing, significantincreases in plant shootlength were found with all the isolates. Maximum shoot length was observed with Pf-31(185.89cm) followed byTh-32(182.64cm),Pf-2(178.82cm) and Th-31(177.64cm). Least shoot length was recorded in case of Th-R(152.60cm).

4.3.1.6.3Stem Diameter

It is evident from the data presented in **Table 4.17** that maximum stem diameter was recorded with Th-32(1.41cm) followed by Th-31(1.38cm), Pf-2(1.36cm) and Pf-31(1.33cm) while least was recorded in case of Th-28(1.26cm).

Table 4.17: Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on growth promotion in PC-5 (45 DAS)

Treatment No.	Treatment	Root length(cm)	Shoot length(cm)	Stem diameter(cm)
T1	Th-R	24.89	152.60	1.27
T2	Th-18	21.12	160.25	1.32
T3	Th-28	23.45	164.89	1.26
T4	Th-31	22.89	177.64	1.38
T5	Th-32	27.12	182.64	1.41
T6	Pf-2	21.89	178.82	1.36
T7	Pf-31	27.1	185.89	1.33
T8	Glomus	22.28	158.59	1.28
T9	Control	14.98	118.23	0.86
	CD at 5%	3.71	9.46	0.09
	CV	9.40	3.32	4.41
	SEM±	1.24	1.15	0.03

This study was aimed to evaluate the effect of *Glomus intraradices* and selected isolates of *Trichoderma harzianum* and *Pseudomonas fluorescens* on growth promotion in

PC-4 and PC-5 cultivars of sorghum when treated with under field conditionat different time intervals.

The data presented in **Table 4.12 to 4.17** clearly show the usefulness of these isolates in growth promotion under field condition. To be an effective PGPR, bacteria must be able to colonize root environment because they need to establish themselves in the rhizosphere at population densities sufficient to produce the beneficial effects. Earlier it has been reported that single inoculation of maize seeds (*Zea mays* L.) by the rhizobacteria *P. fluorescens*improved considerably the maize plants germination and its growth**(Noumavo *et al.*, 2013).** Studies have demonstratedthat the PGPR can stimulate plant growth through the productionof auxins; indole acetic acid (IAA) **(Spaepen *et al.*, 2008)**,gibberellins **(Bottini *et al.*, 2004)** and cytokinins **(Timmusk *et al.*, 1999)**, or by regulating the high levels of endogenous ethylene inthe plant **(Glick *et al.*, 1998)**.The rate of plant growth was higher following treatment with *T.harzianum*, in both cultivars of sorghum.In addition to their biocontrol activities, *Trichoderma* spp. have been reported to promote plant growth **(Chang *et al.*, 1986; Inbar *et al.*, 1994)**.**Harman (2000)** suggestedthat *Trichoderma* spp. are opportunistic plant colonizersthat affect plant growth by promoting abundantand healthy plant roots, possibly via the productionor control of plant hormones **(Baker, 1989; Kleifield and Chet, 1992)**.**Yedidia *et al.*, (2001)**, suggestedthat the induction of increased growth isobtained through a direct effect of *T. harzianum* on root development using an aseptic hydroponic system. AMF are a key functional group of the soil biota that cancontribute to crop productivity and ecosystem sustainability. The mycorrhizosphere helps in enhanced nutrient absorption, soil stability and water retention efficiency**(Bedini *et al.*, 2009)**, biocontrol ability **(Utkhede, 2006)**,improved secondary metabolite synthesis **(Lee and Scagel, 2009)**, tolerance to abiotic stress **(Marulanda *et al.*, 2006)**,phytoremediation **(Gamalero *et al.*, 2009)** and phosphatemobilization. The beneficial effects of AMF on soil healthare essential for the sustainable management of agriculturalecosystems **(Barrios, 2007; Jeffries *et al.*, 2003)**.**Kohler *et al.*, (2007)** reported that the synergistic interactionsbetween phosphate-solubilizing bacteria *B. subtilis*and AMF *G. intraradices* resulted in high phosphataseactivity and enhanced available P in the soil **(Arthurson *et al.*, 2011)**.

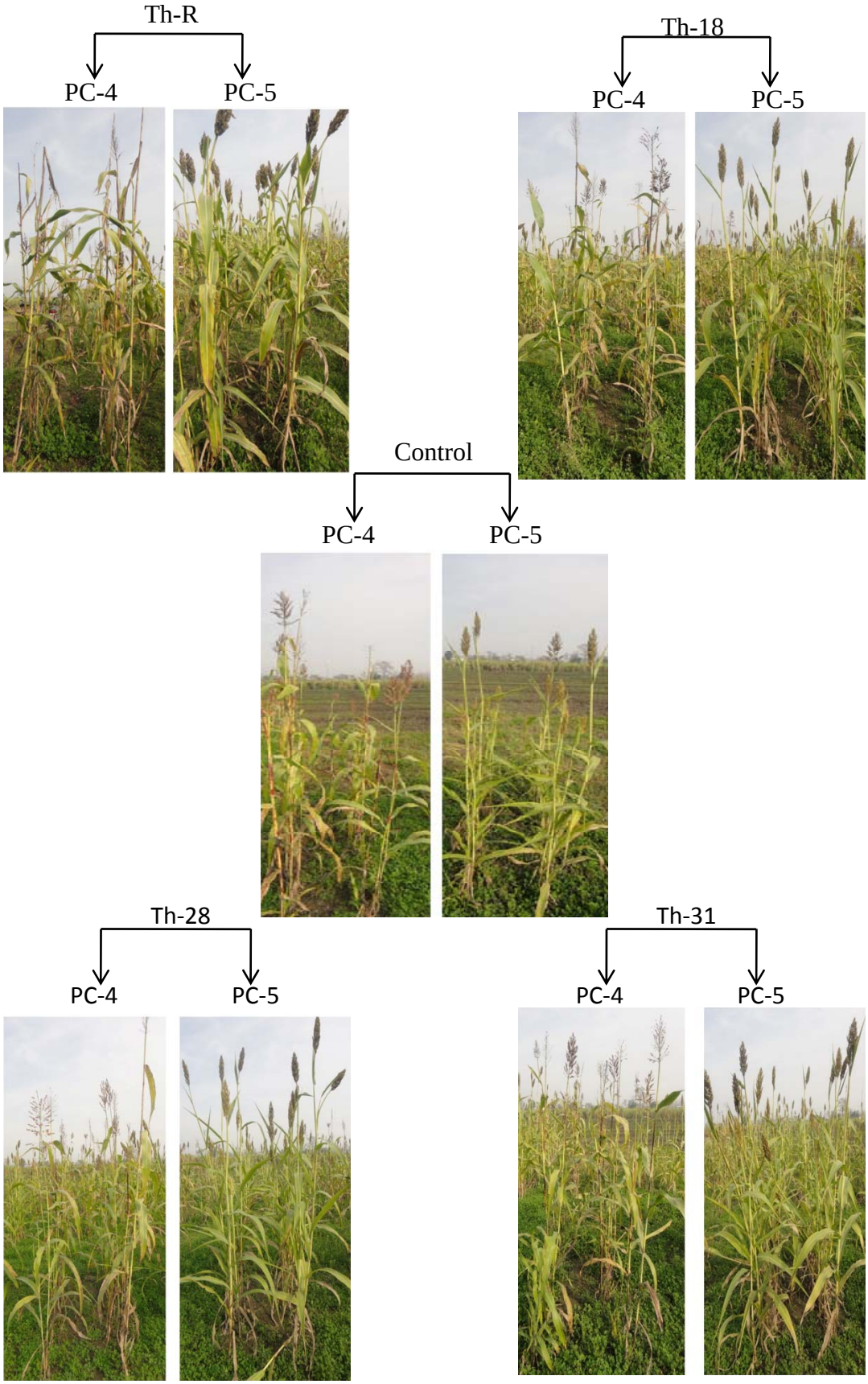


Plate 5(A): Effect of *T. harzianum*, *P. fluorescens and G. intraradices* isolates on growth promotion, disease reduction and green fodder yield of Sorghum cultivars against *G. sorghi* under field condition

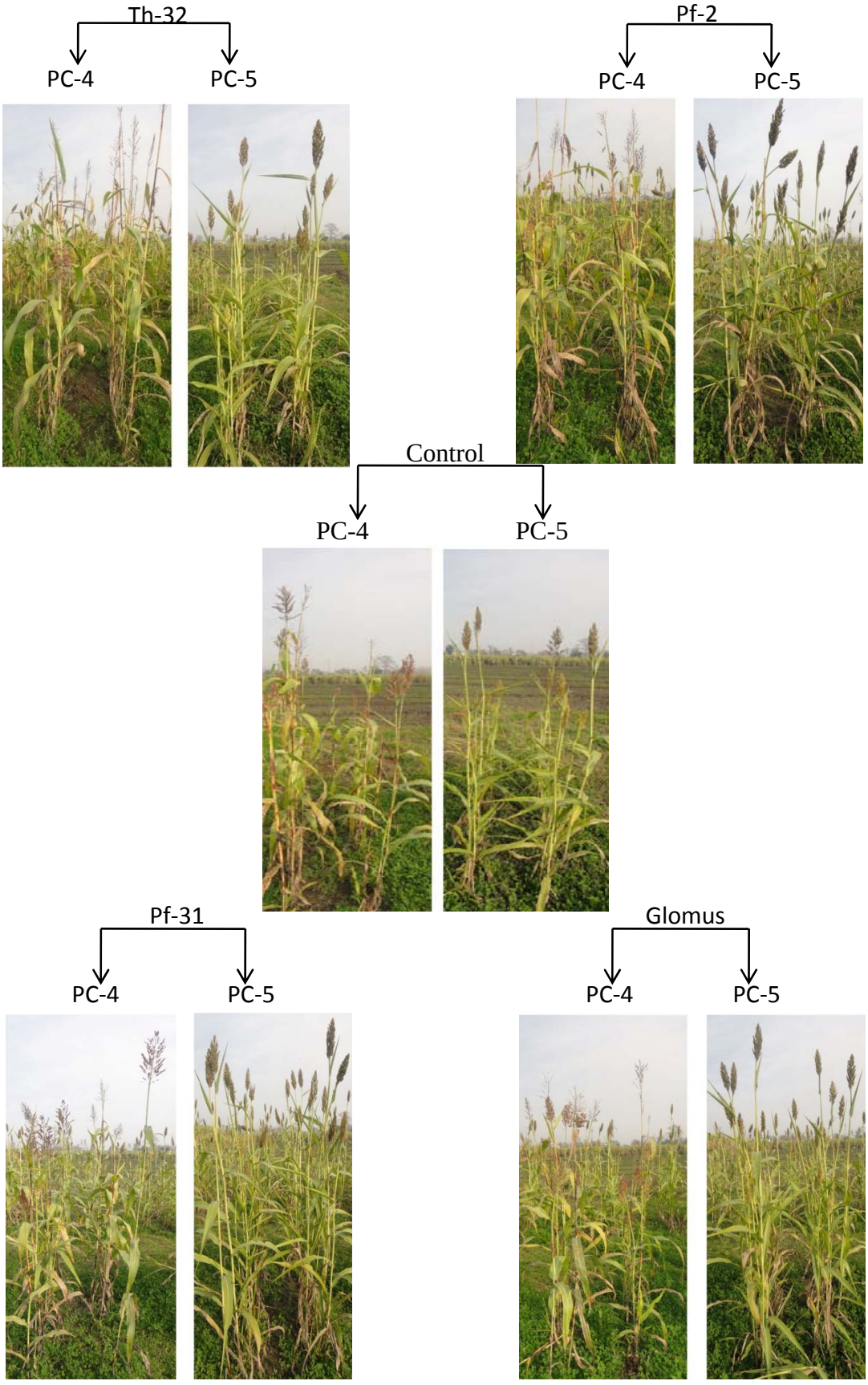


Plate 5(B): Effect of *T. harzianum*, *P. fluorescens and G. intraradices* isolates on growth promotion, disease reduction and green fodder yield of Sorghum cultivars against *G. sorghi* under field condition

4.3.2. Evaluation of the effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on disease reduction, chlorophyll content and green fodder yield of Sorghumagainst *G. sorghi* under field condition.

4.3.2.1 Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on disease reduction, chlorophyll content and green fodder yield on PC-4 against the pathogen

4.3.2.1.1 Disease reduction

Itis evident from data presented in **Table 4.18** that all the isolates reduced the disease severity significantly over control both 45 DAS and 65 DAS. Maximum reduction in disease severity was recorded with Th-32(28.74%) followed by Th-31(27.73%), Pf-2 (27.16%) and Pf-31(26.57%) while least reduction in disease severity was recorded with Th-R (17.24%).

4.3.2.1.2 Chlorophyll content

Total chlorophyll (Chlorophyll a + Chlorophyll b) was observed to be maximum in Th-32(2.356mg/g fr.wt.) followed by Th-31(2.235mg/g fr.wt.), Pf-31(2.150mg/g fr.wt.), Glomus(2.002mg/g fr.wt.) and Pf-2(1.823mg/g fr.wt.) while in control it was 0.438mg/g fr.wt. **Table 4.18.**

4.3.2.1.3 Green fodder yield

Itis evident from data presented in **Table 4.18** that maximum yield was observed with Th-32(27.15 kg/plot) followed by Pf-31(26.89 kg/plot), Glomus (26.04 kg/plot) and Pf-2(25.19 kg/plot) whereas in control it was 15.48kg/plot.

4.3.2.2 Effect of *T. harzianum, P. fluorescens and G. intraradices* isolates on disease reduction, chlorophyll content and green fodder yield on PC- 5 against the pathogen

4.3.2.2.1 Disease reduction

It is evident from data presented in **Table 4.19** that maximum reduction in disease severity was recorded with Th-32(33.81%) followed by Th-31(32.80%),Pf-2(32.15%) and Pf-31(31.80%) while least reduction in disease severity was recorded with Th-R (20.60%).

4.3.2.2.2 Chlorophyll content

Total chlorophyll (Chlorophyll a + Chlorophyll b) was observed to be maximum in Th-31(2.976mg/g fr.wt.), Th-32(2.759mg/g fr.wt.), Th-18(2.321mg/g fr.wt.), Pf-31(2.223mg/g fr.wt.) and Pf-2(2.199mg/g fr.wt.) while in control it was 1.054mg/g fr.wt. **(Table 4.19)**.

4.3.2.2.3 Green fodder yield

It is evident from data presented in **Table 4.19** that maximum yield was observed with Th-32(38.19 kg/plot) followed by Pf-31(37.45 kg/plot), Glomus (37.01 kg/plot), Pf-2(35.46 kg/plot) and Th-18(31.89 kg/plot) whereas in control it was 20.17 kg/plot.

The present study was aimed to evaluate the effect of *Glomus intraradices* and selected isolates of *Trichoderma harzianum* and *Pseudomonas fluorescens* on disease reduction, chlorophyll content and green fodder yield on PC-4 and PC-5 cultivars of sorghum against the pathogen under field condition. The result indicates that on PC-5 cultivar less disease severity was observed as compared to PC-4 cultivar when colonized with bioagents. This may be due to biocontrol activity of bioagents which has earlier been reported by several researches. Recent research suggests that *Trichoderma* spp. can also induce systemic and localized resistance as well as directly attacking or inhibiting the growth of plant pathogens **(Harman *et al.*, 2004; Lo *et al.*, 2000).** In addition, certain *Trichoderma*strains have substantial influence on plantgrowth and development**(Hedge *et al.*, 1962).** Their enhancement ofplant growth has been known for many years and canoccur in natural field soils. In most cases, it isimpossible to separate direct effects on plant growthfrom the control of pathogenic or other deleterious microorganisms that reduce plant growth. However,there were reports indicating that this fungus could also have the potential to stimulate plant growthindependent of any plant disease**(Ozbay *et al.*, 2004; Yedidia *et al.*, 2001).** Seed inoculated with PGPR adapted to stress conditions strongly decreases the level of plant diseases and may help to protect field-workers from exposure to pathogens **(Egamberdiyeva *et al.*, 2008)**. Moreover it has also been observed that there is a correlation between reduction in disease severity and chlorophyll content which has earlier been reported by various researchers.**Gazala, 2009**reported thatthe chlorophyll reduction percentage increased with the increasing levels of infection.As foliar pathogens, *G.sorghi*affects the photosyntheticactivity of its host. It may reduce the rate of photosynthesis in the infected leaves directly via the chloroplasts or chlorophyll content, or

Table 4.18 Effect of disease reduction, chlorophyll content and green fodder yield on PC-4 against *G. sorghi*by *T. harzianum, P. fluorescens and G. intraradices* isolates under field condition.

Treatment No.	Treatment	Disease severity		Mean	Decrease in Disease Severity (%)	Chlorophyll 'a' (mg/g fr. wt.)	Chlorophyll 'b' (mg/g fr. wt.)	Total Chlorophyll (mg/g fr. wt.)	Green fodder yield (kg/plot)
		45 DAS	65 DAS						
T1	Th-R	47.56	57.23	52.40	17.24	0.964	0.114	1.077	20.10
T2	Th-18	43.22	53.64	48.43	23.51	1.324	0.437	1.760	23.24
T3	Th-28	44.41	53.98	49.20	22.30	1.181	0.432	1.613	22.09
T4	Th-31	41.23	50.28	45.76	27.73	1.597	0.638	2.235	23.07
T5	Th-32	40.12	50.11	45.12	28.74	1.768	0.589	2.356	27.15
T6	Pf-2	41.56	50.67	46.12	27.16	1.486	0.337	1.823	25.19
T7	Pf-31	40.96	52.12	46.54	26.57	1.498	0.653	2.150	26.89
T8	Glomus	43.22	53.98	48.60	23.25	1.297	0.705	2.002	26.04
T9	Control	58.23	68.4	63.32		0.278	0.161	0.438	15.48
	CD at 5%	6.63	6.63	-	-	0.03	0.04	-	1.68
	CV	8.61	7.03	-	-	1.46	5.44	-	4.18
	SEM±	2.21	2.21	-	-	0.01	0.01	-	0.56

Table 4.19 Effect of disease reduction, chlorophyll content and green fodder yield on PC-5 against *G. sorghi*by *T. harzianum, P. fluorescens and G. intraradices* isolates under field condition.

Treatment No.	Treatment	Disease severity		Mean	Decrease in Disease Severity (%)	Chlorophyll 'a' (mg/g fr. wt.)	Chlorophyll 'b' (mg/g fr. wt.)	Total Chlorophyll (mg/g fr. wt.)	Green fodder yield (kg/ha)
		45 DAS	65 DAS						
T1	Th-R	37.45	47.23	42.34	20.60	1.058	0.636	1.693	28.45
T2	Th-18	33.12	43.45	38.29	28.20	1.588	0.734	2.321	31.89
T3	Th-28	34.21	43.21	38.71	27.40	1.250	0.657	1.906	31.18
T4	Th-31	31.34	40.32	35.83	32.80	2.049	0.928	2.976	30.89
T5	Th-32	30.45	40.12	35.29	33.81	2.282	0.478	2.759	38.19
T6	Pf-2	31.56	40.79	36.18	32.15	1.783	0.416	2.199	35.46
T7	Pf-31	30.56	42.15	36.36	31.80	1.877	0.347	2.223	37.45
T8	Glomus	33.56	43.15	38.36	28.05	1.312	0.542	1.853	37.01
T9	Control	48.23	58.4	53.32		0.810	0.245	1.054	20.17
	CD at 5%	4.45	6.63	-	-	0.03	0.04	-	1.81
	CV	7.45	8.64	-	-	1.19	4.43	-	3.25
	SEM±	1.46	2.21	-	-	0.01	0.01	-	0.60

indirectly via the photosynthetic enzymes.**Gamal *et al.,* (2007)**reported that when rice plants that weresprayed with *T. harzianum* at 1 x10^8 spore ml^{-1}showed the maximum increase in the total photosyntheticpigments (chlorophyll a and b and carotenoids) against *B. oryzae*.This increase could be attributed to the effect of the biocontrol agent on disease severity and disease index and/or on the chloroplastenzyme activities. **Wall *et al.,* (1989)** estimated the yieldloss as different in sorghums due to severity levelsof gray leaf spot, oval leaf spot and rust. The lossin chlorophyll contents occurred due to developmentof chlorotic and necrotic spots thereby reducing theleaf area for photosynthetic activity. Earlier reportson the reduction of chlorophyll and its componentsin forages (**Patil and Hegde 1995; Ahmad and Gupta 1978**) support the present presumption. A similar observation was recorded with *P.fluorescens* and *G. intraradices* treated plants where content of chlorophyll was significantly increased as compared to control **(Akhtar and Siddiqui, 2008).**In the present study, it was clearly observed that the *Trichoderma*treatment hadpositive impact on growth and yield of sorghum. However the green fodder yield was recorded as highest in those treatments where the disease severity was low and amount of chlorophyll content was higher. Similar to our observations **Haque *et al.*, (2012)** reported that *Trichoderma*-enriched biofertilizer recorded maximum of 108.36% yield increase of mustard over control with T6 isolate. Similarly, themaximum 203.63% yield increase over controlof tomato was noticed in T8, which was 61.82%in T2.Undoubtedly, there is a prospective and potential of *Trichoderma* in crop cultivation to achieveattractive yield.Results of this experiment briefly indicated that *P. florescence* inoculation significantly increased green fodder yield of the two cultivars. A similar result was observed in a study conducted by **Eslamyan *et al.*, (2013)** they observed the potential of *P. fluorescens* in rapeseed growth and yield parameters.

4.4 Determination of the expression of defense response genes during priming and boosting (post inoculation) through *Trichoderma harzianum, Pseudomonas fluorescens* and *Glomus intraradices*, using real time-RT-PCR.

4.4.1 Determination of the expression of defense response genes during priming and boosting through *Trichoderma harzianum*(Th-32)in PC-4 and PC-5 using real time-RT-PCR.

Normalization of data is always required where variations in experimentalconditions contribute greatly to the quantitative difference of measured values observedbetween samples. To compare measurements from gene expression array experiments,quantitative data are commonly normalized using reference genes. The normalization ofrelative quantities with reference genes relies on the assumption that the reference genesare stably express across all tested samples **(Hellmans *et al.*, 2007)**. Ideally normalizationcould be based on a "housekeeping gene" which shows consistent levels of expressionover all conditions.The relative amounts of mRNA detected from target genes or gene of interest (GOI) present in samplescollected 0 to 168 hours post treatment for each cultivar and mock treated plant by each primer pairwere compared based on Ct values.

Comparisons for the expression of PAL, Chitinase and CHS (chalonase synthase) genes, in glasshouse experiments using actin mRNA as a basis fornormalization indicated that the highest amount of fold change was obtained with the PC-5 cultivar of *S.bicolor*treated with Th-32.The PC-4 cultivar showed a very low fold change as compared to untreated control.

4.4.1.1 PAL

During ISR- prime analysis, the expression of sorghum PAL gene(SbPAL) was evaluated in PC-4 and PC-5 when treated with Th-32. SbPAL wasexpressed in both cultivars. The average fold change in the expressionof this gene occurred in between 24 and 96 hours post treatment (HPT). The higher expression of SbPAL was noted in PC-5, whereas it was low in PC-4**(Figure 4.1)**. The maximum fold changein the expression of this gene was 4.88-fold inPC-5, whereas it was 3.24-fold in PC-4. Theexpression of PAL in treated PC-4 was induced at 0 h (0.82-fold) and 24 h (1.75-fold) (**Table 4.20)**. However, the expression of thisgene decreased after 72 h. Similarly in caseof PC-5, the expression of PAL was induced at 0 h (2.04-fold) and 24 h (2.91-fold) while maximum fold change lasted upto 72 hrs (4.88-fold)**(Table 4.21).** In PC-4, after 72 hrs there is decrease in expression of this gene and at 92 hrs it was observed at 1.59-fold as compared to 4.88-fold (72 hrs) but later on it was maintained as 2.88-fold at 168 hrs. A similar trend was observed also in case of PC-5 where fold change was decreased (3.20-fold) after 72 hrs and subsequently it was maintained at 168 hrs (3.81-fold).

