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Original Research Article

Antifungal Activity of a Fungal Isolates against *Pomegranate* Wilt Pathogen *Fusarium*

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A B S T R A C T

Pomegranate wilt complex is an important disease resulting in complete wilting of the plant. Reports suggest that 45 % of crop is severely affected by wilt pathogen and its severity is increasing manifold. Taking into account the hazardous effects of chemical fungicides on the environment, development of an effective and environmentally benign biological agent is the need of the hour. In the present study, fungal pathogen was isolated from wilt infected Pomegranate plant. The isolate could be a putative member of the genus *Fusarium*. Optimum growth of fungal pathogen was observed at pH7 and temperature of 30°C. In primary screening antifungal activity was observed for fungal isolates F1, F5, F6 which were obtained from rhizospheric soil of pomegranate plant. Antifungal activity was observed by dual culture technique against isolated *Fusarium sp*. Using same method, the antifungal activity of a known biocontrol agent, *Trichoderma* was compared with that of fungal isolate F6. Isolate F6 showed significant ($p \leq 0.05$) reduction in growth of *Fusarium* and complete inhibition was observed in 8 days. Antifungal activity of non-volatile metabolites was confirmed by poison agar technique. The effect of volatile metabolites was also tested and significant reduction in growth of pathogen was observed. Antifungal organic metabolites from *Trichoderma sp* and isolate F6 were extracted from dried cell pellets by five different solvents viz, hexane, chloroform, ethyl acetate, methanol and ethanol. The activity of the extracts was tested by well diffusion assay. Growth of *Fusarium* was inhibited by methanolic and ethyl acetate extract. Isolate F6 also exhibited chitinase activity making it a potentially promising biocontrol agent.

Introduction

Pomegranate (*Punica granatum*) is an important fruit crop, belonging to the family Punicaceae. Pomegranate is a good source of carbohydrates and minerals such as calcium, iron and sulphur. It is rich in vitamin C and citric acid is the most predominant organic acid. Glucose (5.46%) and fructose (6.14%) are the main sugars with no sucrose in fruits. The fruits of pomegranate are known to possess

pharmaceutical and therapeutic properties. Sweet varieties are mildly laxative, sour types are good source for curing inflammation of stomach and heartache. The flower buds are very useful in Ayurveda for managing bronchitis. The bark of the stem, root and rind of the fruit is used for slimming, control of dysentery, diarrhoea and killing tape worms.

Successful cultivation of pomegranate in recent years has met with different traumas such as pest and diseases. At present, 45 % crop is severely affected by wilt pathogen and day by day the wilting severity is increasing at faster rate.

Pomegranate wilt complex is an important disease which results in complete wilting of plant. The disease is prevalent in parts of a Maharashtra, Karnataka, Andhra Pradesh, Gujarat and Tamil Nadu states in India.

Affected plants shows yellowing of leaves in some branches, followed by drooping and drying of leaves leading to sudden wilting of the plant, formation of nodules on roots and brown discoloration in the stem. The entire tree dies in few months or a year. Fungal pathogens responsible for wilt disease in Pomegranate Plant are *Ceratocystis fimbriata* and *Fusarium oxysporum* (Telmore and Shaikh, 2015).

Wilt is a destructive disease of many economically important crops caused by the soil – borne fungus *Fusarium oxysporum* and is a common soil pathogen and saprophyte that feeds on dead and decaying organic matter. *Fusarium* wilt diseases are difficult to control which includes chemical, biological and culture control methods and use of disease resistant varieties (National Centre for Integrated Pest Management, New Dehli). Use of chemicals poses threat to the environment. So use of environment friendly biological control is the effective way to combat wilt complex. From several studies, it has been confirmed that *Trichoderma* species have antagonistic and biologically control potential against a diversity of soil borne pathogens (Gaigole *et al.,* 2011; Cigdem Kucuk and Merif Kinvanc, 2009).

Therefore effort has been made in this regard to see the efficacy of some biological agents against the wilt pathogen.