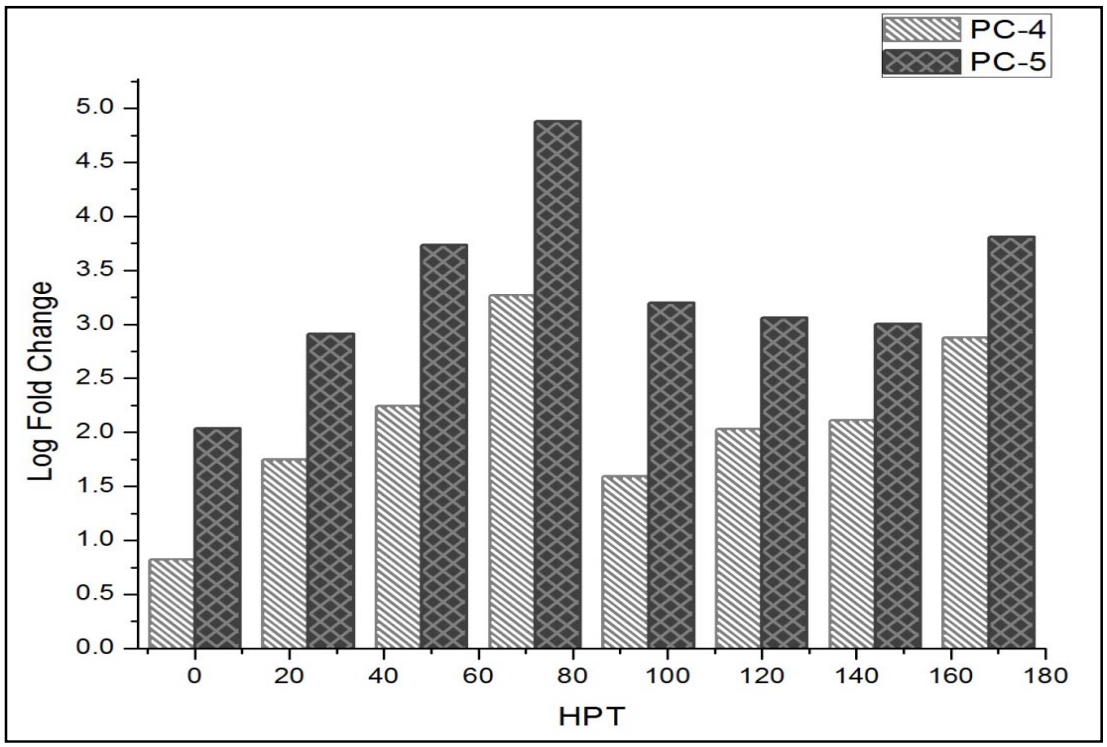


Figure 4.1 Relative gene expression of PAL during priming in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*.

Table 4.20: Threshold values of PAL gene during priming in PC-4 during priming at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	34.47	22.46	31.21	21.94	6.68±0.43	0.82
24	34.47	22.46	29.03	22.82	55.72±0.52	1.75
48	34.47	22.46	26.12	21.56	174.85±0.49	2.24
72	34.47	22.46	21.79	20.53	1722.16±0.32	3.24
96	34.47	22.46	30.67	23.94	38.94±0.37	1.59
120	34.47	22.46	31.29	26.02	107.14±0.31	2.03
144	34.47	22.46	30.36	25.36	128.59±0.25	2.11
168	34.47	22.46	29.56	27.11	754.83±0.15	2.88

Each value represents the mean of three replicates with ±S.D. Mean differences are significant at $P< 0.05$ level.

Table 4.21: Threshold values of PAL gene during priming in PC-5 during priming at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	36.23	21.56	29.84	21.94	108.63±0.24	2.04
24	36.23	21.56	27.82	22.82	812.75±0.24	2.91
48	36.23	21.56	26.12	23.86	5454.89±0.34	3.74
72	36.23	21.56	27.77	29.31	75804.72±0.53	4.88
96	36.23	21.56	31.12	27.07	1573.76±0.20	3.20
120	36.23	21.56	31.72	27.22	1152.06±0.35	3.06
144	36.23	21.56	32.97	28.27	1002.93±0.13	3.00
168	36.23	21.56	30.00	27.98	6427.31±0.40	3.81

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

During ISR- boost (post *G.sorghi* inoculation), the PAL gene was expressed at a very high level. A significant fold change of this gene in PC-5 and PC-4 cultivars of sorghum is an evidence of induction of host defense gene during pathogen infection **(Figure 4.2)**.The greatest expression of this gene was seeninPC-5 cultivar with a fold change of 7.05-fold**(Table 4.23)** while 3.45-foldchange was observed inPC-4 cultivar at 72 HPI **(Table 4.22)**.Upon fungal inoculation, the expression level of PC-4 cultivar increased upto 3.45 and 2.64at 72 and 48 HPIrespectively after that it gradually decreases and reaches below ground level (-0.67-fold) but in case of PC-5 cultivar fold change increases rapidly ranging from 5.92 to 7.05-fold from 24 to 72 HPI but later on a decreasing trend was also observed from 96 to 144 HPI (6.56-3.21 fold).

During GIDR, it is evident that in both cultivars of sorghum prominent downregulation of PAL gene was observed. In PC-4 cultivar -0.84 and -0.86 fold change was observed while 0.62 and 0.37 fold change was observed in PC-5 during 24 and 48 HPI respectively **(Table 4.24 and Table 4.25)**. In PC-5 maximum downregulation was seen at 144 HPI with a fold change of -0.79 while in PC-4 slight upregulation of PAL gene at 144 HPI with a fold change of 0.53 was observed **(Figure 4.3)**.

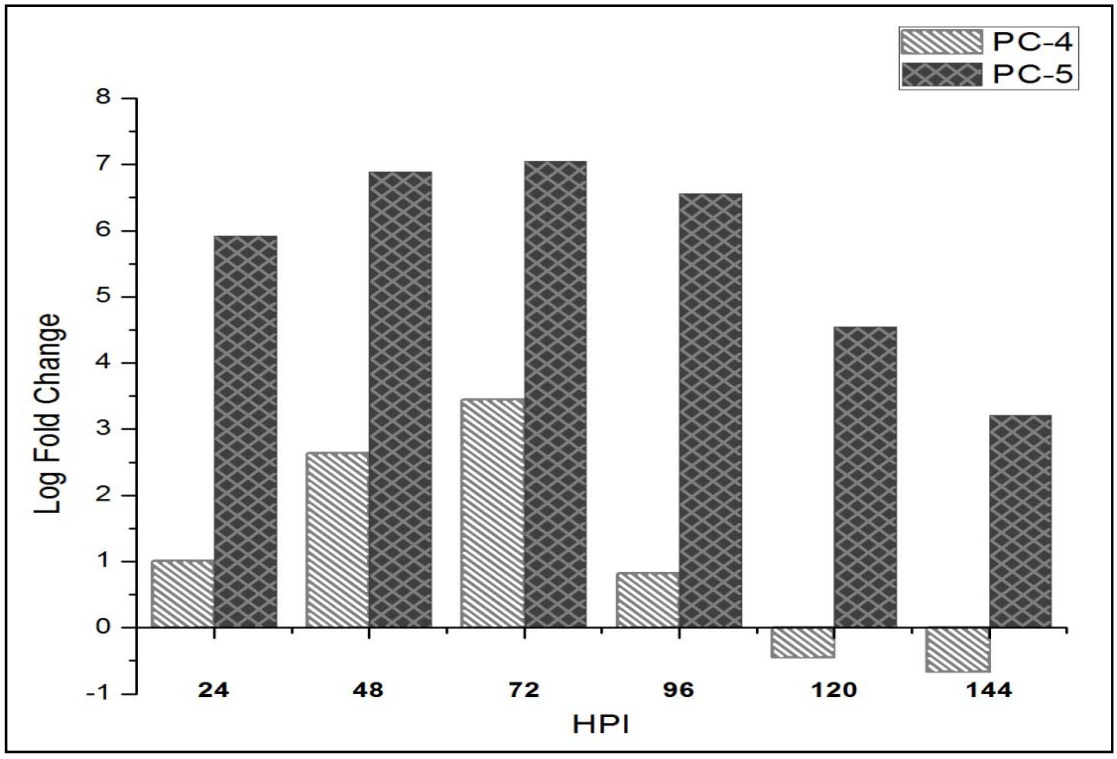


Figure 4.2 Relative gene expression of PAL during boosting in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*.

Table 4.22: Threshold values of PAL gene during boosting in PC-4 at different time intervals.

Hours	CONTROL Ct	CONTROL Ct	TREATMENT Ct	TREATMENT Ct	$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.03	18.80	30.65	18.76	10.13±0.30	1.01
48	33.30	19.29	24.99	19.75	439.586±0.27	2.64
72	33.94	18.84	24.93	21.30	2836.70±0.47	3.45
96	33.85	19.29	30.55	18.71	6.59±0.25	0.82
120	33.14	19.90	34.40	19.64	0.35±0.22	-0.45
144	32.69	19.68	34.05	18.81	0.21±0.19	-0.67

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.23: Threshold values of PAL gene during boosting in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	32.15	17.80	16.97	22.27	822696.06±0.02	5.92
48	33.90	18.67	15.14	22.80	7772784.03±0.10	6.89
72	35.45	19.00	16.97	23.93	11145824.45±0.01	7.05
96	35.32	19.68	18.25	24.39	3601084.44±0.30	6.56
120	35.31	19.97	24.26	24.05	35857.82±0.02	4.55
144	36.85	20.51	31.69	26.02	1629.26±0.16	3.21

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

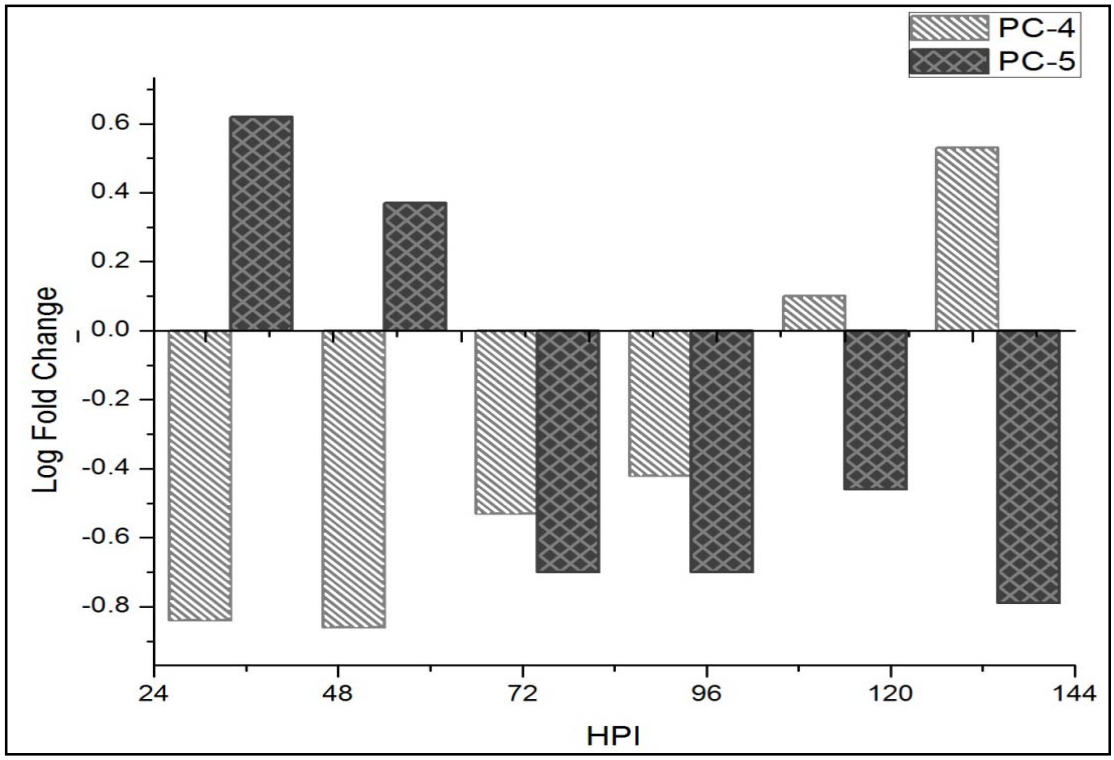


Figure 4.3 Relative gene expression of PAL during GIDR in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*.

Table 4.24: Threshold values of PAL gene during GIDR in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.47	22.46	34.03	19.23	0.14±0.05	-0.84
48	34.47	22.46	33.30	18.45	0.14±0.19	-0.86
72	34.47	22.46	33.94	20.15	0.29±0.04	-0.53
96	34.47	22.46	33.85	20.45	0.38±0.07	-0.42
120	34.47	22.46	33.14	21.45	1.25±0.05	0.10
144	34.47	22.46	32.69	22.45	3.40±0.49	0.53

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.25: Threshold values of PAL gene during GIDR in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	36.23	21.56	32.15	19.53	4.13±0.19	0.62
48	36.23	21.56	33.90	20.45	2.32±0.08	0.37
72	36.23	21.56	35.45	18.45	0.20±0.52	-0.70
96	36.23	21.56	35.32	18.34	0.20±0.08	-0.70
120	36.23	21.56	35.31	19.10	0.34±0.09	-0.46
144	36.23	21.56	36.85	19.56	0.16±0.35	-0.79

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Augmented expression of induced defense is called priming**(Conrath *et al.*, 2002)**.Priming accelerates and increases the plant's ability to activatethe defense that is best adapted to resist the stress situation encountered. Under conditions of diseasepressure,

primed plants exhibit a higher fitness than non-primed plants or defense-expressing plants.Hence, the benefits of priming outweigh its costs in environments where disease occurs.This study showed that the PAL is expressed at a higher level in PC-5during priming through TH-32 treatment. This gene was expressed at lower levelsin susceptible cultivar PC-4. Plant resistance induced by beneficial microorganismshas been associated withfaster and/or stronger activationof defence responses after pathogeninoculation**(Conrath *et al.*, 2006)**. Theexpressionprofiles of *Plasmopara viticola* in grapes provide strongsupport for the view that *Trichoderma*spp. may have a dual effect: it directlystimulates induction of some genes and further reinforcesmodulation of theseand other genes after pathogen inoculation**(Perazzolli *et al.*, 2011).**The dual effect was also reported for support for the view that *Trichoderma*spp. may have a dual effect: it directlystimulates induction of some genes and further reinforcesmodulation of these and other genes after pathogen inoculation**(Perazzolli *et al.*, 2011).**The dual effect was also reported for defencegenemodulation during resistance induced by sulfated laminarin **(Trouvelot *et al.*, 2008)** and for phytoalexin accumulation duringresistance induced by Rheum palmatum extracts **(Godard *et al.*, 2009)**. In another study the expression of PAL gene was found to be up-regulatedin maize colonized by T22**(Shoresh and Harman,2008)**.PAL induction has been linked to defence responses that involve phenylpropanoids in numerous diseases.Typically, accumulation of PAL activity and mRNA is more rapid, higher and longer lasting in incompatible plant–pathogen interactions **(Cui *et al.*, 1996)**. In sorghum, PAL is required for the production of apigeninidin, a phenylpropanoid pathway compound with antifungal properties (i.e., a phytoalexin) **(Nicholson *et al.*, 1987)**. On the second day after *G.sorghi* inoculation, a clear difference in GIDR was observed as compared to ISR-boost, this is possibly as a result of the priming and the subsequent increased inhibition of *G.sorhi* proliferation in *T. harzianum* treated plants. The major induction of the defence system during GIDR was thus limited during ISR-boost, as was the case of various defence processes. Such limited activation of defence response was previously observed for other BCAs **(Wen *et.al.*, 2005)**. In other pathosystem, after the inoculation of *Botritis cinerea* in *Arabidopsis thaliana* colonized with *T. hamatum* T382 significant change in gene expression was observed as compared to *B. cinerea* induced defence plants**(Mathys *et al.*, 2012)**.

4.4.1.2 Chitinase

It is evident from the data presented in **Table 4.26 and Table 4.27**that there was a significant increase in chitinase gene expression during 0 hrs to 72 HPT **(Figure 4.4)**. A high level of expression of this gene was observed at 72 HPT in PC-5 with a value of 3.32 while in PC-4 it was observed 3.24-fold. During 0 to 72 HPT an increasing trend of fold expression was recorded. Interestingly, the expression of chitinase in PC-5 was downregulated with a value of -0.12 while in PC-4 the basal level of expression was maintained with a value of 2.88 at 168 HPT.

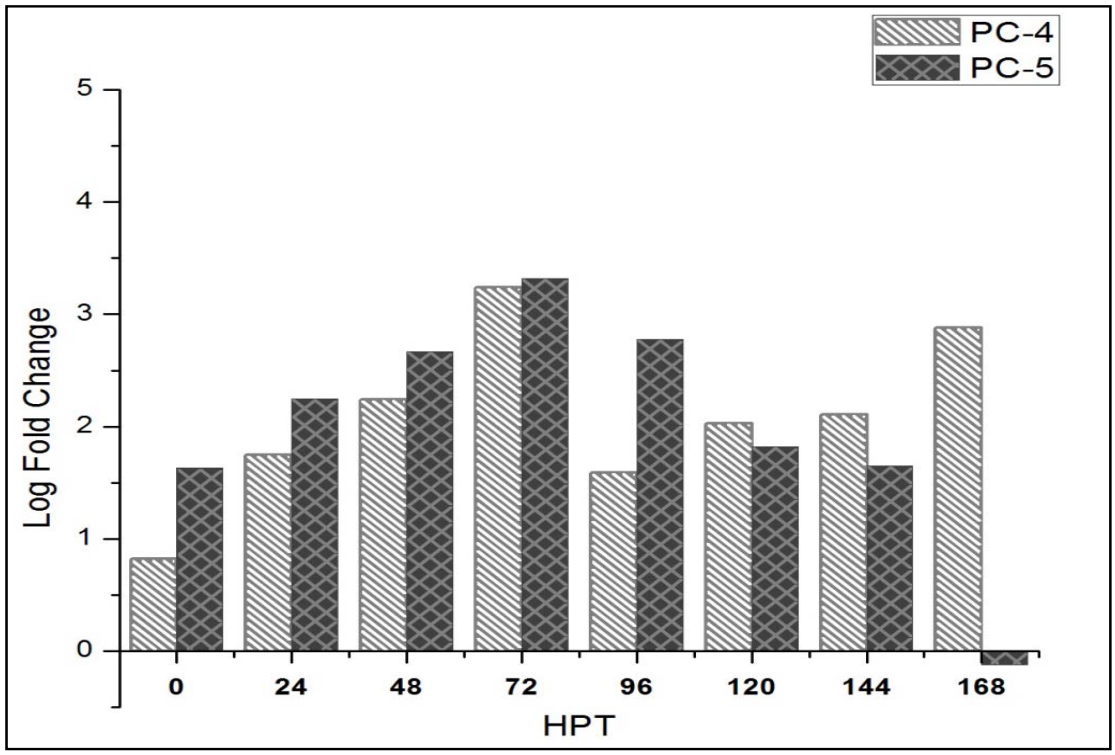


Figure 4.4 Relative gene expression of chitinase during priming in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*.

During ISR-Boost (post *G.sorghi* inoculation) significant fold change in chitinase was observed in PC-5 cultivar while it was moderately expressed in PC-4 cultivar of sorghum **(Figure 4.5)**. This sorghum gene did not show a high level of expression in PC-4. In PC-5 cultivar fold change increases rapidly ranging from 3.14 to 4.12-fold from 24 to 72 HPI. Moreoverat 120 and 144 HPI there was a downregulation of gene observed in PC-4 with a value of -0.79 and -0.89-fold respectively**(Table 4.28)**. After 72 HPI fold expression was retarded in both cultivar but in PC- 5 cultivar it was moderately maintained at 144 HPI with a value of 1.01-fold **Table 4.29.**.

Table 4.26: Threshold values of chitinase gene during priming in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	34.47	22.46	30.73	20.45	3.32±0.14	0.52
24	34.47	22.46	30.90	22.23	10.13±0.03	1.01
48	34.47	22.46	29.49	21.56	16.80±0.31	1.23
72	34.47	22.46	24.43	23.43	2062.24±0.48	3.31
96	34.47	22.46	30.96	22.29	10.13±0.48	1.01
120	34.47	22.46	31.15	22.00	7.26±0.37	0.86
144	34.47	22.46	30.77	21.83	8.40±0.06	0.92
168	34.47	22.46	29.49	20.86	10.41±0.23	1.02

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.27: Threshold values of chitinase gene during priming in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	36.23	21.56	29.76	20.49	42.22±0.30	1.63
24	36.23	21.56	30.14	22.96	179.77±0.15	2.25
48	36.23	21.56	29.65	23.86	471.14±0.18	2.67
72	36.23	21.56	32.12	28.47	2076.59±0.54	3.32
96	36.23	21.56	29.46	24.01	596.34±0.36	2.78
120	36.23	21.56	30.75	22.11	65.34±0.45	1.82
144	36.23	21.56	30.85	21.67	44.94±0.47	1.65
168	36.23	21.56	34.83	19.75	0.75±0.43	-0.12

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

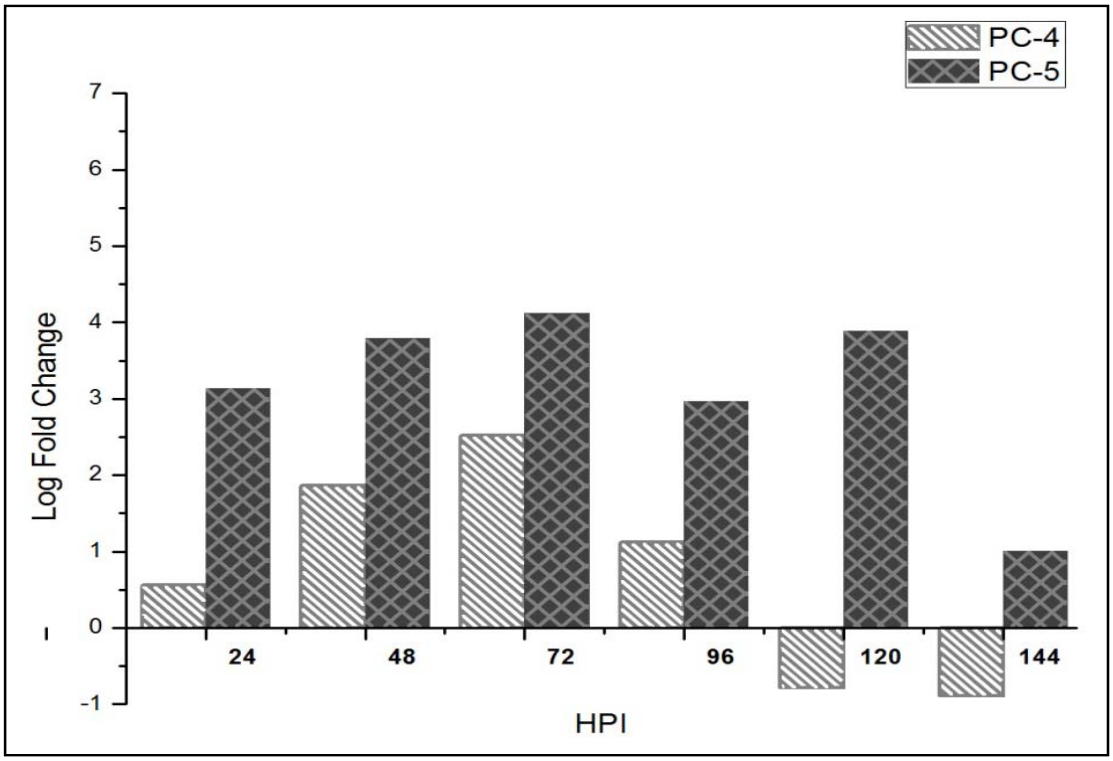


Figure 4.5 Relative gene expression of chitinase during boosting in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*.