Materials and Methods

Isolation and identification of pomegranate wilt pathogen

Three samples were used for isolation of fungal pathogen from wilt infected Pomegranate plant obtained from Natepute Village, Solapur District, Maharashtra State, India.

1. Rhizospheric soil sample of infected plant.
2. Infected nodules formed on roots.
3. The brown discoloured infected stem.

2^{nd} and 3^{rd} samples were cut into small pieces and inoculated in **s**terile potato dextrose agar medium (PDA, Himedia) Dilutions of soil sample were prepared in sterile saline up to 10^{-8} and 0.1 ml of alternate dilution was spread on **s**terile potato dextrose agar plates.

All the plates were incubated at 30^{o}C and observed everyday day for fungal growth. The plate inoculated with brown discoloured bits from infected stem showed white, fluffy, cottony growth which was purified and identified by using slide culture technique. The isolated fungal pathogen identified as *Fusarium sp.*

Isolation of antagonist fungal cultures

The plates on which soil sample was plated for isolation of fungal pathogen, showed some fungal colonies inhibiting growth of other fungal colonies. 6 such fungal colonies were purified and named from F1 to F6. *Trichoderma sp.* is a known biocontrol agent. Hence for comparison 3 Isolates procured from Agriculture College, Pune and named depending upon its spore colour as:-T1 green, T2 white and T3 yellow

Screening by dual culture method

For dual culture method, an agar disc (5mm in diameter) of 7 days old culture of *Fusarium* was placed 1cm away from the periphery of petri dish and a same sized agar disc of 7 days old culture of fungal isolates F1, F5, F6 and *Trichoderma* cultures were placed 1cm away from the edge of the same Petri plate containing PDA on the opposite side of *Fusarium*. As a control an agar disc (5mm in diameter) of 7 days old culture of *Fusarium* was placed 1cm away from the periphery of Petri dish containing PDA. All the plates were incubated at 30^{o}C. Antagonistic activity was checked after incubation by measuring the growth radius of *Fusarium* in the direction of the antagonist colonies (R2) and the growth radius of *Fusarium* colony in the control plate (R1). The two readings were used to calculate percentage inhibition of radial growth (PIRG) using the formula developed by Skidmore and Dickinson (1976) (Rahman and Begum, 2009).

$$PIRG = \frac{R1-R2}{R1} \times 100$$

The growth radius was measured everyday till complete inhibition was observed and PIRG was calculated for all days as shown in figures 1–6.

Poison agar technique using crude metabolites

Fungal isolates F1, F5, F6 and *Trichoderma* cultures were inoculated in 20ml sterile potato dextrose broth and incubated at 30ºC for 28 days. After incubation 1ml broths were filter sterilized through syringe filter assembly and added into 20 ml sterile molten PDA medium, then poured into sterile Petri plates and allowed it to solidify.

Advancing edge of 7 days old agar disc of *Fusarium* was placed in the centre of prepared poison agar plates. An agar disc (5mm) of *Fusarium* was placed in the centre of normal sterile PDA plate as control. All the plates were incubated at 30ºC. The radial mycelial extension of the pathogen for both the test and the control were measured (Rahman and Begum, 2009).

To check effect of volatile metabolites produced by isolate F6 on *Fusarium*

The effect of volatile metabolites produced by fungal isolate F6 against *Fusarium* was tested in the assembly described. Two bases of petri plates containing sterile PDA were inoculated with an agar disc (5mm in diameter) of 7 days old culture cut from advancing edge of pathogen and fungal isolate F6 and bases of inoculated Petri plate were adjusted with each other and attached by tape in which plate containing *Fusarium* was placed at the top. *Fusarium* was inoculated 48 hours earlier, since growth of F6 was more rapid. In the control an agar disc (5mm in diameter) of 7 days old culture cut from advancing edge of pathogen was kept. All the plates were incubated at30°C and observed for reduction in growth diameter of *Fusarium* (Ezizshi and Adekinle, 2006).