Table 4.28: Threshold values of chitinase gene during boosting in PC-4 at different time intervals.

Hours	CONTROL Ct GOI	CONTROL Ct NORM	TREATMENT Ct GOI	TREATMENT Ct NORM	$2^{-(\Delta\Delta ct)}$	Log Fold Change
24	33.23	20.01	35.80	24.43	3.61±0.09	0.56
48	29.56	20.12	24.04	20.79	73.01±0.19	1.86
72	30.25	19.56	22.36	20.05	333.14±0.16	2.52
96	32.56	19.78	32.50	23.43	13.09±0.09	1.12
120	33.12	20.12	34.38	18.75	0.16±0.10	-0.79
144	35.46	23.12	33.96	18.66	0.13±0.17	-0.89

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.29: Threshold values of chitinase gene during boosting in PC-5 at different time intervals.

Hours	CONTROL Ct	CONTROL Ct	TREATMENT Ct	TREATMENT Ct	$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	32.16	20.12	23.56	21.94	1370.04±0.13	3.14
48	30.12	20.45	19.91	22.82	6122.90±0.08	3.79
72	29.45	19.56	20.08	23.86	13034.07±0.10	4.12
96	34.34	20.15	29.13	24.80	929.30±0.15	2.97
120	30.10	20.14	20.98	23.94	7750.10±0.16	3.89
144	33.12	21.01	28.32	19.57	10.27±0.08	1.01

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

During GIDR (Sb+Gs vs Sb) in PC-4 cultivar an initial downregulation with a value of -0.36-fold change was observed at 24 HPI while 24 h later at 48 HPI it was increased upto 0.77-fold change**(Figure 4.6)**. Later on, the basal level of downregulation was maintained for successive hrs upto 120 HPI with a value of 0.40 to -0.30-fold while at 144 HPI it was again slightly upregulated with a value of -0.10-fold **Table 4.30**. In PC-5 cultivar a moderate level of expression was maintained throughout hours post *G.sorghi*inoculation. Moreover maximum fold change was observed at 48 HPI in PC-5with a value of 1.51-fold. Interestingly at 120 and 144 hrs the chitinase expression was basally maintained with a value of 1.42 and 0.77- fold**Table 4.31**.

A time-course expression of chitinase gene in 23-day-old plants of sorghum selected for this studyshowed that their expression levels were significantlyhigher in resistant cultivar. In past too, defenseresponse studies using RT-PCR havebeen carried out in tomato plants colonized with *Trichoderma* spp. and*Bacillus subtilis* (**Hafez *et al.*, 2013).** The most prevalent observation, was an increase in the genetic variations between the control and the treated plants. It was shown that many down-regulated (turned off) and up-regulated genes (turned on) were observed in both samples treated with *T. viride* and *B. subtilis*, at different times. In case of primer ch25, the most induced/suppressed genes were pragmatic with samples 1 and 4 (**Hafez *et al.*, 2013).** Chitinases are key enzymes involved in plant-microbe interactions, and are grouped in the pathogen-related protein type three families (PR-3). Five classes of plant chitinases have been proposed based on their peptidic sequences, conserved domains and specific activities**(Selitrennikoff, 2001)**.

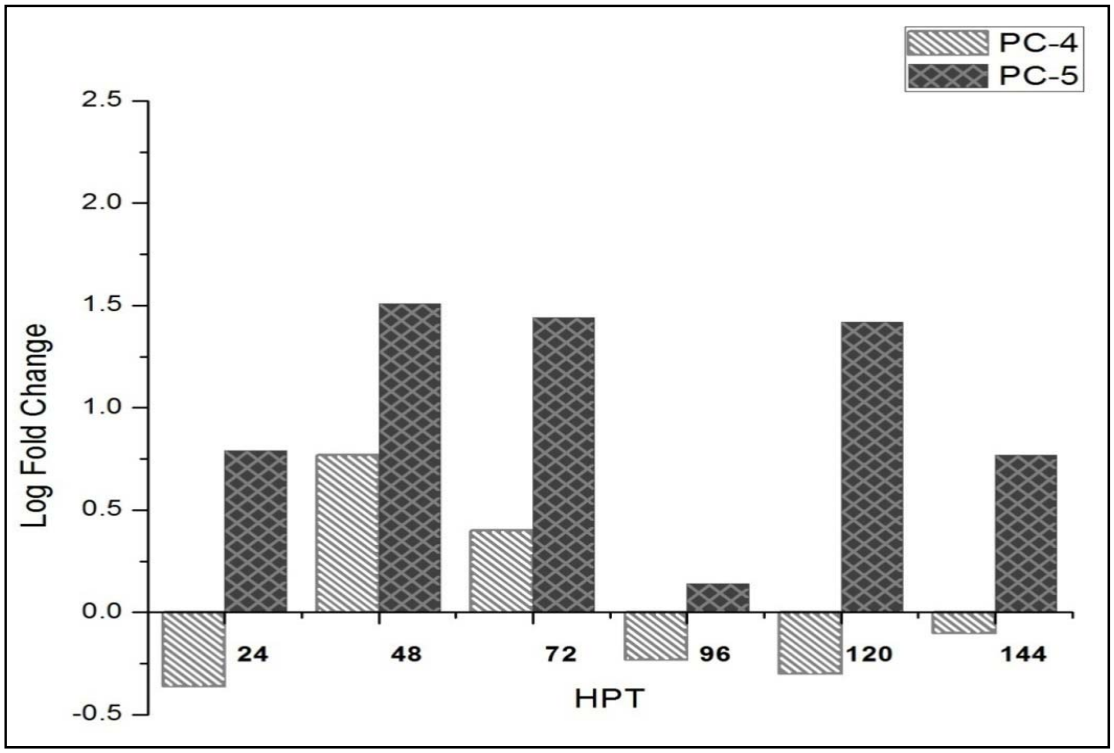


Figure 4.6 Relative gene expression of chitinase during GIDR in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*.

Table 4.30: Threshold values of chitinase gene during GIDR in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.47	22.46	33.23	20.01	0.43±0.08	-0.36
48	34.47	22.46	29.56	20.12	5.94±0.20	0.77
72	34.47	22.46	30.25	19.56	2.50±0.07	0.40
96	34.47	22.46	32.56	19.78	0.59±0.10	-0.23
120	34.47	22.46	33.12	20.12	0.50±0.07	-0.30
144	34.47	22.46	35.46	23.12	0.80±0.09	-0.10

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.31: Threshold values of chitinaseduring GIDR gene in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	36.23	21.56	32.16	20.12	6.19±0.11	0.79
48	36.23	21.56	30.12	20.45	32.00±0.18	1.51
72	36.23	21.56	29.45	19.56	27.47±0.19	1.44
96	36.23	21.56	34.34	20.15	1.39±0.24	0.14
120	36.23	21.56	30.10	20.14	26.17±0.24	1.42
144	36.23	21.56	33.12	21.01	5.90±0.22	0.77

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Because chitin is a primary structural component of the wall of all true fungi, chitinases are considered to play a major role during pathogenic plant-fungal interactions**(Bravo *et al.*, 2003)**. The combined expression of chitinases with other plant-defense proteins, such as glucanases and ribosome-inactivating proteins further enhances the plant's resistance to fungal attack. The time for chitinase induction is also dependent on the specific pathogen-host interaction, and varies from minutes to 15-20 h **(Bravo *et al.*, 2003)**.Similar defense response patterns were observed by**Caldo *et al.*, (2004)** for compatible and incompatibleinteractions involving Mla1-like genes in barley inresponse to powdery mildew; transcripts build-up ofdefense-related genes were same in both resistant andsusceptible varieties except that during later stages ofinfection expression levels get decreased.During GIDR the infected plants consistently showed highergene expression level of chitinase in comparison to thecontrol plants (including mock-inoculation controls).Hence, the chitinase activity can certainly be attributeddue to induction by pathogen.

4.4.1.3. CHS

During ISR-prime, the maximum fold change in the expression ofthis gene was 4.00 and 3.21-fold in PC-5, whereas in PC-4 the values were 3.54 and 2.64-fold at 72 and 48 HPT respectively.The higherexpression of SbCHS was noted in PC-5, whereasit was in PC-4**(Figure 4.7)**. Initially at 0 HPT the least fold change was observed in both PC-4and PC-5 cultivars with a value of 0.92 and 1.11-fold respectively. Later a gradual increase in CHS

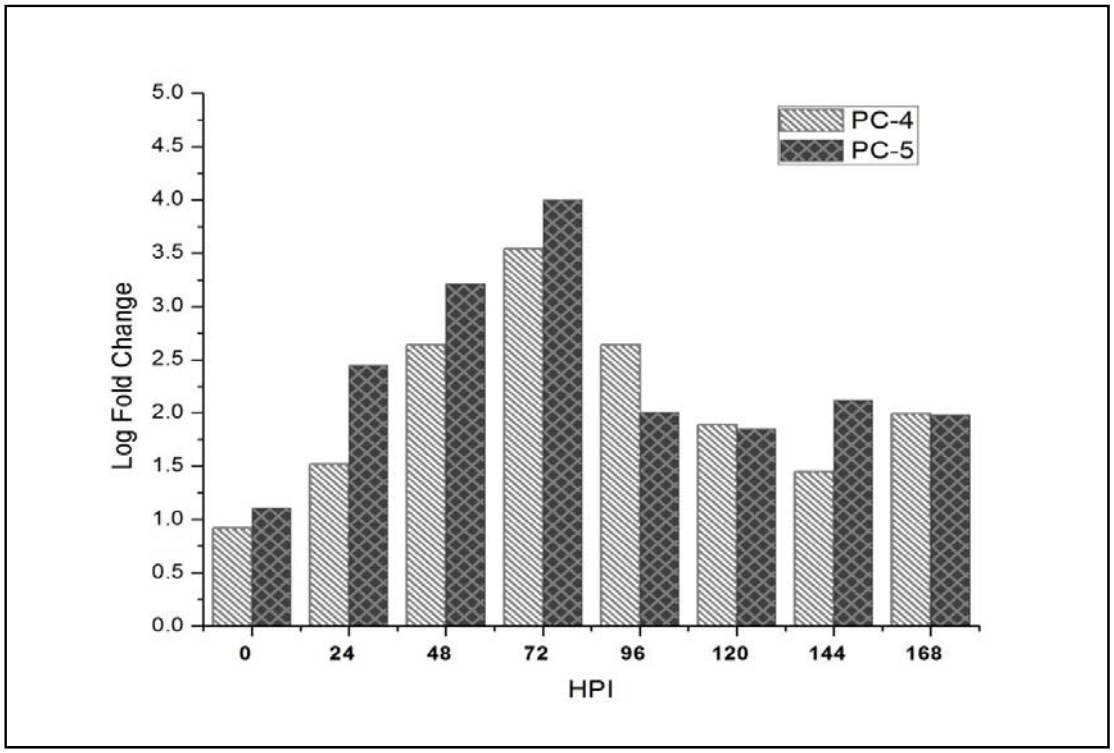


Figure 4.7 Relative gene expression of CHS during priming in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*.

Table 4.32: Threshold values of CHS gene during priming in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	34.47	22.46	30.88	21.94	8.40±0.27	0.92
24	34.47	22.46	27.74	20.79	33.36±0.48	1.52
48	34.47	22.46	24.80	21.56	436.55±0.43	2.64
72	34.47	22.46	20.79	20.53	3444.31±0.29	3.54
96	34.47	22.46	23.43	20.19	436.55±0.17	2.64
120	34.47	22.46	27.56	21.82	77.17±0.30	1.89
144	34.47	22.46	27.64	20.45	28.25±0.47	1.45
168	34.47	22.46	25.60	20.19	97.01±0.54	1.99

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.33: Threshold values of CHS gene during priming in PC-5 at different time intervals.

Hours	CONTROL Ct	CONTROL Ct	TREATMENT Ct	TREATMENT Ct	$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	36.23	21.56	32.91	21.94	13.00±0.41	1.11
24	36.23	21.56	29.34	22.82	284.05±0.44	2.45
48	36.23	21.56	27.86	23.86	1629.26±0.20	3.21
72	36.23	21.56	30.69	29.31	10015.87±0.27	4.00
96	36.23	21.56	35.07	27.07	101.83±0.15	2.01
120	36.23	21.56	35.76	27.22	70.03±0.23	1.85
144	36.23	21.56	35.90	28.27	131.60±0.29	2.12
168	36.23	21.56	36.07	27.98	95.67±0.52	1.98

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

expression was recorded in both cultivars upto 72 HPT with a value ranging from 1.52-3.54-fold in PC-4 and 2.45-4.00-fold PC-5. At 168 HPT both cultivars maintained their CHS basal gene expression with a value of 1.99 and 1.98-fold in PC-4 and PC-5 respectively **(Table 4.32 and Table 4.33)**.

During ISR- boost (post *G.sorghi* inoculation), the CHS gene was expressed at a high level (**Figure 4.8)**.The greatest expression of this gene was seenin PC-5 cultivar with a fold change of 6.13at 96 HPIwhile 4.99-foldchange wasobserved in PC-4 cultivar at 72 HPI. Upon fungal inoculation, the expression level of PC-4 cultivar increased from0.94 at 24 HPI to 4.99-foldat 72 HPI**(Table 4.34& 4.35)**after that it gradually decreases and reaches below ground level (-0.45-fold) at144 HPI but in case of PC-5 cultivar fold change increases rapidly ranging from 4.64 to 6.13-fold from 24 to 96 HPI but later on a decreasing trend was observed from 120 to 144 HPI (3.25-2.12 fold).

During GIDR, it is evident that in PC-4 cultivar of sorghum prominent downregulation of CHS gene was observed while in PC-5 cultivar after initial upregulation upto48 HPI it was downregulated during 72 to 120 HPI **(Figure 4.9)**. In PC-4 cultivar maximum fold change with a value of -0.57 and -0.62-fold wasobserved during 144 and

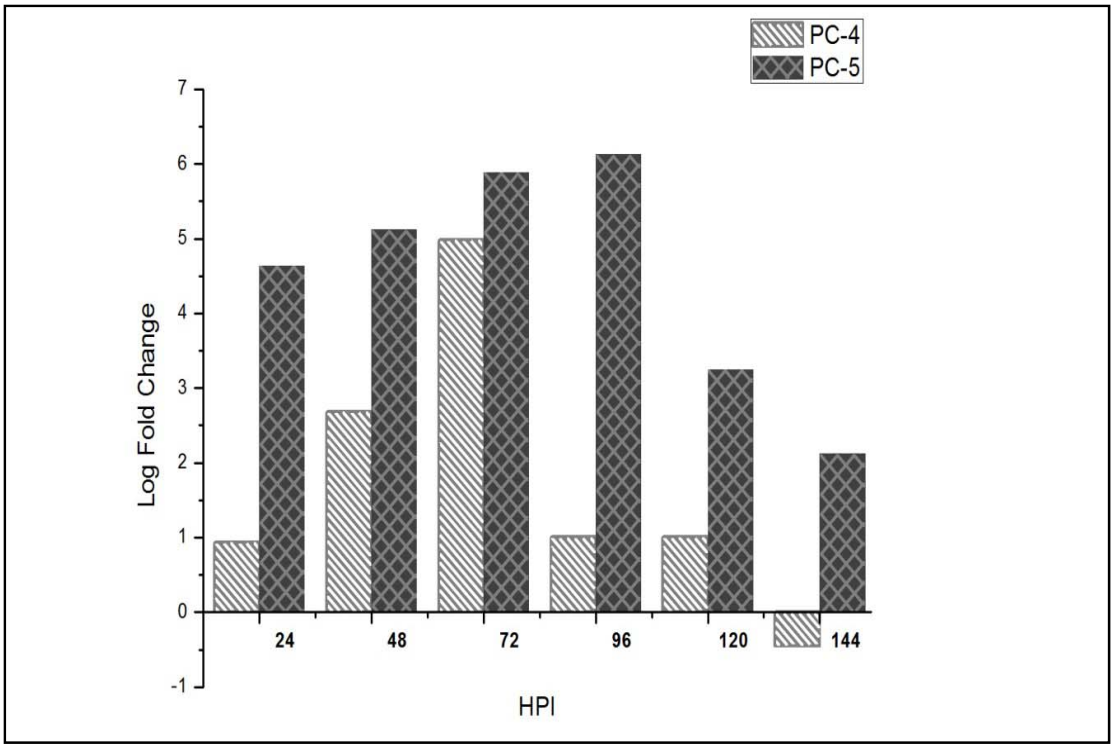


Figure 4.8 Relative gene expression of CHS during boosting in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*.

Table 4.34: Threshold values of CHS gene during boosting in PC-4 at different time intervals.

Hours	CONTROL Ct	CONTROL Ct	TREATMENT Ct	TREATMENT Ct	$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	37.39	20.14	37.27	23.15	8.75±0.08	0.94
48	37.28	20.04	28.42	20.12	491.14±0.14	2.69
72	37.23	20.12	24.54	24.00	97289.74±0.15	4.99
96	35.92	21.84	31.97	21.23	10.13±0.16	1.01
120	38.27	21.65	34.86	21.58	10.13±0.21	1.01
144	35.91	22.01	39.85	24.45	0.35±0.22	-0.45

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.35: Threshold values of CHS gene during boosting in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.15	21.15	22.16	24.56	43237.64±0.12	4.64
48	30.15	20.12	19.92	26.89	131072.00±0.16	5.12
72	35.12	21.34	20.11	25.89	772941.66±0.10	5.89
96	37.45	22.45	20.38	25.76	1364555.84±0.14	6.13
120	36.12	20.89	28.76	24.31	1758.34±0.22	3.25
144	32.15	20.67	29.57	25.12	130.69±0.20	2.12

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

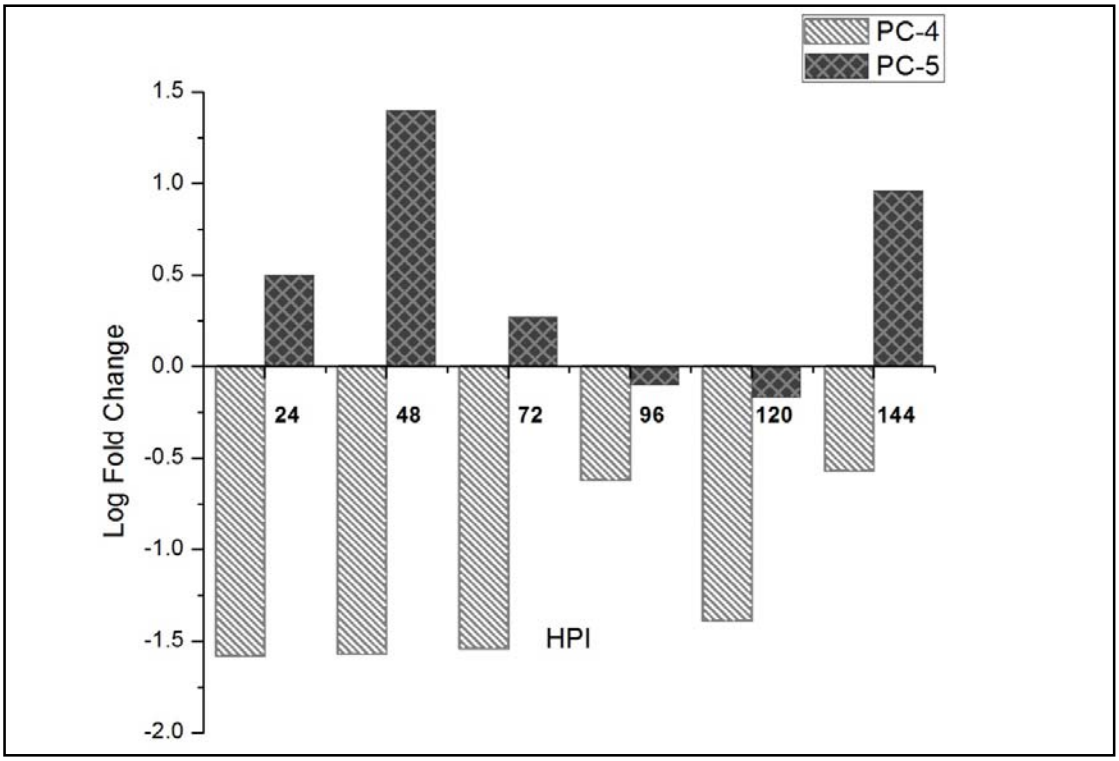


Figure 4.9 Relative gene expression of CHS during GIDR in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

96 HPI respectively. While in PC-5 maximum fold change of 1.40 and 0.96-fold at 48 and 144 HPI respectively was recorded. Least fold change was observed at 120(-0.17-fold) and 96(-0.10-fold) HPI in PC-5 whereas in PC-4 it was -1.58 and -1.57-fold at 24-48 HPI respectively **(Table 4.36 and Table 4.37)**.