Chitinase assay

F6 culture was inoculated in sterile chitin broth and incubated at 30°C. After every 72 hours 1ml aliquot was removed aseptically and assayed to check for chitinase activity. The reaction mixture contained 1 ml of 0.1% colloidal chitin as substrate in sodium acetate buffer (0.05 M, pH 5.2) and 1 ml culture filtrate. The reaction mixture was incubated at 37ºC for 1 hour in a water bath with constant shaking. Suitable substrate and enzyme blanks were included. Chitinase activity was checked by the colorimetric estimation using Nelson Somogyi method for sugar estimation. One unit of chitinase activity was defined as the amount of enzyme, which produces 1µ mole of N-acetyl glucosamine in 1 ml of reaction mixture under standard assay condition (Lenardon *et al.,* 2010).

Extraction of secondary metabolites from isolate F6 and *Trichoderma*

F6 culture and *Trichoderma* (T yellow) were inoculated in sterile potato dextrose broth and incubated at 30^{o}C for 28 days. After incubation broth is filtered through Whatman filter paper no -1 and cell pellet was dried at 60^{o}C till constant weight. 0.5 g dried mass of F6 and T yellow culture was packed in the column made up of silica gel (60–120 µ Loba) and hexane, chloroform, ethyl acetate, methanol, ethanol and water (5ml) were used in the order of increasing polarity for extraction. The fractions obtained were measured and allowed for complete evaporation at 40^{o}C, and then 0.5ml dimethyl sulfoxide was added. These fractions were subjected for thin layer chromatography (TLC) by using readymade silica gel plates (Merck TLC Silica Gel 60 F 254) and Methanol : Hexane : Ethyl acetate : N-butanol solvent system. Spots were developed in iodine chamber. Same extract was used to check antifungal activity by the well diffusion method against *Fusarium* (Lalitha *et al.,* 2012).

Results and Discussion

The samples used for the isolation of fungal pathogen showed varied growth of fungi. Sample of infected brown discoloured stem showed cottony, white, fluffy, mycelial growth on PDA plate with characteristic pink pigmentation. In slide culture observation macroconidia were observed. From the characteristics, the isolated fungal pathogen may be *Fusarium sp. w*hich may be further confirmed by polyphasic approach.

During isolation studies some fungal colonies exhibiting antifungal activity. Six such colonies were purified and checked for its effect against *Fusarium* by using well diffusion method. Fungal Isolates F1, F5 and F6 showed better results than F2, F3 and F4. So for further study fungal isolates F1, F5, F6 were used. Primary screening was done by using dual culture method. For comparison known biocontrol agent i.e. *Trichoderma* was used. In dual culture method, fungal isolates F1, F5, F6 and *Trichoderma* showed inhibition of radial mycelial growth of *Fusarium.* The percentage inhibition of radial growth (PIRG) values for all isolates ranged from 92.40 to 100. The highest PIRG values were observed with isolates F1 and F6 as 100 which were significantly different ($p \leq 0.05$). Colony overgrowth by fungal isolates F1, F5, F6 and *Trichoderma* were varied from 3 to 9 days. As compared to *Trichoderma sp*, where PIRG was found to be 96.38, 92.40, 95.13, for isolate F6 and F1 PIRG was found to be 100 as shown in figure 1–6.

Table.1 Inhibition and Rf value of crude extract fraction of fungal isolate F6

Solvents used	R_f values	Diameter of zone of inhibition in mm.
Hexane	0.780	10
Chloroform	0.8	12
Ethyl acetate	0.783	11
Methanol	0.736	13
Ethanol	0.857	12

Fig.1 Percent inhibition of radial mycelial growth of *Fusarium* by Tricho yellow

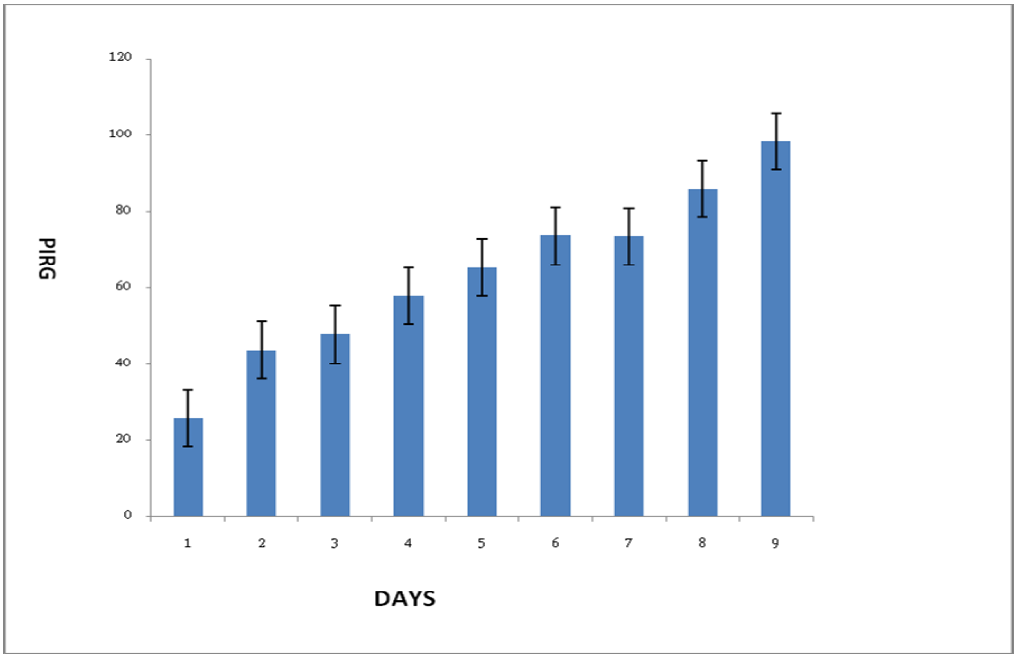


Fig.2 Percent inhibition of the mycelial growth of *Fusarium* by Tricho green

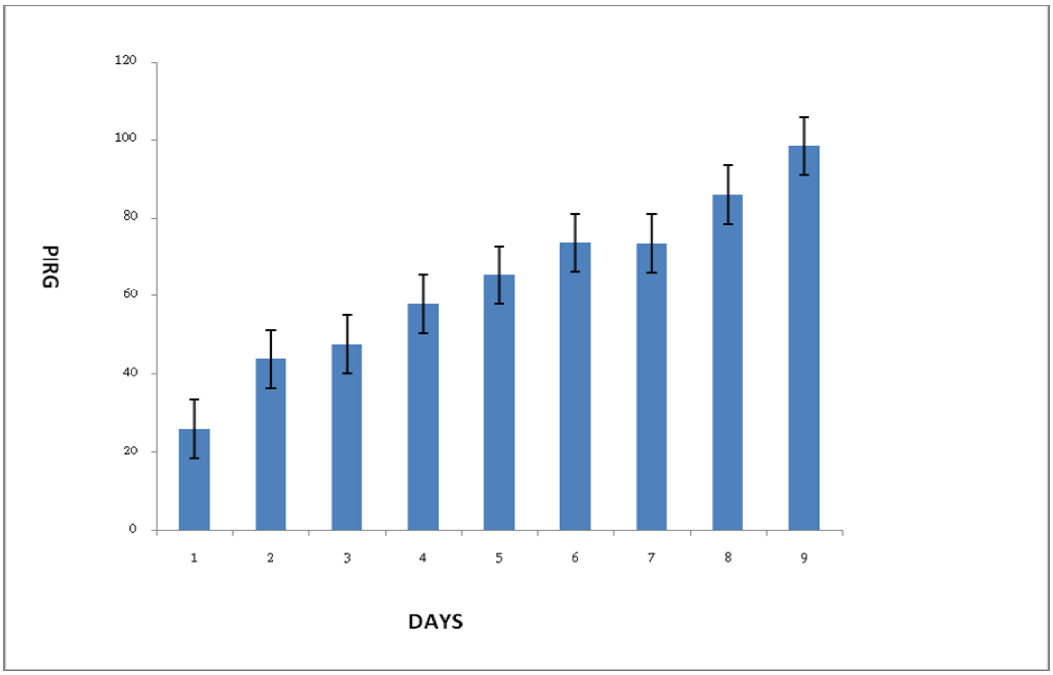