Table 4.36: Threshold values of CHS gene during GIDR in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.47	22.46	37.39	20.14	0.03±0.13	-1.58
48	34.47	22.46	37.28	20.04	0.03±0.20	-1.57
72	34.47	22.46	37.23	20.12	0.03±0.22	-1.54
96	34.47	22.46	35.92	21.84	0.24±0.20	-0.62
120	34.47	22.46	38.27	21.65	0.04±0.17	-1.39
144	34.47	22.46	35.91	22.01	0.27±0.19	-0.57

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.37: Threshold values of CHS gene during GIDR in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	36.23	21.56	34.15	21.15	3.18±0.16	0.50
48	36.23	21.56	30.15	20.12	24.93±0.22	1.40
72	36.23	21.56	35.12	21.34	1.85±0.19	0.27
96	36.23	21.56	37.45	22.45	0.80±0.26	-0.10
120	36.23	21.56	36.12	20.89	0.68±0.21	-0.17
144	36.23	21.56	32.15	20.67	9.13±0.14	0.96

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

SbSTS1 represents the first exampleof a STS gene in monocots. The gene is notconstitutively expressed but inducible following fungal inoculation **(Christine *et al.*, 2005**). This study clearly revealed the effect of *Trichoderma harzainum* application during priming when the both cultivars of sorghum were not under the influence of pathogen.Expression level of STS gene during priming in resistant variety was 60% more than susceptible one. This study also indicated trends towards the expression analysis during different time periods which clearly showed that the expression of this gene was significantly upregulated in both cultivars but marked difference was evident in resistant cultivar.Thereports also postulated that higher expression ofstilbene synthase is related to disease tolerance. The expression of this gene was first notedin sorghum seedlings infected with *Colletotrichum sublineolum***(Lo *et al.,* 2002)**. A fast accumulation ofstilbene synthase-specific mRNA followed by a decreasewas also obtained in transgenic tobacco **(Hain *et al.,* 1993)** and transgenic rice **(Stark-Lorenzen *et al.,* 1997)**. Marked increase in barley occurred after 48 h,but was not observed in transgenic tobacco and rice. Initially the expression of STS gene was upregulated during 0-72HPT but later on a slight decrease in expression was observed. Other reports also support our findings, where the repeated increase was only slight and the stilbenesynthase mRNA synthesis disappeared completely72 h after inoculation. Stilbene synthesis was inducedin grapevine by inoculation with the pathogens*Botrytis cinerea* or *Plasmopara viticola***(Blaich and Bachmann, 1980)**. The level of resistance to *P. viticola*was positively correlated with the capacity of Vitis spp.to synthesize stilbene **(Dercks and Creasy, 1989)**. Theeffectiveness of stilbene synthase genes in increasingresistance in transgenic tomatoes was shown by a 65%reduction in disease incidence following inoculationwith the pathogen *Phytophthora infestans***(Thomzik *et al.,* 1997)**. Results from transgenic rice plantsindicated that the stilbene synthase gene enhancedplant resistance against the rice blast fungal pathogen*Pyricularia oryzae***(Stark-Lorenzen *et al.,* 1997)**.Stilbene synthesis was inducedin grapevine by inoculation with the pathogens*Botrytis cinerea* or *Plasmopara viticola***(Blaich and Bachmann, 1980)**. The level of resistance to *P. viticola*was positively correlated with the capacity of Vitis spp.to synthesize stilbene **(Dercks and Creasy, 1989)**.

4.4.2 Determination of the expression of defense response genes during priming and boosting through *Pseudomonas fluorescens* (Pf-31)in PC-4 and PC-5 cultivars using real time-RT-PCR.

4.4.2.1 PAL

During ISR- prime analysis, the expression of sorghum PAL gene, SbPAL was evaluated in PC-4 and PC-5 when treated with Pf-31, SbPAL wasexpressed in both cultivars. The average fold change in the expressionof this gene occurred between 24 and 120 hours post treatment (HPT). The higherexpression of SbPAL was noted in PC-5, whereasit was low in PC-4**(Figure 4.10)**. The maximum fold changein the expression of this gene was 4.51-fold inPC-5 sample**(Table 4.39)**, whereas it was 3.12-fold in PC-4 samples**(Table 4.38)**. Theexpression of PAL in PC-4 was induced from0.80-fold at 0 hto2.01-fold at 24 h. Maximum fold change was observed at 72 h (3.12-fold). However, the expression of thisgene decreased after 72 h. Similarly in caseof PC-5, the expression of PAL was induced from 1.26-fold at 0 h to 3.45-fold at 24 h while maximum fold change lasted upto 72 hrs (4.51-fold)after Pf-31 treatment. In PC-4, after 72 hrs a transient decrease of gene expression was observed at 96 hrs (1.09-fold) as compared to 3.12-fold

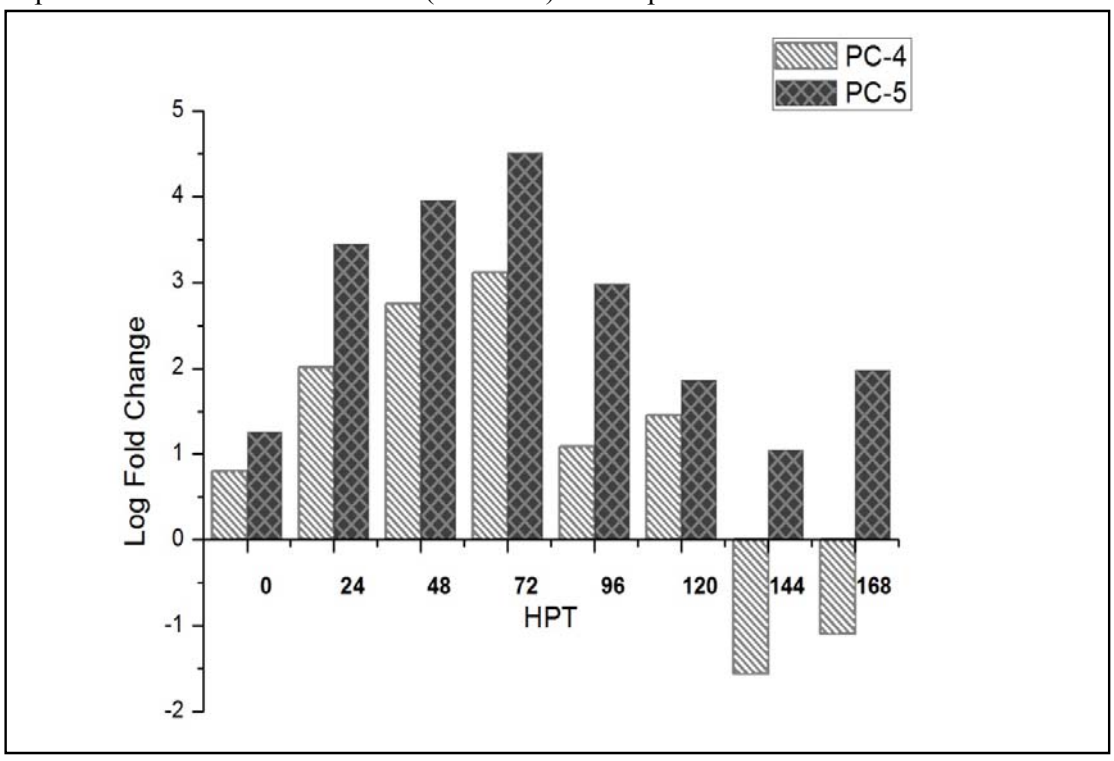


Figure 4.10 Relative gene expression of PAL during priming in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.38: Threshold values of PAL gene during priming in PC-4 at different time intervals

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	34.47	22.46	30.88	21.53	6.32±0.16	0.80
24	34.47	22.46	26.80	21.46	101.83±0.31	2.01
48	34.47	22.46	24.77	21.88	556.41±0.23	2.75
72	34.47	22.46	22.65	21.01	1323.37±0.13	3.12
96	34.47	22.46	28.85	20.45	12.21±0.09	1.09
120	34.47	22.46	28.06	20.86	28.05±0.23	1.45
144	34.47	22.46	36.18	19.00	0.03±0.29	-1.56
168	34.47	22.46	35.75	20.12	0.08±0.28	-1.09

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.39: Threshold values of PAL gene during priming in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	36.23	21.56	32.63	22.15	18.25±0.16	1.26
24	36.23	21.56	27.20	24.00	2836.70±0.20	3.45
48	36.23	21.56	26.06	24.56	9216.48±0.35	3.96
72	36.23	21.56	25.15	25.45	32093.64±0.19	4.51
96	36.23	21.56	28.34	23.56	948.83±0.15	2.98
120	36.23	21.56	31.62	23.12	72.00±0.32	1.86
144	36.23	21.56	33.17	22.00	11.31±0.27	1.05
168	36.23	21.56	31.08	23.00	96.34±0.23	1.98

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

(72 hrs) but later on it was greatly downregulated at 168 hrs (-1.09-fold). While almost similar trend was observed also in case of PC-5 where fold change was downregulated at 96 hrs (2.98-fold) from 4.51-fold at 72 hrs but basal expression was maintained at 168 hrs (1.98-fold).

During ISR- boost (post *G.sorghi* inoculation), the PAL gene was expressed at a very high level. A significant fold change of this gene in PC-5 and PC-4 cultivars of sorghum is an evidence of induction of host defense gene during pathogen infection**(Figure 4.11)**. The greatest expression of this gene was seenin PC-5 cultivar with a fold change of 7.89-fold at 72 HPI**(Table 4.41)** while 2.95-foldchange was observed in PC-4 cultivar at 48 HPI (**Table 4.40)**. Upon fungal inoculation, the expression level in PC-4 cultivar increased from 1.85 to 2.95 between 24 to 48 HPIrespectively after that it decreases and reaches below (0.09-fold)than 1.85-fold (24 HPI) but in case of PC-5 cultivar fold change increases rapidly ranging from 3.64 to 7.89-fold from 24 to 72 HPI. However the expression of PAL in PC-5 dis not decrease as much during 96(7.01-fold) and 120(5.49-fold) HPI, later on it was maintained at 144 HPI (3.10-fold).

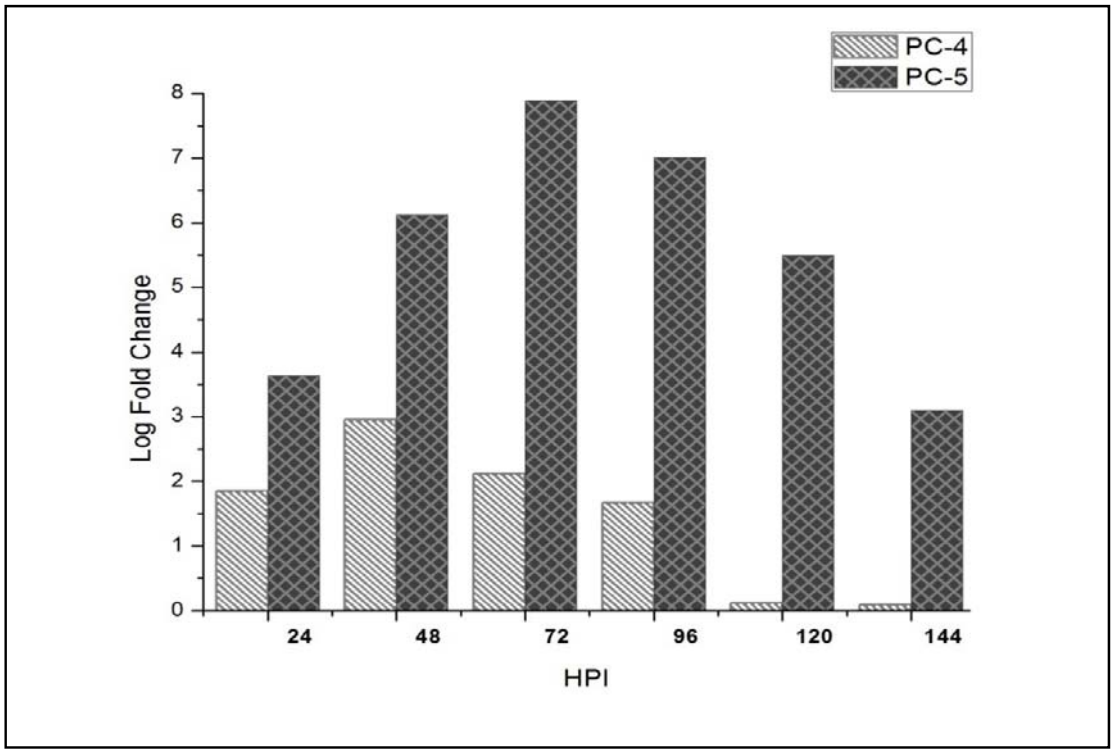


Figure 4.11 Relative gene expression of PAL during boosting in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.40: Threshold values of PAL gene during boosting in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.34	19.23	36.11	27.45	70.36±0.24	1.85
48	32.98	18.45	25.28	20.23	893.51±0.23	2.95
72	33.9	20.15	29.88	23.12	130.39±0.30	2.12
96	33.78	20.45	33.17	25.32	46.96±0.19	1.67
120	33.01	21.45	36.51	25.23	1.33±0.23	0.12
144	32.49	22.45	35.57	25.64	1.24±0.14	0.09

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.41: Threshold values of PAL gene during boosting in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	32.12	32.64	32.15	19.53	4400.14±0.28	3.64
48	30.12	23.24	33.99	20.45	1321122.97±0.20	6.12
72	29.32	20.11	36.01	18.45	77444887.47±0.25	7.89
96	29.35	23.05	35.49	18.34	10208965.41±0.18	7.01
120	30.45	28.43	35.46	19.10	307451.64±0.19	5.49
144	28.35	35.34	36.44	19.56	1260.69±0.24	3.10

Each value represents the mean of three replicates with ±S.E. Mean differences are significant at P< 0.05 level.

During GIDR, it is evident that in both cultivars of sorghum prominent decrease in PAL gene expression was observed **Figure 4.12**. In PC-4 cultivar -0.84 and -0.86 fold change was observed while 0.62 and 0.37 fold change was observed in PC-5 during 24 and 48 HPI respectively **(Table 4.42 and Table 4.43)**. In PC-5 maximum downregulation was

seen at 144 HPI with a fold change of -0.79 while in PC-4 a slight upregulation of PAL gene was observed at 144 HPI with a fold change of 0.53.

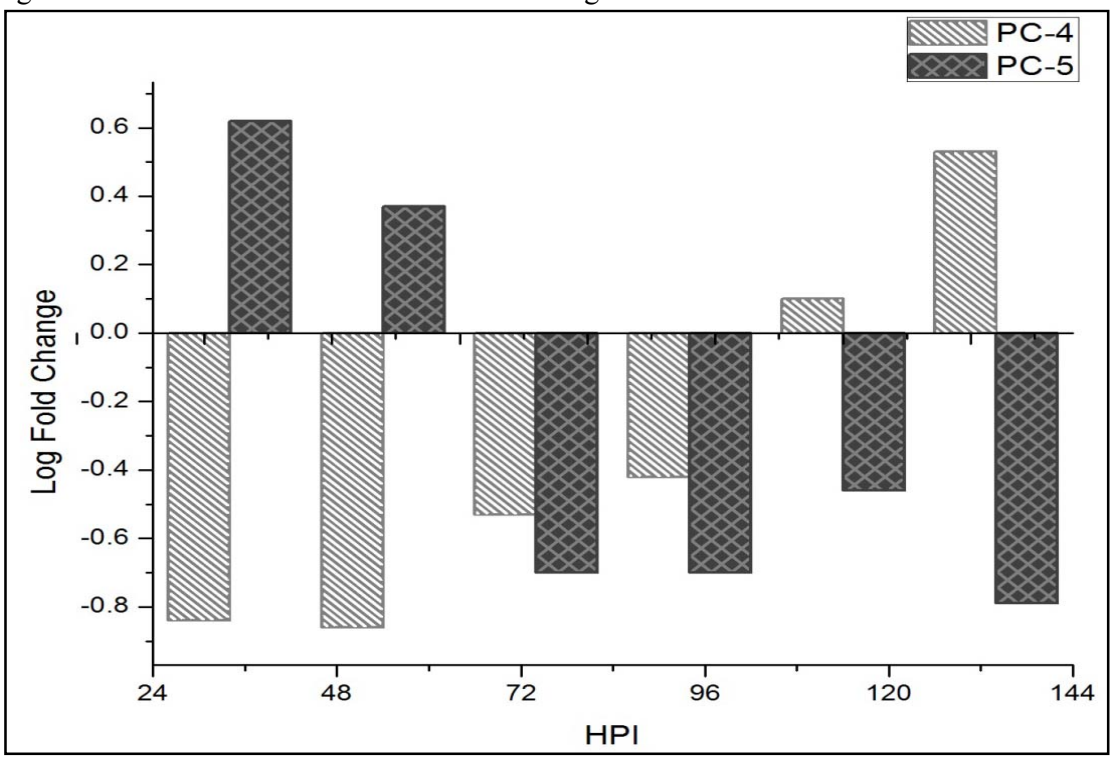


Figure 4.12 Relative gene expression of PAL during GIDR in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.42: Threshold values of PAL gene during GIDR in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.47	22.46	34.03	19.23	0.14±0.05	-0.84
48	34.47	22.46	33.30	18.45	0.14±0.19	-0.86
72	34.47	22.46	33.94	20.15	0.29±0.04	-0.53
96	34.47	22.46	33.85	20.45	0.38±0.07	-0.42
120	34.47	22.46	33.14	21.45	1.25±0.05	0.10
144	34.47	22.46	32.69	22.45	3.40±0.49	0.53

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.43: Threshold values of PAL gene during GIDR in PC-5 at different time intervals.

Hours	CONTROL Ct GOI	CONTROL Ct NORM	TREATMENT Ct GOI	TREATMENT Ct NORM	$2^{-(\Delta\Delta ct)}$	Log Fold Change
24	36.23	21.56	32.15	19.53	4.13±0.19	0.62
48	36.23	21.56	33.90	20.45	2.32±0.08	0.37
72	36.23	21.56	35.45	18.45	0.20±0.52	-0.70
96	36.23	21.56	35.32	18.34	0.20±0.08	-0.70
120	36.23	21.56	35.31	19.10	0.34±0.09	-0.46
144	36.23	21.56	36.85	19.56	0.16±0.35	-0.79

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

It is well known that the defense genes areinducible genes and appropriate stimuli or signals areneeded to activate them. Gene expression patterns werecompared between the treatments and also between thecultivars. During prime analysis, PAL expression was greater in PC-5 cultivar (resistant) than PC-4 cultivar. PAL activity in seedlings treated withonly *P. fluorescens* remained almost unchangedthroughout the experiment but compared to control it wasslightly higher.Induction of PAL by fluorescent pseudomonads was reported also incucumber **(Chen *et al.,* 2000)**, tomato **(Ramamoorthy *et al.,* 2002, Anand *et al.,* 2007)** and mulberry**(Ganeshmoorthi *et al.,* 2008)**.In resistant cv. PC-5 treated with*P. fluorescens*Pf-31and inoculated with *G.sorghi*,we detected maximum expression of PALgenes when compared to control and alsoto the susceptible cv. PC-4. Pre-treatment of tomato plants with *P. fluorescens* triggered the increased PALactivities in response to invasion by*R. solanacearum***(Vanitha and Umesha, 2011)**.**Goswami and Punja (2008)** reported theup-regulation of number of genes involved in hostdefense responses in ginseng (*Panax quinquefolius*)roots challenged with *Fusarium equiseti.* **Peng *et al.,* (2005)**also reported the maximum gene expression of PAL gene in tomato seedlings when exposed to wounding.**Liu *et al.,* (2010)** reported that the *Bacillus cereus* induced the systemic resistance byexpressing defense-related genes in *Lilium formosanum*against leaf blight caused by *Botrytis elliptica.*

4.4.2.2 Chitinase

It is evident from the data presented in **Table 4.44 and Table 4.45**that there was a significant increase in chitinase gene expression during 0 hrs to 96 HPT. Initially at 0 HPT the change in expression of chitinase was less in both PC-5(1.26-fold) and PC-4(0.80-fold) (**Figure 13)**. A high level of expression of this gene was observed at 72 HPT (4.01-fold)in PC-5 treated with Pf-31 while in PC-4 the level of chitinase expression was moderate with a maximum value of 2.89-fold (72 HPT). During 0 to 72 HPT an increasing trend of fold expression was recorded.Interestingly, the expression of chitinase in PC-5 and PC-4 was significantly downregulated with a value of -0.98-foldand -0.96-fold at 168 HPT.

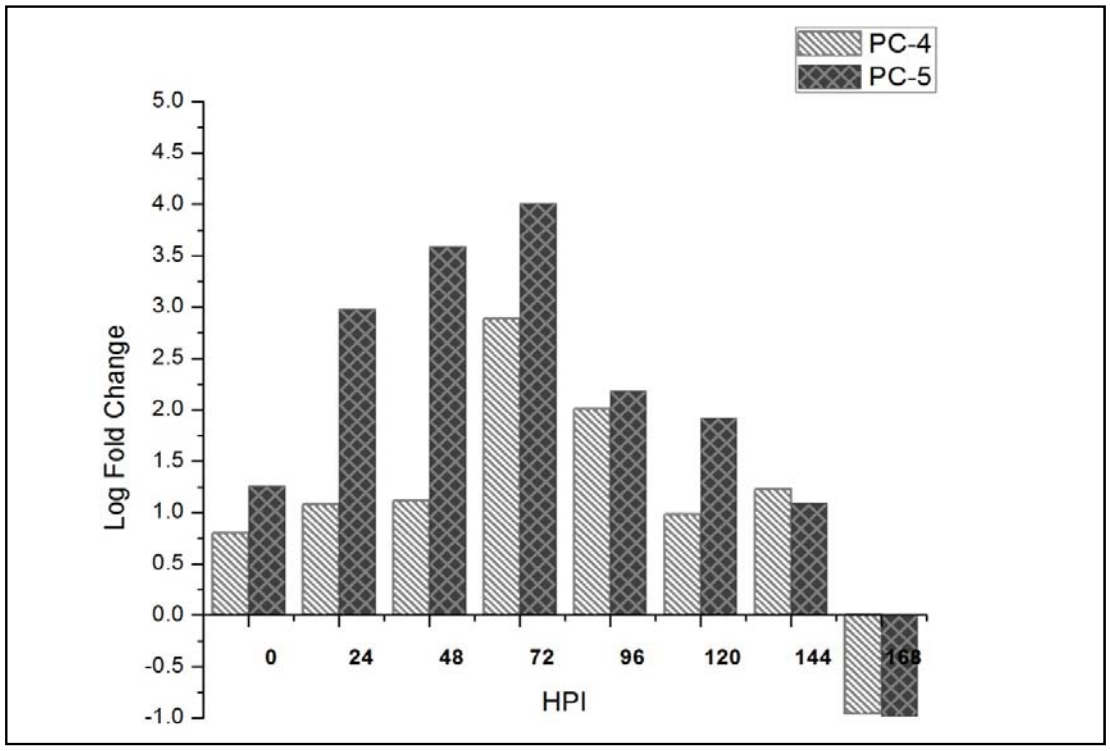


Figure 4.13 Relative gene expression of chitinase during priming in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

During ISR-Boost (post *G.sorghi* inoculation) significant fold change in chitinase was observed in PC-5 cultivar while it was moderately expressed in PC-4 cultivar of sorghum **(Figure 4.14)**. This sorghum gene did not show a high level of expression in PC-4. Increasing fold change remained upto 72 HPI (2.98-fold) in PC-4 **(Table 4.46)**while in PC-5 cultivar, fold change increased rapidly ranging from 2.99 to 7.01-fold from 24 to 96 HPI **(Table 4.47)**. Moreoverat 96(1.85-fold), 120(1.02-fold) and 144(0.78-fold) HPI there

Table 4.44: Threshold values of chitinase gene during priming in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	34.47	22.46	34.70	25.35	6.32±0.11	0.80
24	34.47	22.46	30.56	22.13	11.96±0.19	1.08
48	34.47	22.46	29.52	21.23	13.18±0.25	1.12
72	34.47	22.46	23.98	21.56	770.69±0.30	2.89
96	34.47	22.46	27.68	22.34	101.83±0.29	2.01
120	34.47	22.46	33.10	24.35	9.58±0.27	0.98
144	34.47	22.46	30.22	22.31	17.15±0.17	1.23
168	34.47	22.46	36.43	21.23	0.11±0.24	-0.96

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.45: Threshold values of chitinase during priming gene in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	36.23	21.56	36.60	26.12	18.25±0.21	1.26
24	36.23	21.56	28.01	23.23	948.83±0.23	2.98
48	36.23	21.56	27.31	24.56	3875.05±0.24	3.59
72	36.23	21.56	25.90	24.56	10297.45±0.27	4.01
96	36.23	21.56	28.65	21.23	152.22±0.25	2.18
120	36.23	21.56	32.86	24.56	82.71±0.31	1.92
144	36.23	21.56	36.94	25.89	12.30±0.19	1.09
168	36.23	21.56	38.17	20.23	0.10±0.28	-0.98

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

was a downregulation of gene in PC-4 whereas in PC-5 it was maintained at 144 HPI with a value of 2.24-fold.

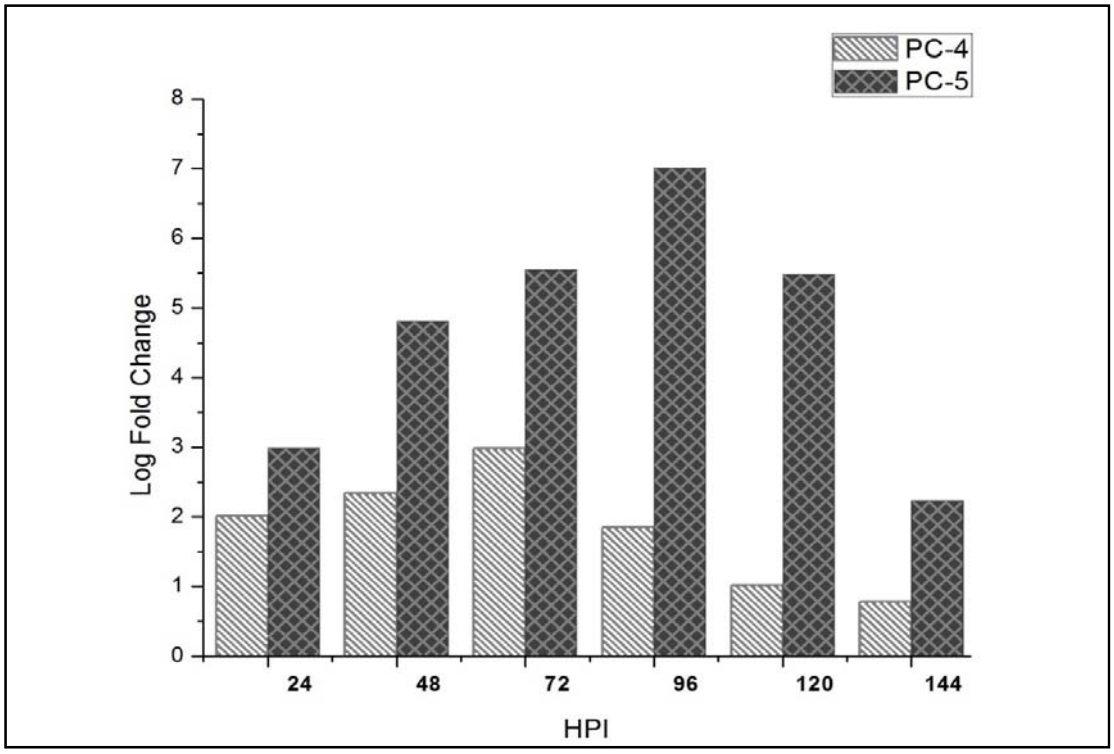


Figure 4.14 Relative gene expression of chitinase during boosting in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.46: Threshold values of chitinase gene during boosting in PC-4 at different time intervals.

Hours	CONTROL Ct GOI	CONTROL Ct NORM	TREATMENT Ct GOI	TREATMENT Ct NORM	$2^{-(\Delta\Delta ct)}$	Log Fold Change
24	33.23	20.01	34.45	21.23	102.54±0.28	2.01
48	29.56	20.12	31.33	21.89	219.79±0.25	2.34
72	30.25	19.56	30.79	20.10	962.07±0.31	2.98
96	32.56	19.78	35.90	23.12	71.01±0.25	1.85
120	33.12	23.12	37.56	24.56	10.41±0.23	1.02
144	35.46	20.12	37.79	25.45	6.02±0.21	0.78

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.47: Threshold values of chitinase gene during boosting in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	32.16	20.12	30.57	28.45	968.76±0.18	2.99
48	30.12	20.45	21.13	27.45	65083.31±0.18	4.81
72	29.45	19.56	21.02	29.56	353169.20±0.29	5.55
96	34.34	20.15	20.78	29.89	10327587.87±0.30	7.01
120	30.10	20.14	21.28	29.56	309590.14±0.28	5.49
144	33.12	21.01	33.79	29.13	174.85±0.18	2.24

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

During GIDR (Sb+Gs vs Sb) in PC-4 cultivar an initial downregulation with a value of -0.36-fold change was observed at 24 HPI while 24 h later at 48 HPI it was increased upto 0.77-fold change**(Figure 4.15)**. Later on, the basal level of downregulation was maintained for successive hrs upto 120 HPI with a value of 0.40 to -0.30-fold while at 144 HPI it was again slightly upregulated with a value of -0.10-fold. In PC-5 cultivar a moderate level of expression was maintained throughout hours post *G.sorghi*inoculation. Moreover maximum fold change was observed at 48 HPI in PC-5with a value of 1.51-fold. Interestingly at 120 and 144 hrs the chitinase expression was basally maintained with a value of 1.42 and 0.77- fold**(Table 4.48& 4.49)**.

Inducing the plant's own defense mechanisms by prior application of a biologicalinducer is thought to be a novel plant protectionstrategy.The use of fluorescent pseudomonads forcontrolling soil-borne diseases has been well documented **(Paulitz and Loper, 1991; Weller and Cook, 1986)**. Present study on induction of defense genes revealedthat higher accumulation of chitinase SbCHI was observedin bacterized sorghum resistant cultivar PC-5 challenge inoculatedwith *G. sorghi*.Accumulation of this gene was started one day after challenge inoculation. *P. fluorescens* could act as strong elicitors of plant defense reactions **(M'Piga *et al.*, 1997).Ramamoorthy*et al.*, (2002)**also reported that expression of *β*-1,3-glucanase, chitinase and TLP have been inducedby *P. fluorescens*

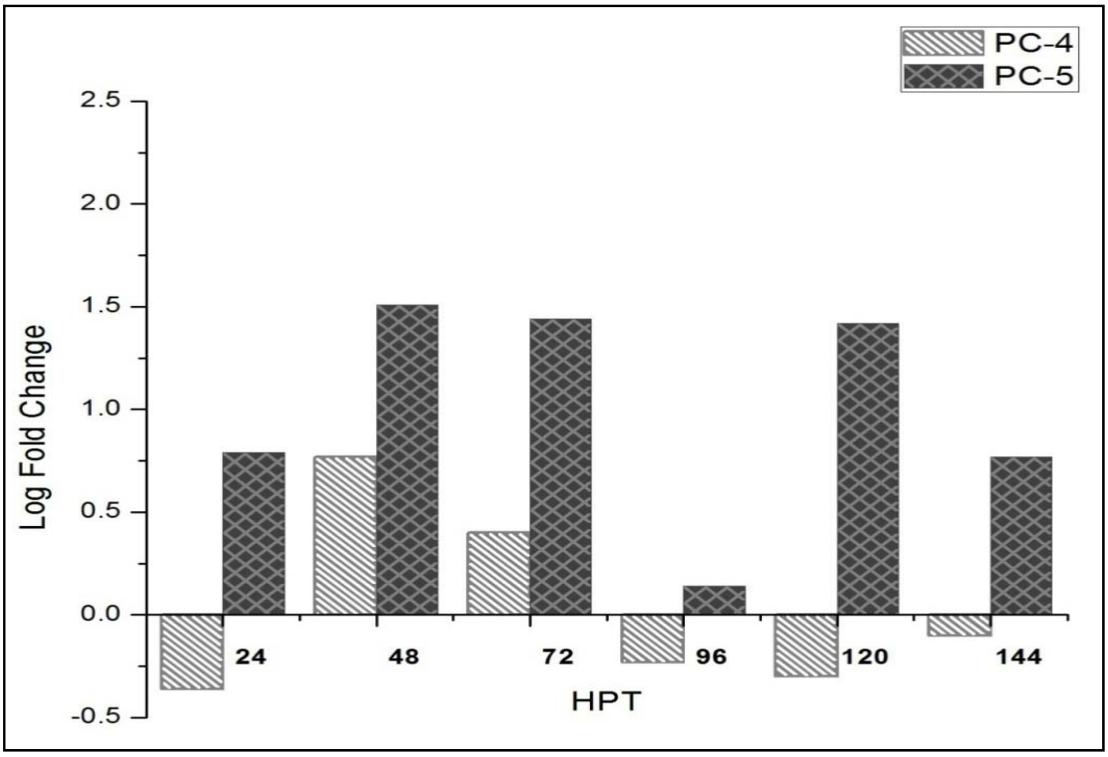


Figure 4.15 Relative gene expression of chitinase during GIDR in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.48: Threshold values of chitinase gene during GIDR in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.47	22.46	33.23	20.01	0.43±0.08	-0.36
48	34.47	22.46	29.56	20.12	5.94±0.20	0.77
72	34.47	22.46	30.25	19.56	2.50±0.07	0.40
96	34.47	22.46	32.56	19.78	0.59±0.10	-0.23
120	34.47	22.46	33.12	20.12	0.50±0.07	-0.30
144	34.47	22.46	35.46	23.12	0.80±0.09	-0.10

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.49: Threshold values of chitinaseduring GIDR gene in PC-5 at different time intervals.

Hours	CONTROL Ct GOI	CONTROL Ct NORM	TREATMENT Ct GOI	TREATMENT Ct NORM	$2^{-(\Delta\Delta ct)}$	Log Fold Change
24	36.23	21.56	32.16	20.12	6.19±0.11	0.79
48	36.23	21.56	30.12	20.45	32.00±0.18	1.51
72	36.23	21.56	29.45	19.56	27.47±0.19	1.44
96	36.23	21.56	34.34	20.15	1.39±0.24	0.14
120	36.23	21.56	30.10	20.14	26.17±0.24	1.42
144	36.23	21.56	33.12	21.01	5.90±0.22	0.77

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

isolate Pf1. Chitinase isoform Chi2has been induced in *P. fluorescens* isolate Pf1-treatedplants challenged with the pathogen. Induction of 33kDa TLP was observed in bacterized plants and plantsinoculatedwith the pathogen.Thus present study clearly indicates that the activities of chitinase enzymesin sorghum have been inducedby *P. fluorescens* isolate Pf-31 in response to challengeinoculation with*G.sorghi*.**Maurhofer *et al.*, (1994)** reported that induction of systemic resistance by *P. fluorescens* was correlatedwith the accumulation of *β*-1,3-glucanase andchitinase. Ineffective isolates of *P. fluorescens* did nottrigger accumulation of *β*-1,3-glucanase and chitinaseand did not induce systemic resistance in tobaccoagainst tobacco mosaic virus. In pea, seed treatment with *P. fluorescens* isolate 63-28 induced the accumulationof hydrolytic enzymes such as chitinases and*β*-1,3-glucanase at the site of penetration of fungalhyphae of *F. oxysporum* f. sp. *pisi*. These enzymes actupon the fungal cell wall resulting in degradation andloss of inner contents of cells **(Benhamou *et al.*, 1996)**. However, in contrast to these results, **Pieterse *et al.*, (1998)** and **Van Wees *et al.*, (1999)** reported that ISR by rhizobacteria is independentof PR-proteins accumulation in *Arabidopsis*.Over expression of cloned rice TLP gene in transgenicrice enhances resistance *to Rhizoctonia solani* causingsheath blight disease **(Datta *et al.*, 1999)**.

4.4.2.3 CHS

During ISR-prime,higherexpression of SbCHS was noted in PC-5as compared to PC-4**(Figure 4.16)**. Maximum fold change in the expression of SbCHS gene was observed at 96 HPT (3.91-fold) and 72 HPT (3.49-fold) in PC-5 **(Table 4.51)**, whereas it was maximum at 72 (2.79-fold) and 48 (2.25-fold) in PC-4**(Table 4.50).**Initially at 0 HPT the least fold change was observed in both PC-4(0.74-fold) and PC-5(0.98-fold). Later a moderate increase in CHS expression was recorded in PC-5 upto 96 HPT (3.91-fold) while in PC-4 less increase was recorded which lasted only upto 72 HPT (2.79-fold). After that it peaks transiently upto 2.79-fold at 72 HPT and return to 1.28-fold at 96 HPT in PC-4 while in case of PC-5 it retain upto 3.91-fold at 96 HPT and return to 2.12-fold at 120 HPT. Interestingly in PC-5 a moderate level of downregulation was observed at 168 HPT (-0.91-fold) while a basal level of expression was maintained in case of PC-4 at 168 HPT (1.21-fold).

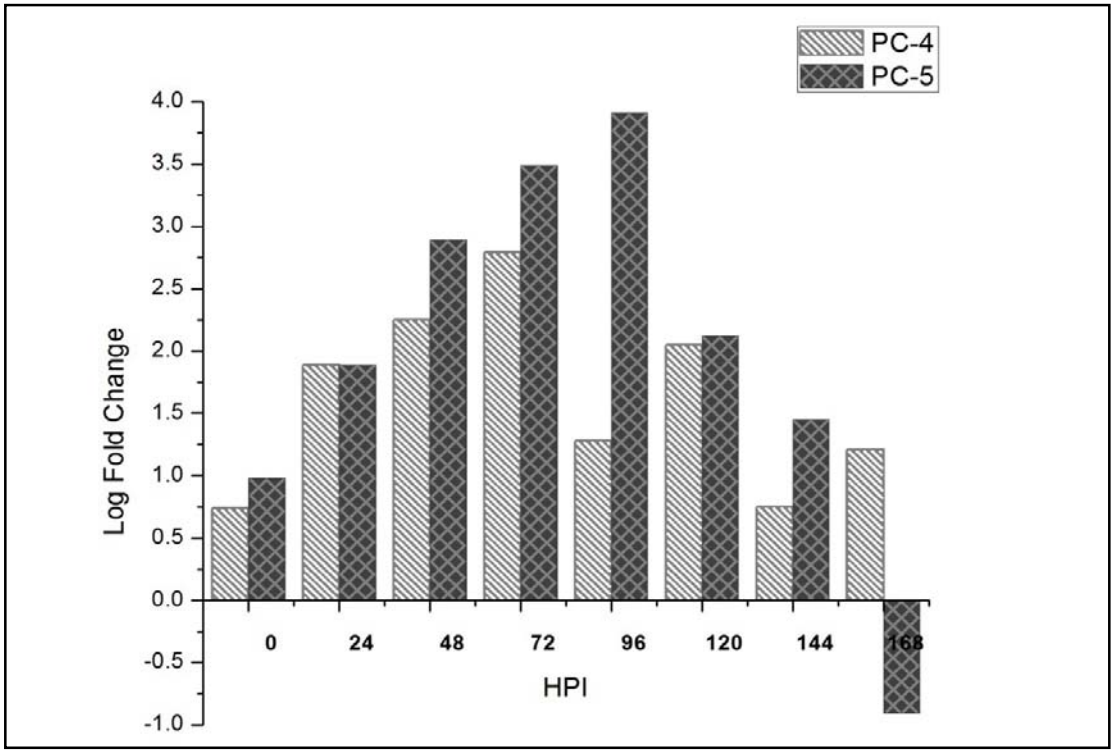


Figure 4.16 Relative gene expression ofCHS during priming in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

During ISR- boost (post *G.sorghi* inoculation), the CHS gene expressed at a high level in both cultivars (**Figure 4.17)**.The greatest expression of this gene was seeninPC-5 cultivar with a change of 7.01-fold **(Table 4.53)**while 3.01-foldchange inPC-4 cultivar at

Table 4.50: Threshold values of CHS gene during priming in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	34.47	22.46	32.68	23.12	5.46±0.21	0.74
24	34.47	22.46	31.84	26.12	78.25±0.34	1.89
48	34.47	22.46	26.78	22.23	176.07±0.28	2.25
72	34.47	22.46	25.87	23.12	613.11±0.26	2.79
96	34.47	22.46	33.09	25.34	19.16±0.19	1.28
120	34.47	22.46	27.35	22.15	112.21±0.17	2.05
144	34.47	22.46	32.65	23.12	5.58±0.20	0.75
168	34.47	22.46	28.12	20.12	16.11±0.14	1.21

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.51: Threshold values of CHS gene during priming in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	36.23	21.56	35.31	23.89	9.51±0.10	0.98
24	36.23	21.56	33.94	25.56	78.25±0.25	1.89
48	36.23	21.56	26.31	21.23	770.69±0.26	2.89
72	36.23	21.56	23.49	20.42	3104.19±0.27	3.49
96	36.23	21.56	23.24	21.56	8135.41±0.24	3.91
120	36.23	21.56	27.75	20.12	131.60±0.27	2.12
144	36.23	21.56	34.42	24.56	28.05±0.13	1.45
168	36.23	21.56	38.59	20.89	0.12±0.20	-0.91

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

72 HPI **(Table 4.52)**. Upon fungal inoculation, the expression level of PC-4 cultivar increased from1.98 to 3.01-foldat 24 to 72 HPIrespectively after that it gradually decreased and reache below ground level (-0.34-fold) at 144 HPI but in case of PC-5 cultivar fold change increased rapidly ranging from 3.25 to 7.01-fold from 24 to 72 HPI but later on a decreasing trend was observed from 96 to 144 HPI (6.89-3.01 fold).

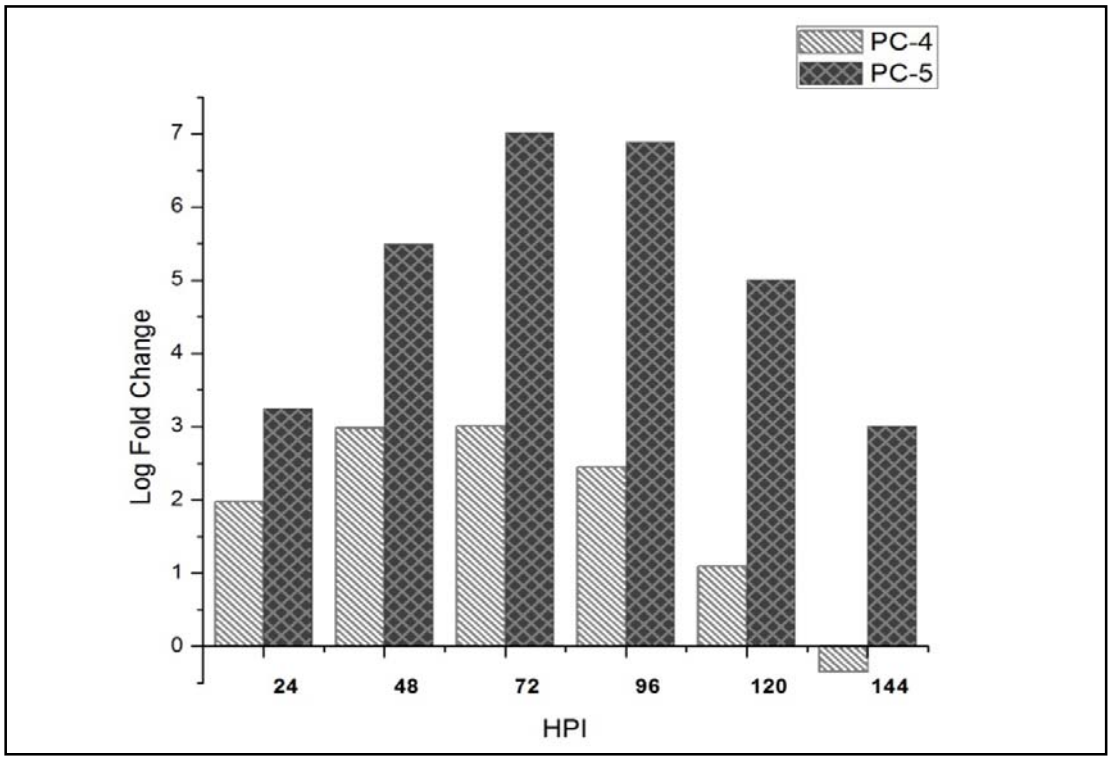


Figure 4.17 Relative gene expression ofCHS during boosting in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

During GIDR, it is evident that in PC-4 cultivar of sorghum prominent downregulation of CHS gene was observed while in PC-5 cultivar after initial upregulation upto 48 HPI it was downregulated during 72 to 120 HPI **(Figure 4.18)**. In PC-4 cultivar maximum fold change with a value of -0.57 and -0.62-fold was observed during 144 and 96 HPI respectively **(Table 4.54)** while in PC-5 maximum fold change of 1.40 and 0.96-fold at 48 and 144 HPI respectively was recorded **(Table 4.55)**. Least fold change was observed at 120(-0.17-fold) and 96(-0.10-fold) HPI in PC-5 whereas in PC-4 it was -1.58 and -1.57-fold at 24-48 HPI respectively.

Inthis study, it is demonstrated that*P. fluorescens*Pf-31 is effective in inducing and/or priming defenceresponses, and triggered resistance of sorghum against*G. sorghi*.Earlier it had been clearly demonstrated thattreatment of grapevine cells or roots by

Table 4.52: Threshold values of CHS gene during boosting in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	37.39	20.14	32.12	21.45	95.67±0.18	1.98
48	37.28	20.04	30.56	23.21	948.83±0.18	2.98
72	37.23	20.12	28.56	21.45	1024.00±0.29	3.01
96	35.92	21.84	31.01	24.35	282.09±0.30	2.45
120	38.27	21.65	33.14	20.15	12.38±0.28	1.09
144	35.91	22.01	35.14	20.12	0.46±0.18	-0.34

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.53: Threshold values of CHS gene during boosting in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.15	21.15	25.12	25.12	1782.89±0.23	3.25
48	30.15	20.12	28.56	28.56	307451.64±0.21	5.49
72	35.12	21.34	28.56	28.56	10256250.01±0.31	7.01
96	37.45	22.45	28.45	28.45	7772784.03±0.25	6.89
120	36.12	20.89	22.13	22.13	101421.22±0.15	5.01
144	32.15	20.67	26.45	26.45	1016.93±0.25	3.01

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Pseudomonas spp.and their cellular extracts induced and/or primed theexpression of cellular defence responses, resulting in anenhanced level of induced resistance against *B.cinerea*.Our results also provide evidence for the greater induction of stilbene gene expression whensorghum was colonized with*P. fluorescens* Pf-31 thus tiggering ISR.