Fig.3 Percent inhibition of the mycelial growth of *Fusarium* by Tricho white

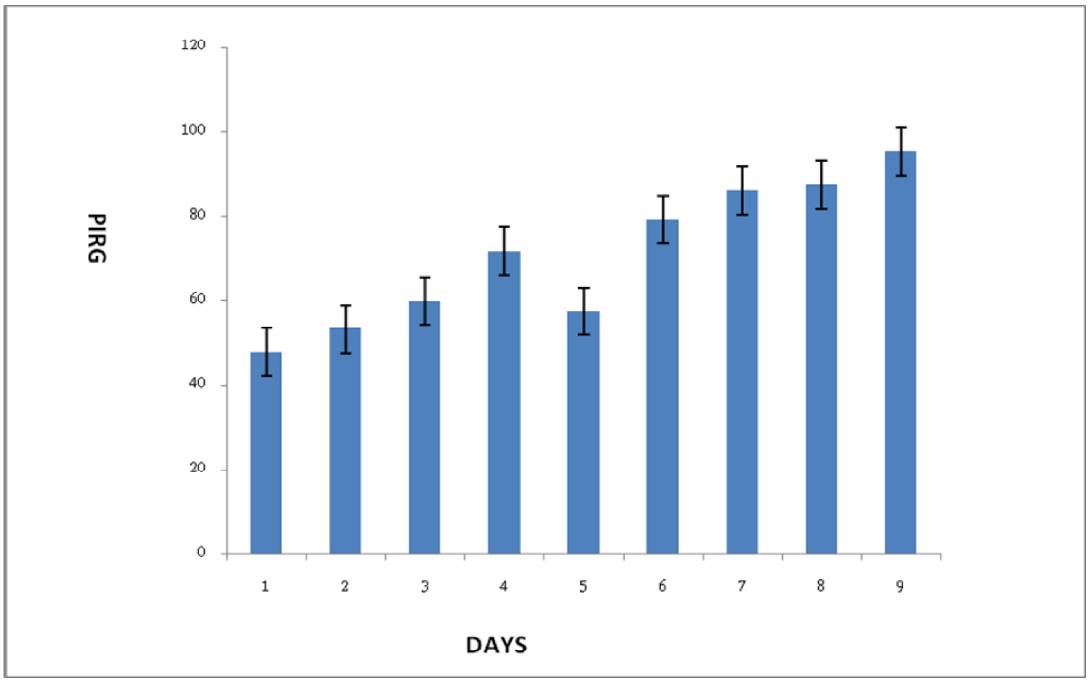


Fig.4 Percent inhibition of the mycelial growth of *Fusarium* by fungal isolate F1