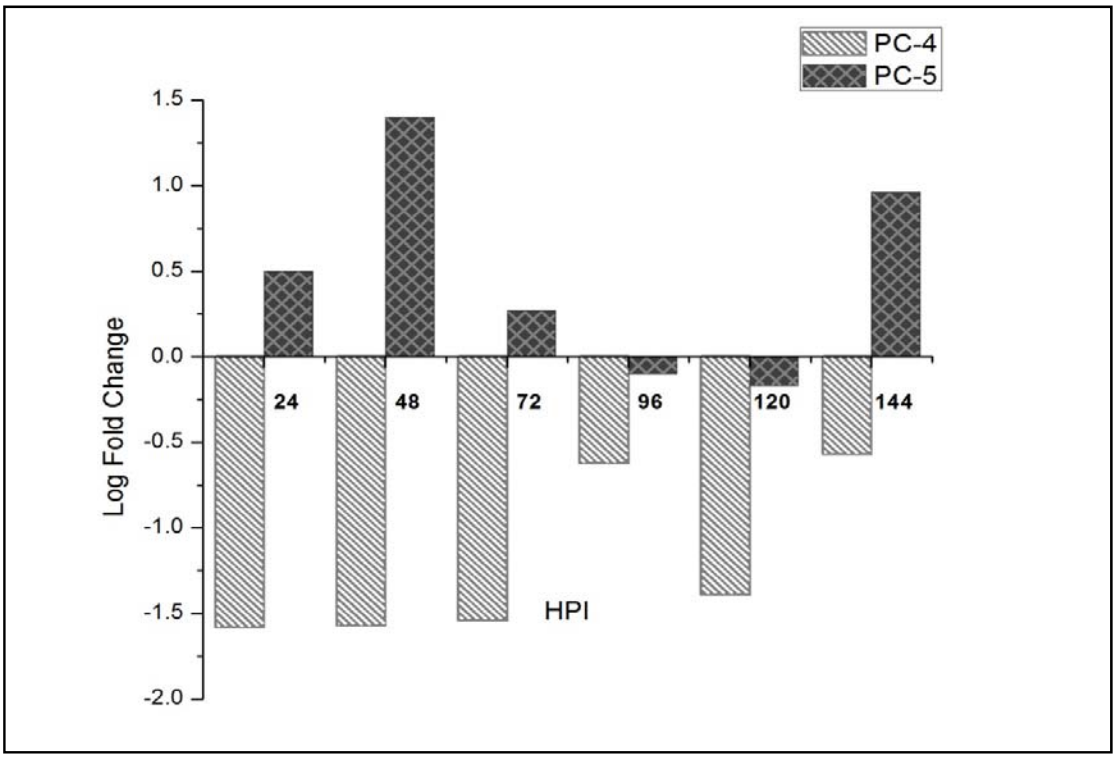


Figure 4.18 Relative gene expression ofCHS during boosting in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Stress induction of CHS (stilbene) transcripts hasbeen reported in a number of systems **(Dixon and Paiva, 1995)**.Most of rhizobacteria induced a small and transientincrease in the amount of stilbene, in grapevine suspensioncells and leaves. This phytoalexin have been shown topossess biological activity against a wide range of pathogensand can be considered as markers for plant diseaseresistance **(Langcake, 1981; Coutos-Thevenot *et al.*, 2001; Jeandet *et al.*, 2002)**.According to the priming concept **(Conrath *et al.*, 2002)**,the enhanced stilbene synthase accumulation in grapevine leavesafter root treatments with *P. fluorescens* CHA0, Q2-87 and*P. putida* CWS358, or their corresponding extracts suggestsa pathogen-dependent activation of defence responses ingrapevine plants.**De Vleesschauwer *et al.*, (2006)** previously uncovered priming as a crucial facet of the resistancemechanism underlying *P. aeruginosa* 7NSK2-mediated ISRagainst *M. oryzae*.

Table 4.54: Threshold values of CHS gene during GIDR in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.47	22.46	37.39	20.14	0.03±0.13	-1.58
48	34.47	22.46	37.28	20.04	0.03±0.20	-1.57
72	34.47	22.46	37.23	20.12	0.03±0.22	-1.54
96	34.47	22.46	35.92	21.84	0.24±0.20	-0.62
120	34.47	22.46	38.27	21.65	0.04±0.17	-1.39
144	34.47	22.46	35.91	22.01	0.27±0.19	-0.57

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.55: Threshold values of CHS gene during GIDR in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	36.23	21.56	34.15	21.15	3.18±0.16	0.50
48	36.23	21.56	30.15	20.12	24.93±0.22	1.40
72	36.23	21.56	35.12	21.34	1.85±0.19	0.27
96	36.23	21.56	37.45	22.45	0.80±0.26	-0.10
120	36.23	21.56	36.12	20.89	0.68±0.21	-0.17
144	36.23	21.56	32.15	20.67	9.13±0.14	0.96

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

4.4.3 Determination of the expression of defense response genes during priming and boosting through *G. intraradices* in PC-4 and PC-5 using real time-RT-PCR.

4.4.3.1 PAL

During ISR- prime analysis, the expression of sorghum PAL gene, SbPAL was evaluated in PC-4 and PC-5 when treated with *Glomus*, SbPAL expressed in both cultivars. Initially (0 to 72 HPT) the expression of PAL in both cultivars was less as compared to 96 to 168 HPT. Fold change during 0 to 72 HPT was ranging from 0.82 to 1.09-fold in PC-4 **(Table 4.56)**. Interestingly in PC-5 at 0 HPT the value of fold change was 1.4-fold but at 24 HPT **(Table 4.57)** it decreased slightly (1.09-fold) later it followedincreasing trend. Moreover in both cultivars significant PAL expression was last upto 144 HPT while at 168 HPT it reduces transiently with a value of 2.11 and 2.65-fold in PC-4 and PC-5 respectively. The higherexpression of SbPAL was noted in PC-5, as compared toPC-4**(Figure 4.19)**. The maximum fold changein the expression of this gene was 3.92-fold and 3.02-fold at 144 HPT inPC-5 and PC-4 sample respectively. However, during 96 to 144 HPT in both cultivars a similar trend of increase in expression was observed. In PC-5 moderate level of expression was recorded as compared to control.

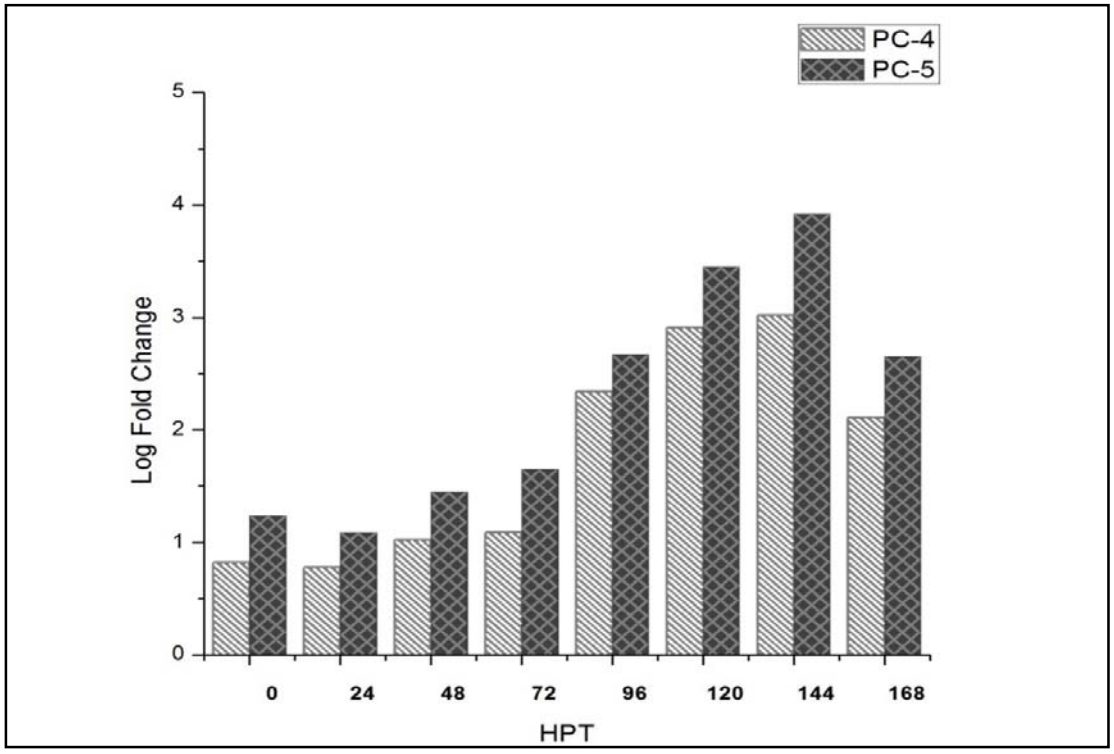


Figure 4.19 Relative gene expression of PAL during priming in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

During ISR- boost (post *G.sorghi* inoculation), the PAL gene was expressed at a very high level. A significant fold change of this gene in PC-5 and PC-4 cultivars of sorghum is an evidenceof induction of host defense gene during pathogen infection

Table 4.56: Threshold values of PAL gene during priming in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	34.47	22.46	30.17	20.89	6.63±0.12	0.82
24	34.47	22.46	30.98	21.56	6.02±0.12	0.78
48	34.47	22.46	29.62	21.01	10.56±0.29	1.02
72	34.47	22.46	29.92	21.54	12.38±0.50	1.09
96	34.47	22.46	26.14	21.89	216.77±0.51	2.34
120	34.47	22.46	22.82	20.48	814.63±0.17	2.91
144	34.47	22.46	22.48	20.49	1038.29±0.15	3.02
168	34.47	22.46	26.44	21.45	129.79±0.22	2.11

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

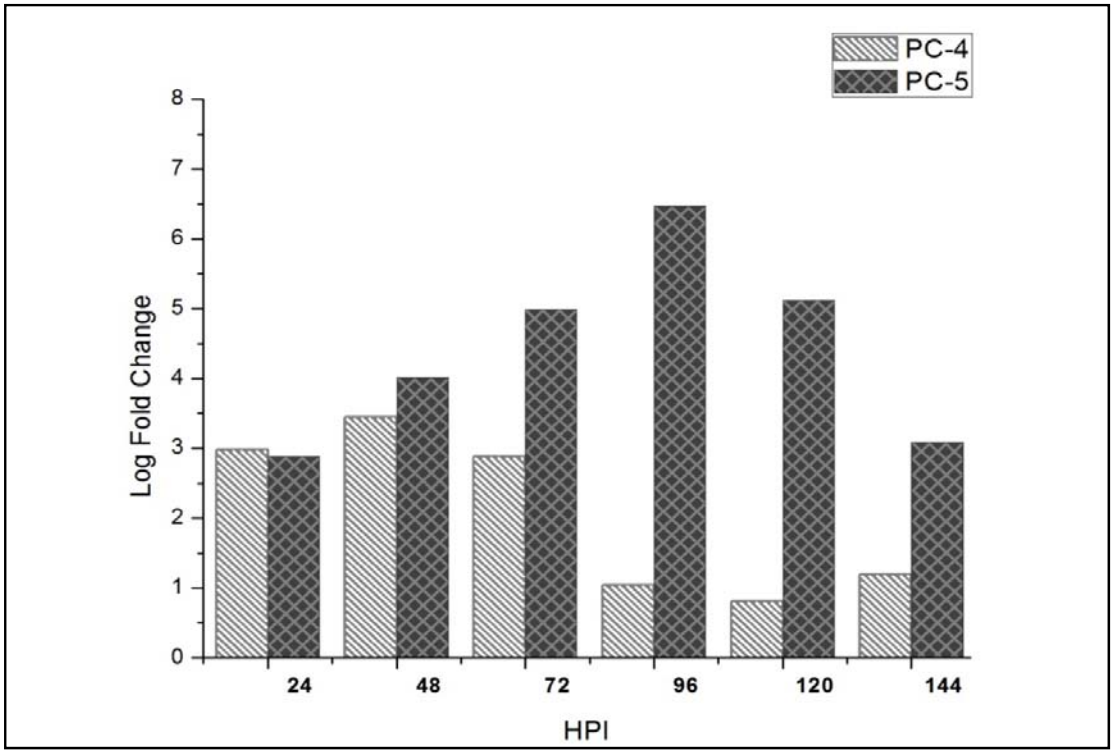
Table 4.57: Threshold values of PAL gene during priming in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	36.23	21.56	32.69	22.15	17.51±0.08	1.24
24	36.23	21.56	33.17	22.13	12.38±0.14	1.09
48	36.23	21.56	34.41	24.56	28.250±0.23	1.45
72	36.23	21.56	33.07	23.89	44.94±0.30	1.65
96	36.23	21.56	29.26	23.45	464.65±0.29	2.67
120	36.23	21.56	25.78	22.56	2797.65±0.32	3.45
144	36.23	21.56	24.22	22.56	8248.98±0.12	3.92
168	36.23	21.56	29.44	23.56	442.64±0.24	2.65

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

(Figure 4.20). The greatest expression of this gene was seeninPC-5 cultivar with a change of 6.47-fold at 96 HPIwhile a maximum 3.45-foldchange was observed inPC-4 cultivar at 48 HPI **(Table 4.58& 4.59)**. Upon fungal inoculation, the expression level in PC-4 cultivar increased from 2.98 at 24HPI to 3.45 at 48 HPIafter that it decreased gradually and reached0.81-fold at 120 hrsbut in case of PC-5 cultivar fold change increased rapidly ranging from 2.88-foldat 24 HPI to 6.47-flod at 96 HPI. However expression of PAL in PC-5 did not decrease as much after 96 HPI. There was a slight decrease in fold change at 120 HPT (5.12-fold) later it was maintained at 144 HPT (3.09-fold).

Figure 4.20 Relative gene expression of PAL during boosting in moderately resistant



(PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

During GIDR, it is evident that in both cultivars of sorghum prominent downregulation of PAL gene was observed. In PC-4 cultivar -0.84 and -0.86 fold change was observed while 0.62 and 0.37 fold change was observed in PC-5 during 24 and 48 HPI respectively **(Table 4.60 and Table 4.61)**. In PC-5 maximum downregulation was seen at 144 HPI with a fold change of -0.79 while in PC-4 slight upregulation of PAL gene at 144 HPI with a fold change of 0.53 was observed **(Figure 4.21)**.

Table 4.58: Threshold values of PAL gene during boosting in PC-4 at different time intervals.

Hours	CONTROL Ct	CONTROL Ct	TREATMENT Ct	TREATMENT Ct	$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.03	19.23	25.60	20.69	946.64±0.28	2.98
48	33.30	18.45	23.96	20.58	2843.27±0.25	3.45
72	33.94	20.15	28.36	24.15	763.60±0.31	2.88
96	33.85	20.45	30.11	20.15	10.88±0.17	1.04
120	33.14	21.45	29.78	20.78	6.45±0.09	0.81
144	32.69	22.45	29.71	23.45	15.82±0.12	1.20

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.59: Threshold values of PAL gene during boosting in PC-5 at different time intervals.

Hours	CONTROL Ct	CONTROL Ct	TREATMENT Ct	TREATMENT Ct	$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	32.15	32.15	26.19	23.15	767.13±0.20	2.88
48	33.90	33.90	23.59	23.45	10179.18±0.24	4.01
72	35.45	35.45	24.99	24.56	97065.21±0.29	4.99
96	35.32	35.32	20.94	25.46	2972681.22±0.35	6.47
120	35.31	35.31	24.76	25.56	131983.68±0.27	5.12
144	36.85	36.85	29.19	22.15	1217.75±0.23	3.09

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

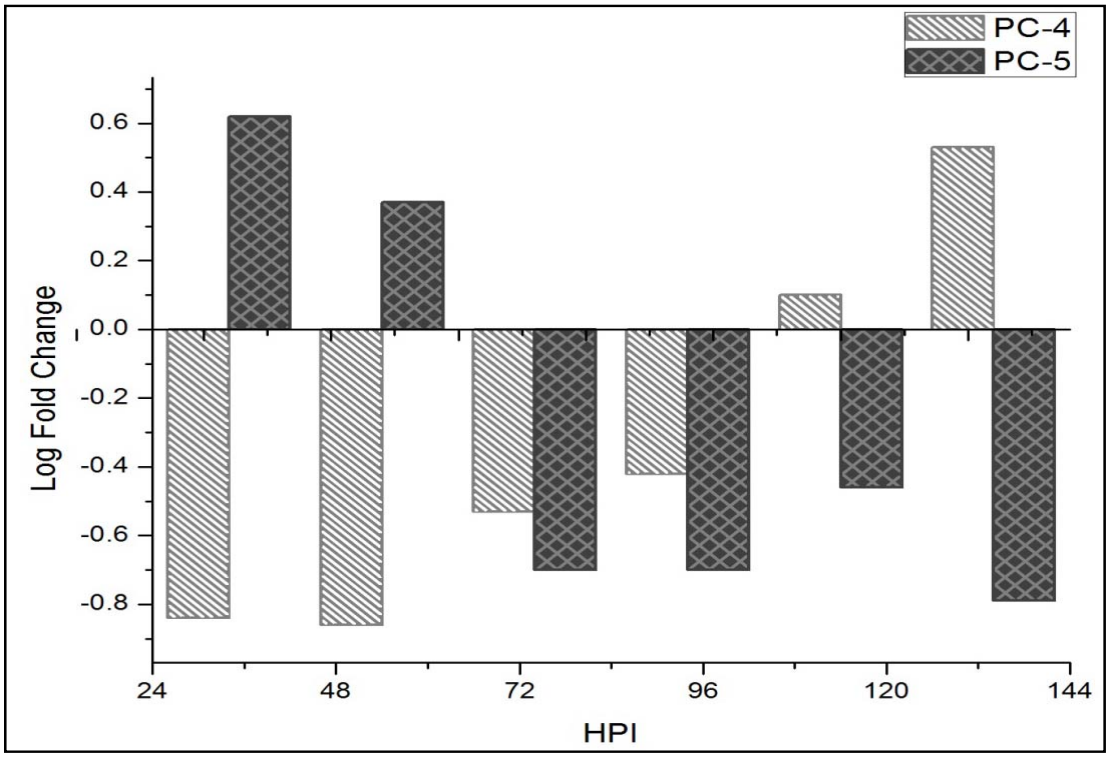


Figure 4.21 Relative gene expression of PAL during GIDR in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.60: Threshold values of PAL gene during GIDR in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	Ct GOI	Ct NORM	Ct GOI	Ct NORM		
24	34.47	22.46	34.03	19.23	0.14±0.05	-0.84
48	34.47	22.46	33.30	18.45	0.14±0.19	-0.86
72	34.47	22.46	33.94	20.15	0.29±0.04	-0.53
96	34.47	22.46	33.85	20.45	0.38±0.07	-0.42
120	34.47	22.46	33.14	21.45	1.25±0.05	0.10
144	34.47	22.46	32.69	22.45	3.40±0.49	0.53

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.61: Threshold values of PAL gene during GIDR in PC-5 at different time intervals.

Hours	CONTROL Ct GOI	CONTROL Ct NORM	TREATMENT Ct GOI	TREATMENT Ct NORM	$2^{-(\Delta\Delta ct)}$	Log Fold Change
24	36.23	21.56	32.15	19.53	4.13±0.19	0.62
48	36.23	21.56	33.90	20.45	2.32±0.08	0.37
72	36.23	21.56	35.45	18.45	0.20±0.52	-0.70
96	36.23	21.56	35.32	18.34	0.20±0.08	-0.70
120	36.23	21.56	35.31	19.10	0.34±0.09	-0.46
144	36.23	21.56	36.85	19.56	0.16±0.35	-0.79

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

The effect of the AM symbiosis on reducing the severity of diseases caused by soil-bomepathogens has received considerable attention. Numerous mechanisms have beenpostulated to explain this phenomenon such as the stimulation of plant defense responsesby the AM fungus which would allow the plant to more effectively respond to asubsequent pathogenic attack. This has lead many researchers to investigate how the AMsymbiosis effects plant defense mechanisms. Initial research focused on how the AMsymbiosis alone influences plant defense mechanisms, with more recent studiesinvestigating the defense responses ofAM plants during a pathogenic infection. With fewexceptions, these studies only investigated the state ofthe plant's defense responses and at different time point during priming and boosting (after infection with the pathogen). The lack ofknowledge on the spatial and temporal effects ofthe AM symbiosis during a pathogenic infection formed the basis ofthis thesis, which investigated how the AM fungus *G.intraradices* may alter defense responses in sorghumduring a pathogenic infection by *G. sorghi,* and if the AM symbiosis affords sorghum protection against *G.sorghi*. It is weIl known that pathogens or elicitors cause activation of defense mechanisms no tonly at the site of infection or elicitor treatment, but also in distant uninfecte tissues.**(Lawton and Lamb, 1987; Lafitte *et al.,* 1993)** Consistent with these reports,*G.sorghi* infection often resulted in a systemic activationof all defense-related

genes studied. Transcript levels of PALshowed a very similar spatial andtemporal pattern of accumulation in response to *G.sorghi*infection. Similar results werefound with PAL, CHS and CHI transcripts, using the same cDNA clones, in bean cellsuspension cultures as well as in entire plants in response to fungal elicitors and infectionby C. *lindemuthianium,* another highly virulent fungal pathogen ofbeans **(Cramer *et al.,* 1985; Mehdy and Lamb, 1987; Bolwell *et al.,* 1988)**. In this study, it is demonstrated that*G. intraradices*is effective in inducing and/or priming defenceresponses, and triggered resistance of sorghum against*G. sorghi*.AMF was also found to induce several defence enzymes and phytoalexins such as PhenylalanineAmmonia Lyase (PAL), Rishitin andsolavetivone **(Engström *et al.*, 1999)**,hydroxyproline-rich glycoproteins **(Lambais, 2000; Garcia-Garrído and Ocampo, 2002)**,isoflavonoid (-)-medicarpin, medicarpin-3-O-glycoside and formononetin in alfalfa**(Volpin *et al.*, 1995; Harrison and Dixon, 1993)**. They are lowmolecular weight, anti-microbial compoundsthat are both synthesized by andaccumulated in plants after encounteringpathogens **(Paxton, 1981)**.AMF colonizationin roots stimulates the phenylpropanoidpathway **(Morandi, 1996),** which can be dueto the induction of PAL activity as observedby **Kapoor (2008)**.

4.4.3.2 Chitinase

Initially at 0 and 24 HPT the change in expression of chitinase is less in both PC-4(0.52, 0.89-fold) **(Table 4.62)** and PC-5(0.82, 0.45-fold) **(Table 4.63) (Figure 4.24)**.Higher level of expression of this gene in PC-5 was 3.15-fold at 72 hrs while in PC-4 it was observed 3.45 at144 hrs. During 0 to 72 hrs there is an increasing trend of fold expression is recorded in both cultivars. Interestingly, the expression of chitinase in PC-5 and PC-4 is significantly downregulated with a value of -0.81-fold and -2.01-fold at 168 HPT.

During ISR-Boost (post *G.sorghi* inoculation) significant fold change in chitinase was observed in PC-5 cultivar while it was moderately expressed in PC-4 cultivar of sorghum **(Figure 4.23)**. In PC-5 a maximum fold change of 7.24 was observed at 72 HPI **(Table 4.65)** while in PC-4 it is 3.45-fold at 24 HPI **(Table 4.64)**. The significant change in expression of chitinase gene during different time frame in both cultivars was distinct. InPC-5 cultivar, fold change increases rapidly ranging from 3.41-fold at 24 HPI to 7.24-fold at 72 HPI. However in PC-4 a decreasing trend in chitinase expression was observed during 24 to 144 HPT with a fold change of 3.45 to 0.85-fold respectively. Interestingly in both cultivars expression was decreased at 144 HPI.

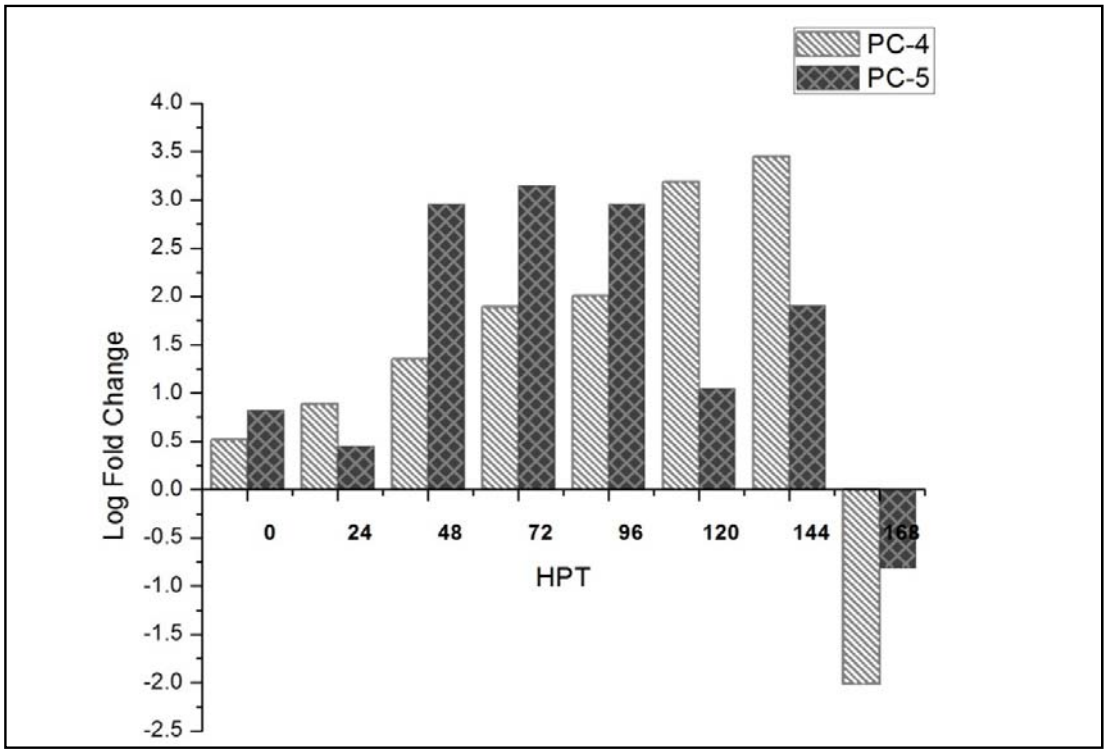


Figure 4.22 Relative gene expression of chitinase during priming in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.62: Threshold values of chitinase gene during priming in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	34.47	22.46	32.39	22.12	3.34±0.27	0.52
24	34.47	22.46	32.18	23.13	7.78±0.18	0.89
48	34.47	22.46	27.64	20.12	22.47±0.13	1.35
72	34.47	22.46	27.17	21.45	78.25±0.14	1.89
96	34.47	22.46	26.57	21.23	101.83±0.18	2.01
120	34.47	22.46	24.31	22.89	1541.37±0.09	3.19
144	34.47	22.46	23.67	23.13	2836.70±0.16	3.45
168	34.47	22.46	38.81	20.12	0.01±0.11	-2.01

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.63: Threshold values of chitinase gene during priming in PC-5 at different time intervals.

Hours	CONTROL Ct	CONTROL Ct	TREATMENT Ct	TREATMENT Ct	$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	36.23	21.56	34.08	22.13	6.59±0.23	0.82
24	36.23	21.56	35.74	22.56	2.81±0.21	0.45
48	36.23	21.56	26.00	21.15	903.89±0.24	2.96
72	36.23	21.56	24.76	20.56	1418.35±0.18	3.15
96	36.23	21.56	26.30	21.45	903.89±0.17	2.96
120	36.23	21.56	32.42	21.23	11.16±0.19	1.05
144	36.23	21.56	29.88	21.56	81.57±0.16	1.91
168	36.23	21.56	37.51	20.14	0.15±0.14	-0.81

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

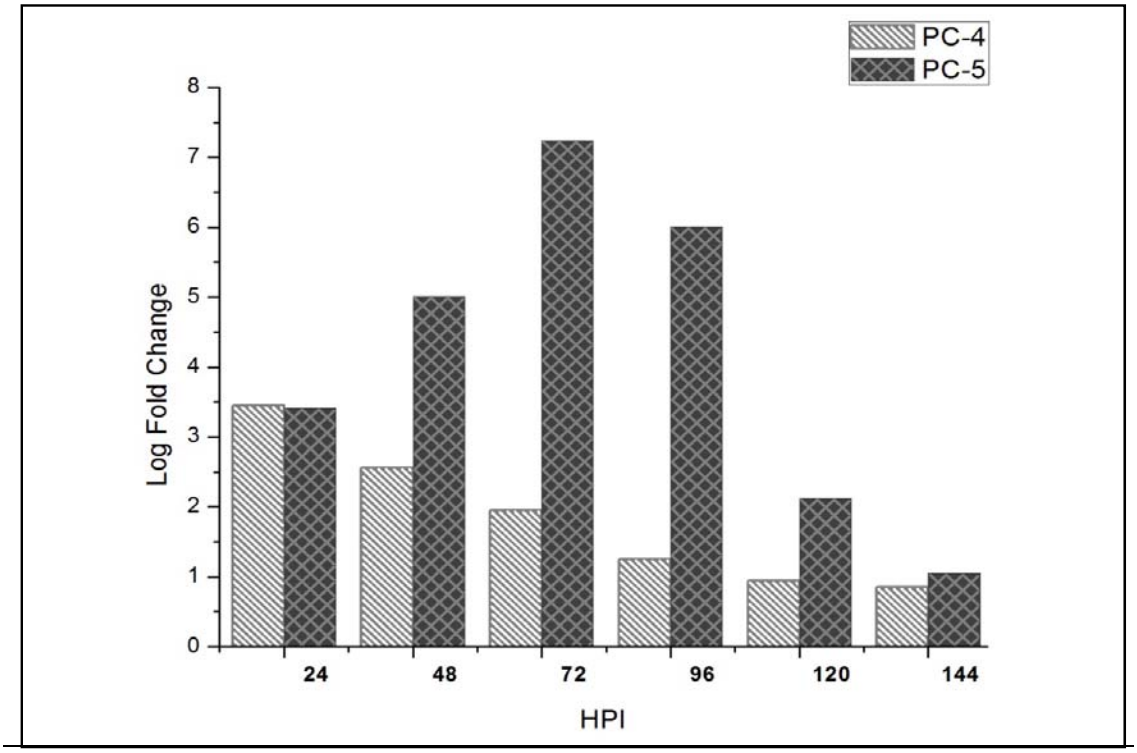


Figure 4.23 Relative gene expression of chitinase during boosting in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.64: Threshold values of chitinase gene during boosting in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	33.23	20.01	23.88	22.13	2836.70±0.08	3.45
48	29.56	20.12	22.18	21.23	359.54±0.11	2.56
72	30.25	19.56	25.65	21.45	89.88±0.17	1.95
96	32.56	19.78	30.77	22.15	17.88±0.26	1.25
120	33.12	20.12	35.54	25.65	8.63±0.20	0.94
144	35.46	23.12	36.28	26.78	7.16±0.23	0.85

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.65: Threshold values of chitinase gene during boosting in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	32.16	20.12	28.94	28.23	2574.36±0.24	3.41
48	30.12	20.45	24.26	31.23	102126.66±0.29	5.01
72	29.45	19.56	20.08	34.23	17248887.74±0.21	7.24
96	34.34	20.15	24.34	30.12	1026996.62±0.24	6.01
120	30.10	20.14	30.06	27.15	132.51±0.26	2.12
144	33.12	21.01	33.80	25.18	11.24±0.23	1.05

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

During GIDR (Sb+Gs vs Sb) in PC-4 cultivar an initial downregulation with a value of -0.36-fold change was observed at 24 HPI while 24 h later at 48 HPI it was increased upto 0.77-fold change**(Figure 4.24)**. Later on, the basal level of downregulation was maintained for successive hrs upto 120 HPI with a value of 0.40 to -0.30-fold while at 144 HPI it was again slightly upregulated with a value of -0.10-fold. In PC-5 cultivar a moderate level of expression was maintained throughout hours post *G.sorghi*inoculation.

Moreover maximum fold change was observed at 48 HPI in PC-5with a value of 1.51-fold. Interestingly at 120 and 144 hrs the chitinase expression was basally maintained with a value of 1.42 and 0.77- fold**(Table 4.66& 4.67)**.

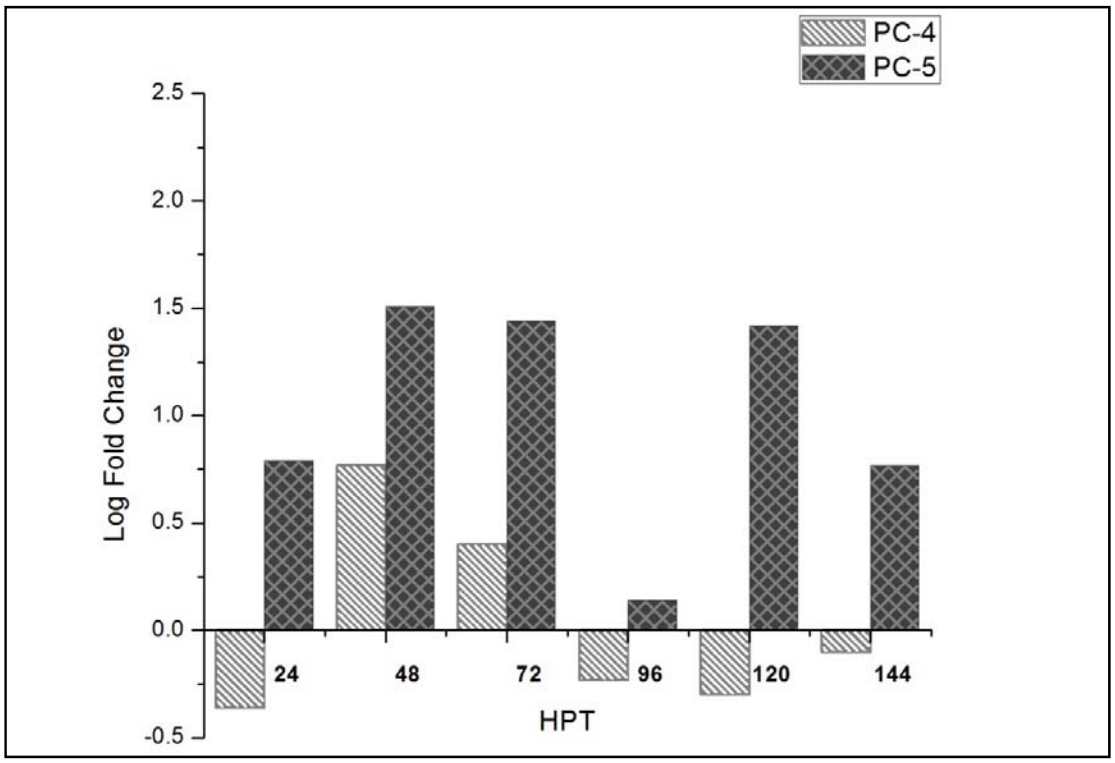


Figure 4.24 Relative gene expression of chitinase during GIDR in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.66: Threshold values of chitinase gene during GIDR in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.47	22.46	33.23	20.01	0.43±0.08	-0.36
48	34.47	22.46	29.56	20.12	5.94±0.20	0.77
72	34.47	22.46	30.25	19.56	2.50±0.07	0.40
96	34.47	22.46	32.56	19.78	0.59±0.10	-0.23
120	34.47	22.46	33.12	20.12	0.50±0.07	-0.30
144	34.47	22.46	35.46	23.12	0.80±0.09	-0.10

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.67: Threshold values of chitinaseduring GIDR gene in PC-5 at different time intervals.

Hours	CONTROL Ct	CONTROL Ct	TREATMENT Ct	TREATMENT Ct	$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	36.23	21.56	32.16	20.12	6.19±0.11	0.79
48	36.23	21.56	30.12	20.45	32.00±0.18	1.51
72	36.23	21.56	29.45	19.56	27.47±0.19	1.44
96	36.23	21.56	34.34	20.15	1.39±0.24	0.14
120	36.23	21.56	30.10	20.14	26.17±0.24	1.42
144	36.23	21.56	33.12	21.01	5.90±0.22	0.77

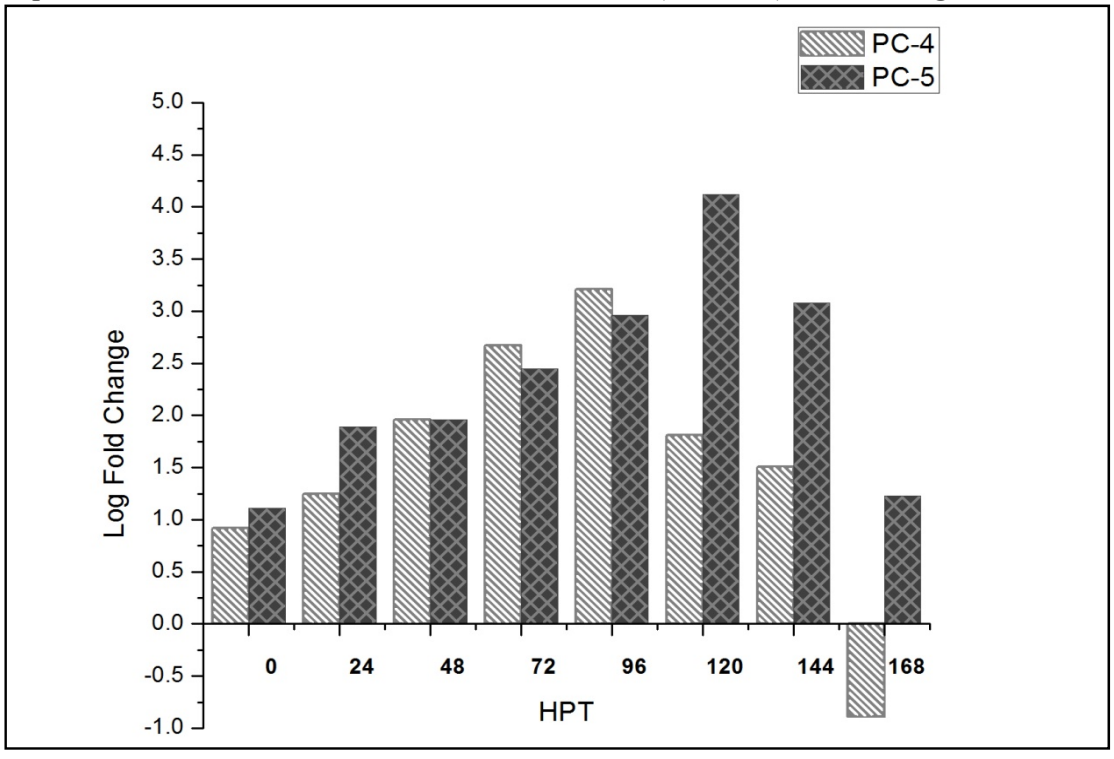
Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

The results ofthe present study indicate that during a pathogenic interaction, thepresence of an AM symbiosis alters the defense responses in the distant tissues such as leaves that are uncolonized by the AM fungus. Theeffect of the AM symbiosis on the levels oftranscripts ofdefense-related genes iscomplex and may cause transcript levels to increase, decrease or remain unchangeddepending on the time after infection with the pathogen, the plant tissue, as well as thetranscript studied. This supports previous work that has shown that the AM symbiosis canhave a variety of effects on plant defense mechanisms. This is one of the first studies toinvestigate the spatial and temporal effects of the AM symbiosis on plant defenseresponses during a pathogenic infection and the complexity ofthe results substantiate theneed for further studies. Chitinases are amongst the most widely studied defence-related plantproteins, and there are many reports of their differentialexpression in mycorrhizal roots **(Dumas-Gaudot *et al.*, 2000)**. **Li *et al.*, (2006)** have described transcriptional activationof a class III chitinase gene in mycorrhizal grapevineroots which is further enhanced during a defence responseagainst the root-knot nematode *Meloidogyne incognita*.Stimulation of plant defense responses as a result ofAM symbiosis had been postulated as a possible cause for the reduction in disease severity observed in AM plants**(Rosendahl, 1985; Caron *et al.*, 1986; St-Arnaud *et al.*, 1994; Niemira *et al.*, 1996)**. Although the result ofthe present study shows that the AM interaction systemically anddifferentially effects the expression of chitinase genes during a pathogenicinfection. Chitinases are synergistically induced during attack by fungal

pathogens and by fungal elicitors. Their induction is generally considered to be part of a non-specific defence response initiated in plants after pathogen attack, but also a consequence of various physical, chemical and environmental stresses **(Sahai and Manocha, 1993)**.Previous studies have identified transitory increases in chitinase and glucanase activity in mycorrhizal roots **(García-Garrido and Ocampo, 2002)**, and there is one report of a mycorrhizal specific class iii chitinase identified in *Medicago truncatula***(Salzer *et al.*, 2000)**.

4.4.3.3 CHS

During ISR-prime,higherexpression of SbCHS was noted in PC-5, whereasthe least was in PC-4**(Figure 4.25)**. Initially at 0 hrs the least fold change was observed in both PC-4(0.92-fold) **(Table 4.68)** and PC-5(1.11-fold) **(Table 4.69)**. Later a moderate increase in CHS expression was recorded in PC-5 upto 96 hrs (2.96-fold) while in PC-4 it was recorded at 72 hrs (2.67-fold). Maximum fold change in the expression of SbCHS gene was observed at 120 hrs (4.12-fold) and 144 hrs (3.08-fold) in PC-5, whereas maximum fold change in PC-4 was at 96 hrs (3.21-fold) and 72 hrs (2.67-fold). In PC-4 downregulation of SbCHS was observed at 168 hrs (-0.89-fold) while a basal level of expression was maintained in case of PC-5 at 168 hrs (1.23-fold). **Figure 4.25**



Relative gene expression of CHS during priming in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.68: Threshold values of CHS gene during priming in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	34.47	22.46	29.84	20.89	8.34±0.23	0.92
24	34.47	22.46	29.98	22.13	17.88±0.22	1.25
48	34.47	22.46	27.95	22.45	91.14±0.23	1.96
72	34.47	22.46	26.30	23.15	464.65±0.30	2.67
96	34.47	22.46	25.33	23.98	1618.00±0.30	3.21
120	34.47	22.46	28.44	22.45	64.89±0.27	1.81
144	34.47	22.46	29.16	22.16	32.22±0.24	1.51
168	34.47	22.46	35.10	20.15	0.13±0.24	-0.89

Each value represents the mean of three replicates with ±S.D. Mean differences are significant at P< 0.05 level.

Table 4.69: Threshold values of CHS gene during priming in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
0	36.23	21.56	33.30	22.31	12.82±0.20	1.11
24	36.23	21.56	32.94	24.56	78.25±0.26	1.89
48	36.23	21.56	32.94	24.78	91.14±0.23	1.96
72	36.23	21.56	27.98	21.45	282.09±0.27	2.45
96	36.23	21.56	24.98	20.15	916.51±0.29	2.96
120	36.23	21.56	22.42	21.45	13307.94±0.17	4.12
144	36.23	21.56	26.20	21.75	1192.69±0.28	3.08
168	36.23	21.56	32.73	22.15	17.03±0.26	1.23

Each value represents the mean of three replicates with ±S.D. Mean differences are significant at P< 0.05 level.

During ISR- boost (post *G.sorghi* inoculation), the CHS gene was expressed at a high level in both cultivars (**Figure 4.26)**.The greatest expression of this gene was seenon the PC-5 cultivar with a fold change of 7.41-fold at 72 HPI **(Table 4.71)**while 3.85-foldchange was observed on the PC-4 cultivar at 72 HPI **(Table 4.70)**. Upon fungal inoculation, the expression level of PC-4 cultivar increased from2.15-fold at 24 HPIto 3.85-foldat 72 HPIafter it wasgradually decreased and reaches below ground level (-0.45fold) at

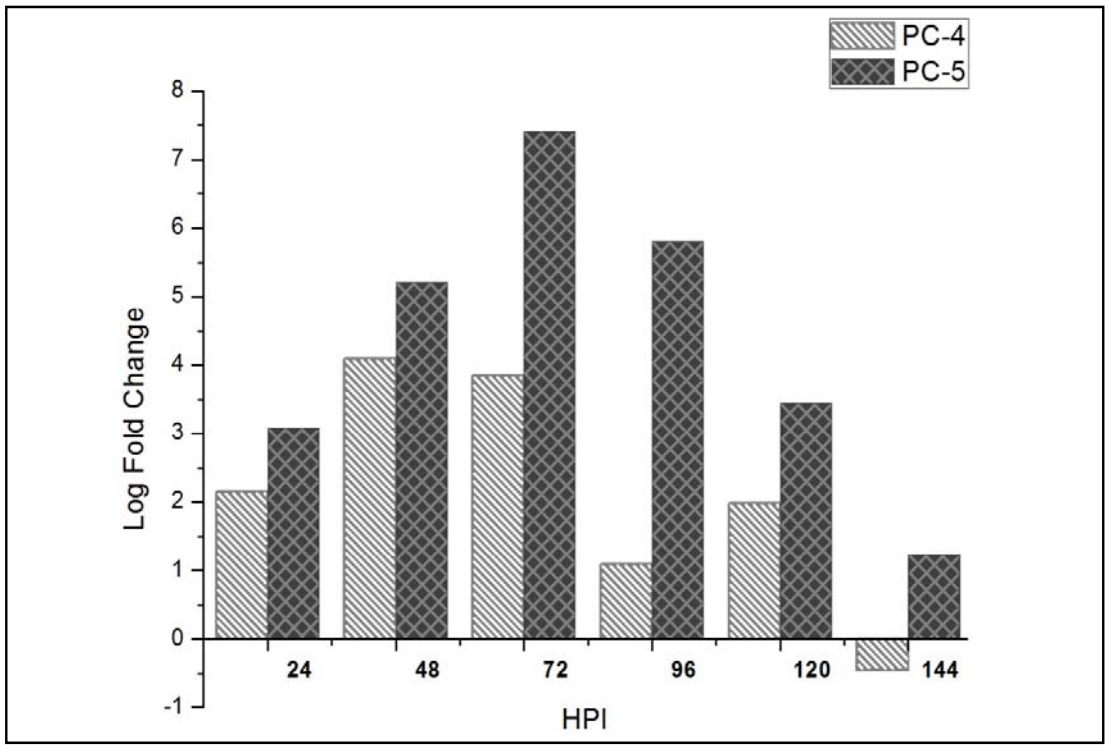


Figure 4.26 Relative gene expression of CHS during boosting in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

144 HPI but in case of PC-5 cultivar fold change increased rapidly ranging from 3.08-fold at 24 HPI to 7.41-fold at 72 HPI but later on a decreasing trend was also observed from 96 to 144 HPI (5.81-1.23fold).

During GIDR, it is evident that in PC-4 cultivar of sorghum prominent downregulation of CHS gene was observed while in PC-5 cultivar after initial upregulation upto 48 HPI it was downregulated during 72 to 120 HPI **(Figure 4.27)**. In PC-4 cultivar maximum fold change with a value of -0.57 and -0.62-fold was observed during 144 and 96 HPI respectively. While in PC-5 maximum fold change of 1.40 and 0.96-fold at 48 and 144 HPI respectively was recorded. Least fold change was observed at 120(-0.17-fold) and

Table 4.70: Threshold values of CHS gene during boosting in PC-4 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	37.39	20.14	31.42	21.30	140.07±0.28	2.15
48	37.28	20.04	31.79	28.14	12330.98±0.24	4.09
72	37.23	20.12	25.77	21.45	7082.29±0.26	3.85
96	35.92	21.84	37.05	26.59	12.30±0.25	1.09
120	38.27	21.65	37.48	27.45	96.34±0.27	1.98
144	35.91	22.01	35.54	20.14	0.35±0.18	-0.45

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.71: Threshold values of CHS gene during boosting in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.15	21.15	28.23	25.45	1192.69±0.28	3.08
48	30.15	20.12	20.17	27.45	162490.97±0.22	5.21
72	35.12	21.34	20.06	30.89	25606380.42±0.21	7.41
96	37.45	22.45	25.14	29.45	649963.87±0.22	5.81
120	36.12	20.89	28.32	24.56	2836.70±0.25	3.45
144	32.15	20.67	33.56	26.15	16.80±0.24	1.23

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

96(-0.10-fold) HPI in PC-5 whereas in PC-4 it was -1.58 and -1.57-fold at 24-48 HPI respectively **(Table 4.72 and Table 4.73)**.