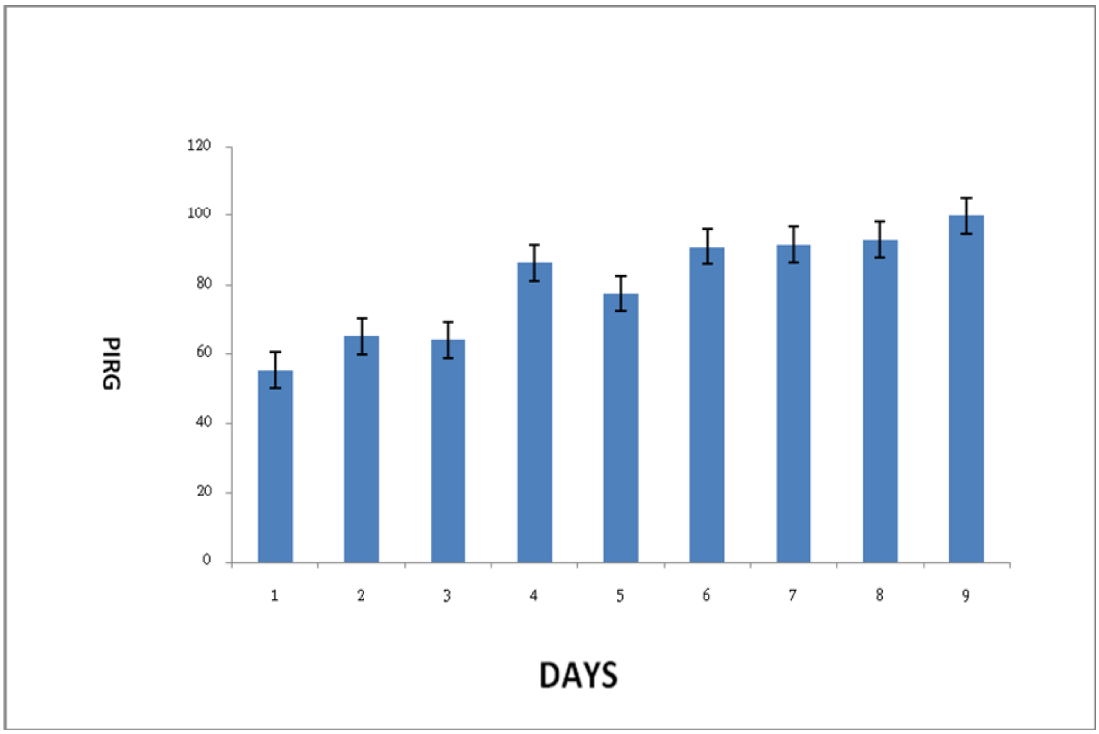


Fig.5 Percent inhibition of the radial mycelial growth of *Fusarium* by fungal isolate F5

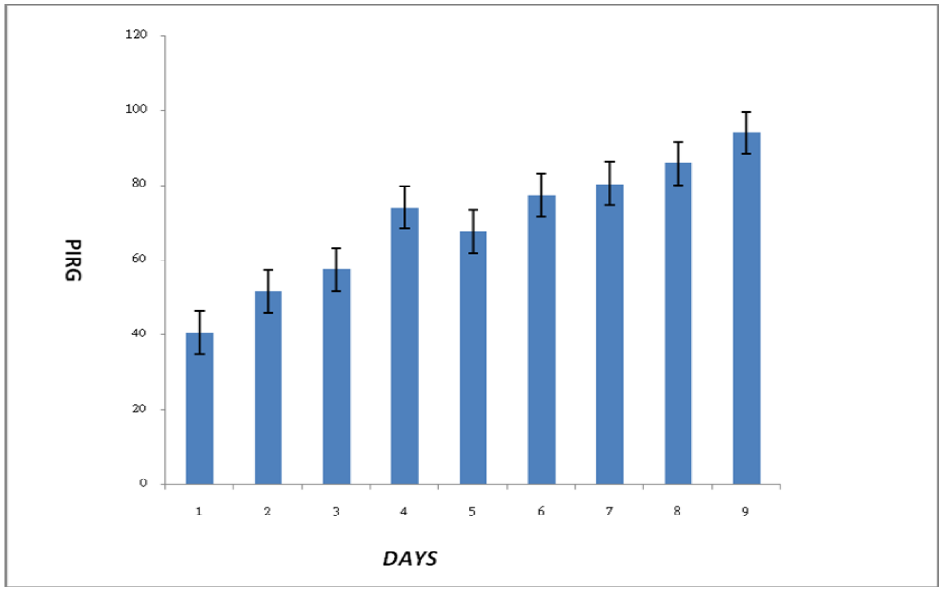


Fig.6 Percent inhibition of the radial mycelial growth of *Fusarium* by fungal isolate F6