G. intraradices induces the expression of chalcone synthase (an antimicrobial compound), the first enzyme in flavonoidcompound, such as phytoalexin, in *M.truncatula* **(Bonanomi *et al.*, 2001)**. On the contrary, RNA blot analysis revealed slightly higher

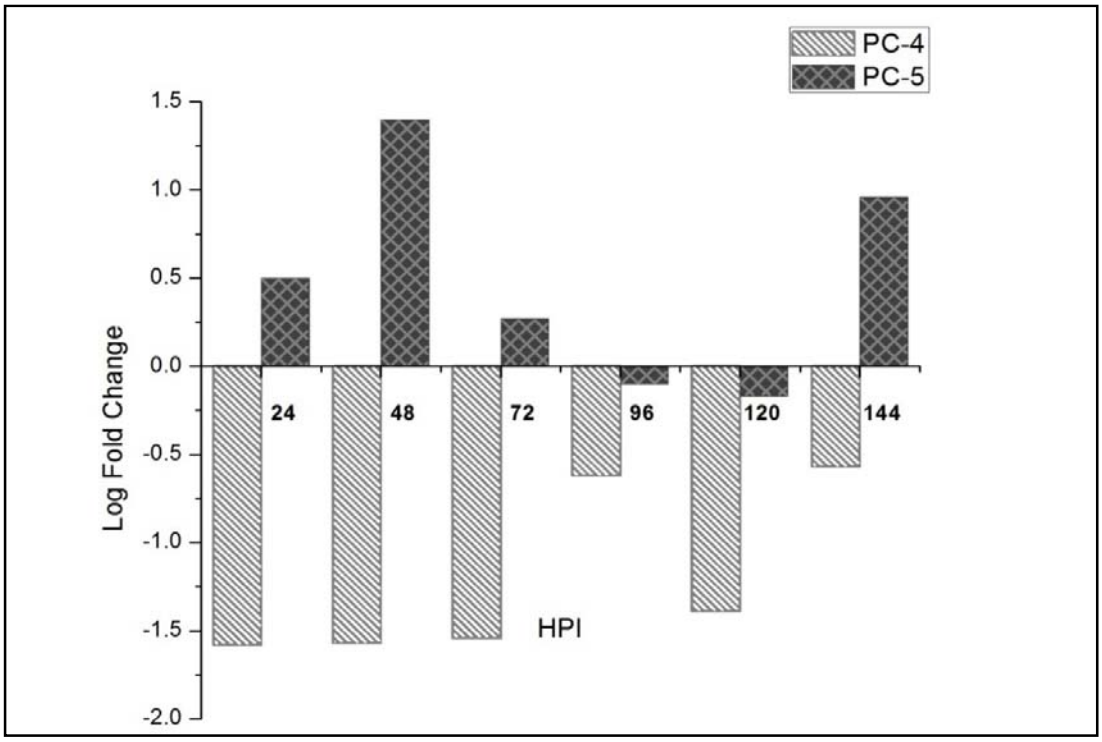


Figure 4.27 Relative gene expression of CHS during GIDR in moderately resistant (PC-5) and a susceptible (PC-4) cultivar of *S. bicolor*

Table 4.72: Threshold values of CHS gene during GIDR in PC-4 at different time intervals.

Hours	CONTROL Ct	CONTROL Ct	TREATMENT Ct	TREATMENT Ct	$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	34.47	22.46	37.39	20.14	0.03±0.13	-1.58
48	34.47	22.46	37.28	20.04	0.03±0.20	-1.57
72	34.47	22.46	37.23	20.12	0.03±0.22	-1.54
96	34.47	22.46	35.92	21.84	0.24±0.20	-0.62
120	34.47	22.46	38.27	21.65	0.04±0.17	-1.39
144	34.47	22.46	35.91	22.01	0.27±0.19	-0.57

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

Table 4.73: Threshold values of CHS gene during GIDR in PC-5 at different time intervals.

Hours	CONTROL Ct		TREATMENT Ct		$2^{-(\Delta\Delta ct)}$	Log Fold Change
	GOI	NORM	GOI	NORM		
24	36.23	21.56	34.15	21.15	3.18±0.16	0.50
48	36.23	21.56	30.15	20.12	24.93±0.22	1.40
72	36.23	21.56	35.12	21.34	1.85±0.19	0.27
96	36.23	21.56	37.45	22.45	0.80±0.26	-0.10
120	36.23	21.56	36.12	20.89	0.68±0.21	-0.17
144	36.23	21.56	32.15	20.67	9.13±0.14	0.96

Each value represents the mean of three replicates with ±SD. Mean differences are significant at P< 0.05 level.

accumulation of chalcone synthase in*G. intraradices*-colonized roots of dark redkidney bean (*Phaseolus vulgaris* L. cvMoncalm) when compared with the noncolonizedones. This may be due to the factthat different plants may have their specificrequirements. With the plausible inductionof the genes by AMF in plants, it may play animplicate role in the biochemical reactionswith proteins, secondary metabolites andother chemicals involved in plant defenceresponse. It has been well reported that, withthe AMF-colonized roots, the above groundeffect against phytopathogen is quiteapparent **(Sensoy *et al.*,2007; Ozgonen *et al.*, 2010; Al-Askar andRashad, 2010; Kapoor, 2008)**.

SUMMARY AND CONCLUSION

CHAPTER 5 SUMMARY AND CONCLUSION

Zonate leaf spot is one of the destructive foliar diseases of sorghum caused by *Gloeocercospora sorghi.* The experiments were conducted on this pathogen to study isolation and purification of the test fungus, pathogenicity test, screening of bioagents for their growth promotion and biocontrol activity *in vitro* and glasshouse conditions, evaluation of potential bioagents in disease reduction, growth promotion, determination of chlorophyll content and green fodder yield under field conditions. Expression of defence response genes through real time PCR (RT-PCR) based method during bioagents treatment (Priming) and subsequent infection by *G. sorghi* (Boosting) under glasshouse condition was also determined.

The salient findings of the study are summarized as below:

1. The characteristic zonate leaf spot lesions were roughly circular or semicircular with alternating bands of dark purple or red color and tan or straw color, to give a concentric or zonate appearance.
2. The fungus was isolated from the disease samples collected from field following the standard procedure for fungal isolation.
3. The pathogenicity of the test fungus was established and it proved to be a potential pathogen.
4. Microscopic studies of the test pathogen were also done for the structures it produces. Hyaline, septate hyphae were produced by the fungus. Sporodochia of pink color were observed. The fungus also produced hyaline septate conidiophores aggregated together in clusters. The conidia were born in pinkish to salmon slimy matrix, were curved, tapering from base to apex and elongated to filiform with dimensions 1.4-3.2 × 20-195 µ. The fungus produced sclerotia on oat meal agar medium which were tiny, black and visible to naked eyes.
5. Thirteen isolates of *Trichoderma harzianum* and five isolates of *Pseudomonas fluorescens* were tested for their antagonistic potential *in vitro* by dual culture technique. TH-32 isolate brought maximum inhibition of radial growth (85.78 %) of the test pathogen.

6. Two cultivars of sorghum viz. susceptible PC-4 (Pant Chari) and moderately resistant PC-5 (Pant Chari) were used for paper towel method to study seed germination and vigour index by *T. harzianum* and *P. fluorescens in vitro*. In PC-4 cv. Th-31 isolate brought highest germination (88.67%), maximum shoot length (6.27 cm) and increased plant fresh weight (4.50gm) while increased root length (4.77cm) and maximum vigour index (950.7 VI) was observed with Pf-31 isolate. In PC-5 cv. Th-32 isolate brought highest germination (89.33%). Pf-31 isolate brought maximum root length (5.37cm), increased plant fresh weight (5.00gm) and dry weight (0.893gm) and maximum vigour index (1004.1 VI).
7. *T. harzianum, P. fluorescens* and *Glomus intraradices* isolates were evaluated for their effect on plant growth promotion and disease reduction under glasshouse condition. In PC-4 cv. Th-25 isolate brought maximum seed germination (85.67%) 15 DAS. Pf-31 increased maximum root length (5.24cm) and shoot length (16.30cm). Moreover, maximum stem diameter was observed with Pf-31(0.65cm) and Th-32 (0.65cm) isolate. In PC-5, Th-32 and Pf-31 brought maximum germination percentage (89.67%), increased root length (6.12cm), shoot length (18.11cm) and stem diameter (0.72cm).
8. Thirty days after sowing in PC-4 cv. Th-28 isolate performed best with maximum root length (15.40cm). Shoot length (55.73cm) and stem diameter (2.11cm) was observed maximum when treated with Pf-31. In PC-5 cv. Th-31 was most effective in increasing root length (17.13cm). Similarly shoot length (68.52cm) and stem diameter (2.26cm) was also observed maximum when treated with Pf-31.
9. At 45 days after sowing in PC-4 cv. Th-28 isolate performed best with maximum root length (18.75cm). Whereas, maximum shoot length (71.71cm) was recorded with Th-32 isolate, Pf-31 isolate was found most effective in increasing stem diameter (2.27cm). In PC-5 cv. Th-32 was most effective in increasing root length (18.99cm). Shoot length (82.81cm) and stem diameter (2.31cm) was observed to be maximum when treated with Th-31 and Pf-31 isolates respectively.
10. Effect of *G. intraradices* and isolates of *T. harzianum* and *P. fluorescens* on reduction of disease severity under glasshouse condition was assessed 45 and 65 DAS in both PC-4 and PC-5 cultivars. All treatments significantly reduced the disease severity as compared to control. Maximum reduction in disease severity

(45.30%) was observed with Th-32 isolate followed by Pf-31(44.10%) in PC-4. while in PC-5. maximum reduction in disease severity (43.04%) was recorded with Th-32 followed by Pf-2(41.21%) isolate.

11. Potential isolates Th-R, 18, 28, 31, 32, Pf-2, Pf-31 and Glomus were selected as per growth promotion and disease reduction experiments *in vitro* and under glasshouse conditions were further evaluated for growth promotion, disease reduction, chlorophyll content and green fodder yield in both cultivars of sorghum against the pathogen under field condition.
12. In field, 15 days after sowing in PC-4. Pf-31 isolate brought maximum seed germination (89.67%). Root length (7.12cm) and shoot length (25.89cm). Maximum Stem diameter (0.75cm) was recorded with Th-32. In PC-5, Th-32 brought maximum germination percentage (89.26%), shoot length (28.98cm) and stem diameter (0.91cm). However Pf-31 was best in increasing root length (10.89cm).
13. 30 days after sowing in PC-4 cv. Pf-31 isolate performed best with maximum root length (23.89cm) and increased shoot length (74.12cm). However maximum stem diameter (1.01cm) was observed with Th-32. In PC-5 cv. Pf-31(24.98cm) exhibited maximum root length. However maximum shoot length (86.89cm) and stem diameter (0.99cm) was observed with Th-32.
14. At 45 days after sowing in PC-4 cv. Th-32 isolate performed best with maximum root length (26.89cm) and stem diameter (1.35cm). However maximum shoot length (172.12cm) was recorded with Pf-31 isolate. In PC-5 cv. Th-32 isolate was most effective in increasing root length (27.12cm) and stem diameter (1.41cm). However shoot length (185.89cm) was observed maximum when treated with Pf-31 isolate.
15. Effect of *G. intraradices* and selected isolates of *T. harzianum* and *P. fluorescens* on reduction of disease severity under glasshouse condition was assessed 45 and 65 DAS in both PC-4 and PC-5 cultivars. All treatments significantly reduced the disease severity of zonate leaf spot as compared to control. Th-32 resulted maximum reduction of disease severity by (28.74%) and (33.81%) in both PC-4 and PC-5 cultivars respectively.

16. Both cultivars of sorghum were assessed for total chlorophyll content present on leaves. In PC-4. Th-32 treated plants were observed with highest amount of total chlorophyll content (2.356mg/g fr.wt) while in PC-5 it was highest in Th-31treated plants (2.976mg/g fr.wt.).

17. Green fodder yield estimation in both cultivars of sorghum treated with bioagents was estimated. Plants treated with Th-32 were recorded with maximum green fodder yield in PC-4 (27.15 kg/plot) as well as in PC-5 (38.19 kg/plot).

18. Real time polymerase chain reaction (RT-PCR) was used to quantify the responses in moderately resistant (PC-5) and susceptible (PC-4) cultivars of sorghum during priming with bioagents and boosting with additional pathogen inoculation at different time intervals. Maximum fold change was observed in PC-5 than PC-4.

19. PC-5 treated with Th-32 recorded maximum PAL gene expression during priming and boosting as compared to PC-4. The expression of chitinase was moderate during priming in PC-4 while it was high in PC-5. After pathogen inoculation rapid increase was observed upto 72 HPI in both cultivars. Maximum fold change of CHS gene was recorded at 72 HPT during priming in both cultivars. During boosting at 92 HPI maximum fold change was recorded in PC-5 while it was at 72 HPI in PC-4.

20. Pf-31 treated plants during priming were recorded with moderate level of PAL gene expression. Greatest expression of this gene was recorded in moderately resistant cultivar than susceptible cultivar at 72 HPT. The expression of PAL gene during boosting in PC-5 was observed maximum at 72 HPI while it decreased in PC-4. The expression of chitinase in both cultivars was higher at 72 HPT during priming. Interestingly both cultivars observed a downregulation of this gene at 168 HPT. During boosting maximum fold change was recorded in PC-5 at 96 HPI while in PC-4 moderate level of expression was observed at 72 HPI. CHS expression during priming was higher at 96 HPT in PC-5 while in PC-4 it was at 72 HPT. During boosting in PC-5 higher level of expression was recorded while a moderate level was observed in PC-4 at 72 HPI.

21. Expression of PAL gene in plants treated with *G. intraradices* was observed maximum during priming at 144 hrs. During boosting expression of this gene was high at 96 HPI in PC-5 while a moderate level was recorded at 48 HPI in PC-4.

Interestingly the expression of chitinase during priming in PC-4 was higher at 144 hrs while it was maximum in PC-5 at 72 hrs. Maximum increase in fold change was observed at 72 HPI in PC-5 while less fold change was recorded in PC-4. The level of expression of CHS in PC-5 during priming was maximum at 120 hrs. PC-4 recorded maximum level of expression of this gene at 96 hrs. During boosting higher level of CHS expression was observed in PC-5 at 72 HPI while in PC-4 it was occurred at 48 HPI.

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ABSTRACT

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the prime food and fodder crops of the world. The yield potential of this crop is affected by a number of diseases. Among them zonate leaf spot is one of the important destructive foliar disease caused by *G. sorghi*. Considering the destructive nature of this disease and availability of only little information on biological control, present investigations were carried out with objectives to study the (1) screening of biocontrol agents *in vitro* and glasshouse for growth promotion and control of disease, (2) effect of selected biocontrol agents on growth promotion, disease reduction, chlorophyll content and green fodder yield in field conditions, (3) expression pattern of defence genes in Pant Chari-4 and Pant Chari-5 cultivar of sorghum during priming and boosting through RT-PCR.

Among all isolates tested *in vitro* for antagonism against the pathogen, TH-32 was most effective in reducing radial growth. In PC-4, maximum seed germination and vigour index was recorded in Th-31(88.67%) and Pf-31(950.7VI) respectively. In PC-5, maximum seed germination and vigour index was recorded in Th-32(89.33%) and Pf-31(1004.1VI) respectively. Bioagents screening in glasshouse on growth promotion of PC-4 cv. revealed that maximum root, shoot length and stem diameter was observed in Th-28(18.75cm), Th-32(71.71cm) and Pf-31(2.27cm) respectively. While in PC-5 cv. Th-32(18.99cm), Th-31(82.82cm) and Pf-31(2.31cm) treatments recorded maximum root, shoot length and stem diameter. Th-32 reported maximum reduction in disease severity in both PC-4(45.30%) and PC-5(43.04%) cultivars under glasshouse conditions. Further promising isolates were evaluated for growth promotion, disease reduction, chlorophyll content and green fodder yield in both cultivars in field condition. Th-32 was found best in increasing root length (26.89cm) and stem diameter (1.35cm) in PC-4 while it was 27.12cm (root length) and 1.41cm (stem diameter) in PC-5. However significantly increased shoot length of 172.12cm and 185.89cm was recorded with Pf-31 in PC-4 and PC-5 respectively at 45DAS. Among all isolates Th-32 reported maximum reduction in disease severity in both PC-4(28.74%) and PC-5(33.81%). Maximum chlorophyll content in PC-4 was observed in Th-32 treated plants (2.356 mg/g fr.wt.) while Th-31 recorded maximum (2.976 mg/g fr.wt.) in PC-5. Green fodder yield was observed maximum in Th-32 treated plots of both PC-4 (27.15kg/plot) and PC-5(38.19kg/plot) cultivars.

Expression of defence genes was determined during priming with bioagents and boosting (post inoculation) with additional inoculation of *G. sorghi*. Samples were collected at different time intervals during priming and boosting for RNA extraction. Gene expression was measured for PAL, chitinase and CHS using real time polymerase chain reactions (RT-PCR). The response of sorghum cultivars to *G. sorghi* infection involves multiple defense genes. Real time PCR used to study the expression of sorghum defence in glasshouse grown plants showed that PAL was highly expressed during priming and boosting in moderately resistant cultivar PC-5 leaves. The expression of some other defense genes like chitinase and CHS was variable in both cultivars.

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ज्वार *(सोर्घम बाईकोलर* (एल) मोएंक) विश्व की प्रमुख खाद्य एवं चारे की फसल है। इस फसल की उत्पादन क्षमता को बहुत से रोग प्रभावित करते हैं। इनमें से मंडलीय पर्ण चित्ती एक महत्वपूर्ण एवं विनाशकारी पर्ण रोग है, जो *ग्लिओसर्कोस्पोरा सोर्घाई* नामक कवक द्वारा होती है। इस रोग की विनाशकारी प्रकृति तथा जैव नियंत्रण एवं रोग विरोधी स्रोतों की उचित जानकारी उपलब्ध न होने के कारण वर्तमान परीक्षण किए गये, (1) विकास को बढ़ावा देने एवं रोग की रोकथाम के लिए पात्रे और काँच घर में जैव अभिकर्ताओं की स्क्रीनिंग, (2) क्षेत्र की परिस्थितियों में विकास को बढ़ावा ,रोग की रोकथाम, क्लोरोफिल एवं हरा चारा उपज पर चुने गये जैव अभिकर्ताओं का प्रभाव, (3) आर. टी.पी.सी.आर. के माध्यम से प्राइमिंग एवं बूस्टिंग के दौरान ज्वार के पन्तचरी–4 और पन्तचरी–5 किस्मों में रक्षा जीन का अभिव्यक्ति पैटर्न।

रोगाणु के खिलाफ विरोध के लिए पात्रे में सभी परीक्षण विलगों में से टी.एच.–32 रेडियल वृद्धि को कम करने में सबसे प्रभावी था। पी.सी.–4 में, अधिकतम बीज अंकुरण और विगर इन्डैक्स, टी.एच.–31 (88.67%) एवं पी.एफ.–31 (950.7 वी आई) क्रमशः में दर्ज की गयी थी। पी.सी.–5 में अधिकतक बीज अंकुरण और विगर इन्डैक्स, टी.एच.–32 (89.33%) एवं पी.एफ.–31 (1004.1 वी आई) क्रमशः में दर्ज की गयी थी। विकास को बढ़ावा देने के लिए काँच घर में जैव अभिकर्ताओं एजेंटों की स्क्रीनिंग के दौरान, पी.सी.–4 किस्म में अधिकतम जड़–प्ररोह लम्बाई और तने के व्यास टी.एच.–28(18.75सेमी), टी.एच.– 32(71.71 सेमी) एवं पी.एफ.-31(2.27 सेमी) विलगो क्रमशः के साथ पता चला, जबकि पी.सी.–5 में अधिकतम जड़ –प्ररोह लम्बाई और तने का व्यास टी.एच.–32 (18.99 सेमी), टी. एच.–31 (82.82 सेमी) एवं पी.एफ.–31 (2.31 सेमी) उपचारों में पता चला। काँच घर परिस्थितियों में टी.एच.–32 का उपचार दोनो, पी.सी.–4 (45.30%) और पी.सी.–5 (43.04%) किस्मों में रोग की रोकथाम करने में अधिकतक पाया गया। काँच घर परीक्षण के बाद अच्छे पाये गये विलगो का क्षेत्र की परिस्थितियों में हरा चारा उपज को दोनो किस्मों (पी.सी.–4 एवं पी.सी.–5) के लिए मूल्यांकन किया गया। टी.एच.–32, पी.सी.–4 के जड़ की लम्बाई (26.89 सेमी) एवं तने का व्यास (1.35 सेमी) को बढ़ाने में सबसे अच्छा मिला और यही पी.सी.–5 में 27.12 सेमी. (जड़ की लम्बाई) एवं 1.41 सेमी. (तने का व्यास) मिला। हालांकि बोने के 45 दिन बाद, पी.एफ.–31 दोनों पी.सी.–4 (172.12सेमी) पी. सी.–5 (185.89 सेमी) में प्ररोह की लम्बाई को बढ़ाने में सबसे अच्छा मिला। सभी विलगो में से टी.एच. –32 दोनों पी.सी.–4 (28.74%) और पी.सी.–5 (33.81%) में रोग की गम्भीरता को कम करने में सबसे अधिक प्रभावी पाया गया। टी.एच.–32 उपचारित पी.सी.–4 पौधों में अधिकतम क्लोरोफिल (2.356 मिलीग्राम/ग्राम ताजा वजन पायी गयी जबकि यही पी.सी.–5 (2.976 मिलीग्राम/ग्राम ताजा वजन) में टी.एच.–31 के उपचार पर पायी गयी। चारे की उपज टी.एच.–32 उपचारित पी.सी.–4 (27.15 किलोग्राम /भूखंड) और पी.सी.–5 (38.19 किलोग्राम /भूखंड) में अधिकतम पायी गयी।

रक्षा जीन कि अभिव्यक्ति, जैव नियंत्रणों द्वारा प्राइमिंग और बूस्टिंग (पोस्ट टीकाकरण) *जी. सोर्घाई* का अतिरिक्त टीके के साथ निर्धारित किया गया था। आर.एन.ए. निकासी के लिए प्राइमिंग और बूस्टिंग के नमूनो को अलग–अलग समय अन्तराल पर एकत्रित किया गया था। आर.टी.पी.सी. आर. से पी.ए.एल., काइटिनेज और सी.एच.एस. जीन की अभिव्यक्ति का माप लिया गया था। *जी. सोर्घाई* संक्रमण से ज्वार के व्यासों की प्रतिक्रिया में कई रक्षा जीन षामिल होते है। ज्वार रक्षा की अभिव्यक्ति पी.सी.आर. द्वारा प्राइमिंग और बूस्टिंग के समय अध्ययन करने पर पी.ए.एल. की अभिव्यक्ति मध्यम प्रतिरोधी किस्मों (पी.सी.–5) में सबसे अधिक पाई गयी। काइटिनेज और सी.एच. एस. जैसे कुछ अन्य रक्षा जीनों की अभिव्यक्ति दोनों किस्मों में परिवर्तनषील पाई गयी।

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