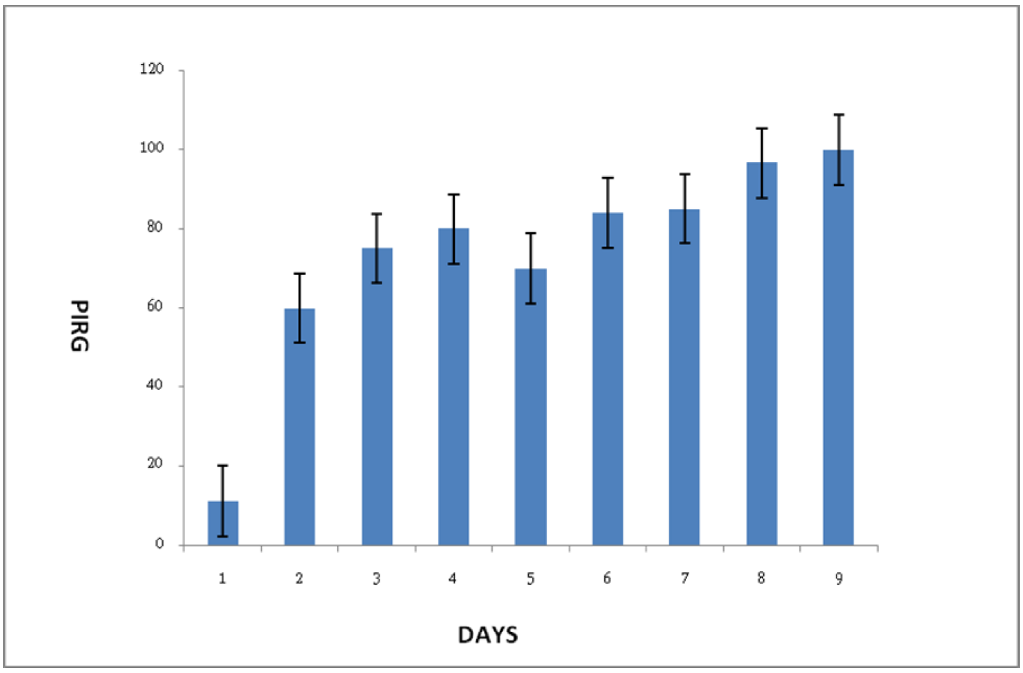
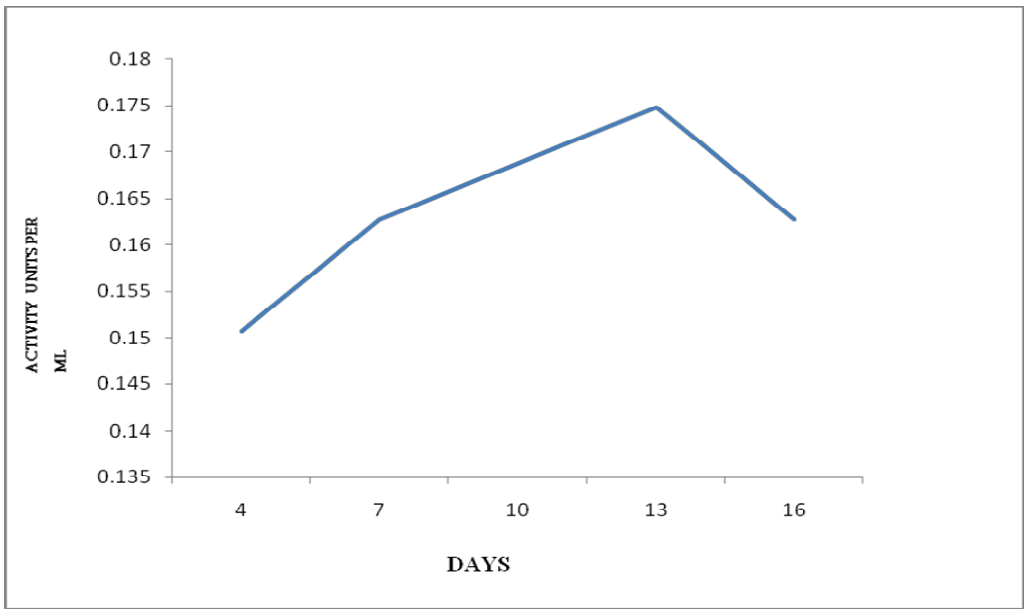


Fig.7 Chitinase activity shown by fungal isolate F6



This study demonstrated that isolate F6 showed 100% inhibition of *Fusarium* in 8 days. The growth rate of fungal isolate F6 was faster than other isolates i.e. it showed spore formation within 2 days. So for further studies fungal isolate F6 was used.

Activity of non volatile metabolites produced by fungal isolates was checked by poison agar method. In this method as compared to control showing growth of 8 cm. in 3 days, growth diameter of *Fusarium* inoculated in the plate containing metabolites of F6, F5, F1 was 1.63cm, 3cm and 1.89 cm. respectively. F6 culture showed 99 percent inhibition of *Fusarium.*

Trichoderma sp. is known to produce volatile metabolites which have antifungal activity. Similarly fungal isolate F6 was also showed presence of volatile metabolites by inhibiting, growth of *Fusarium* which was present in upper base of the assembly described in method compared to control.

Fungal Isolate F6 also showed 0.15-0.18 *U*/ml of chitinase enzyme activity. Maximum activity was observed on 13th day. As shown in figure 7.

In solvent extraction from dried cell pellet of F6, activity was observed in all eluted fractions given in Table 1. Methanol: ethyl acetate (75:25) extract showed maximum activity i.e. 13mm zone of inhibition. R_f values of components separated by TLC were given in Table 1.

Pomegranate is an important fruit crop envisaged by its role in the economy, is affected by many fungal, bacterial, viral and nematode diseases. Among the different diseases wilt complex is most prevalent fungal disease caused by *Fusarium*. Many factors which limit the successful cultivation and marketing of quality Pomegranate of which disease play important role. Among the diseases soil borne wilt caused by *Fusarium* has become serious in recent years causing heavy economic loss. Mycelial interaction is a basic method to assess antagonistic properties of microorganisms.

The results of dual culture method revealed that stronger antagonism was noticed in case of fungal isolates F6, F1 than *Trichoderma* isolates. In this comparative study known *Trichoderma* isolates showed 96% inhibition of *Fusarium* where as isolates F1, F6 shows 100% inhibition of *Fusarium.* In present investigation complete inhibition of *Fusarium* was observed in dual culture method whereas some growth of *Fusarium* was present on the plates with *Trichoderma* isolates. Morphologically these two fungal isolates are completely different from *Trichoderma*. Fungal Isolate F6 sporulates within 48 hours as compared to other isolates and in case of *Trichoderma* sporulation was observed after 96 hours. So growth rate of isolate F6 was faster than *Trichoderma*. The present study is to explore potential of these isolates to be used as biocontrol agent. *Trichoderma* is known to produce different non-volatile, volatile metabolites along with chitinase enzyme to exhibit antifungal activity. Similarly these isolates were used to check for production of non nonvolatile, volatile antifungal metabolites along with presence of chitinase enzyme. Isolate F6 also showed presence of non-volatile and volatile antifungal metabolites.

Among the metabolites, hydrolytic enzymes like chitinase are thought to be closely related to mycoparasitism in order to be able to degrade phytopathogen cell walls; the antagonistic *Trichoderma* induces the production of extracellular hydrolytic enzymes responsible for the directly attack against the pathogen. In the present study, chitinase activity is exhibited by isolateF6 which is responsible for complete removal

of growth of *Fusarium*. Attempts have made in the present investigation to extract antifungal compound and all the extract showed antifungal activity. The results suggested that the many active compounds must be present in isolate F6.Current study explored potential of isolate F6 as antagonistic fungus and has promising future to be used as biocontrol agent.

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