ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com
Journal DOI: 10.21474/IJAR01

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Growth stimulation and induction of systemic resistance in chilli against anthracnose disease by *Penicillium citrinum* AVGE5.

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Manuscript Info

Manuscript History:

Received: 18 March 2016
Final Accepted: 26 April 2016
Published Online: May 2016

Key words:
chilli, *Acorus calamus*, *P citrinum.* , *Colletotrichum* sp, IAA, Ammonia, and Phosphate solubilisation.

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Abstract

The concept of biological control is getting more importance now days than the chemicals because of its eco friendly nature. The plant growth promoting endophytic fungi are known to enhance growth and induce systemic response in plants. Efficacy of *Penicillium citrinum* AVGE5, an endophytic fungus was evaluated for in vitro fungal antagonism to *Colletotrichum* sp, along with growth stimulation and induction of systemic resistance in chilli seedlings against anthracnose disease. The activity of extracellular hydrolytic enzymes amylase, lipase and protease was also high in this culture fluid. This isolate exhibited plant growth promoting characters such as Production of IAA(975 ppm), Ammonia(116µg/ml) and Phosphate solubilisation. The levels of Phosphate solubilisation, IAA and Ammonia(15.76µg/ml) respectively. 7days old Fungal culture broth of *P. citrinum* AVGE5 was incorporated into plastic pots containing chilli seeds var.LCA 334 and growth data was recorded periodically at an interval of 2 weeks. *P.citrinum* AVGE5 inhibited mycelium growth of *C. acutatum* and *C. gloeosporioides* under in vitro conditions and significantly increased number of leaves, root and shoot length, leaf surface area and seedling vigour index in chilli plant compared to control. This study revealed, application of *P.citrinum* AVGE2 resulted in overall improvement in growth of chilli seedlings and induced systemic resistance.

Introduction:-

Chilli (Capsicum annuum L.) is considered as an important tropical and subtropical crop on the basis of its high consumption, nutritional and cash value to farmers and consumers both in developed and developing countries. Among them, India is the largest consumer and exporter of chilli in the international market and exports dry chilli, chilli powder and olio resins to over 90 countries (Singhal 1999). The major constraint to chilli production in India is fruit rot disease caused by *Colletotrichum capsici* (Syd.). Losses varying from 10–60% have been reported in India (Patil et al. 1993; Pandey and Pandey 2003). Although chemicals are available for the management of the disease, a continuous, inappropriate, non-discriminative use of chemicals is known to cause undesirable effects such as residual toxicity, development of resistance, environmental pollution, health hazards to humans and animals. In an attempt to modify this condition some alternative methods of control have been adopted. The plant pathologists of late have focused their attention on developing environmentally safe, long lasting and effective bio control methods by gradually replacing chemicals for the management of plant diseases. A number of plant species have been reported to possess natural substances that are toxic to many fungi causing plant diseases. Crude extract from rhizome, leaves and creeping branches of Sweetflag (*Acorus calamus* L.), palmorosa (*Cymbopogonmartinii*) oil, *Ocimum sanctum* leaf extract and neem (*Azadirachia indica*) oil could restrict growth of the anthracnose fungus among the bio fungicides used against the fungus *Colletotrichum* sp on chilli fruit (Jeyalakshmi, C.,& Seetharaman, K., 1998; Korpraditskul *et al*.,1999 & Charigkapakorn, N., 2000).

Plant growth promoting microbes are heterogeneous groups of microbes associated with plants in diverse ways. The plant associated microbes colonize the rhizosphere (rhizospheric microbes), phyllosphere (epiphytes) and inside of the plant tissue (endophytes). The word "endophyte" means "inside the plant" (derived from the Greek words "endon" meaning "within" and "phyton" meaning "plant"). Although there are diverse meanings for the term, endophytes are most commonly defined as those organisms whose "infections are inconspicuous, the infected host tissues are at least transiently symptomless and the microbial colonization can be demonstrated to be internal". In the last decade the study of endophytic microbes has become very important as these microbes are native to the host plants and most of the endophytes are beneficial to the host plants in terms of production of plant growth regulating hormones, solubilization of insoluble minerals and their antagonistic behavior against plant pathogens and pests.

Endophytic fungi live inside the plant without causing any overt negative effect on the host; rather they protect the host plant from pest and diseases (Saikkonen K *et al*.,2004).The ability of endophytic micro flora especially fungi provide protection against wide range of plant pathogens and insect herbivores (Clay K *et al* 1985, Clay K 1988) and hence drawn the attention to study the endophytes for better health of crop plants and their antifungal bioactivity (Kumar S, Kaushik N 2013;Clarke B B,*et al* 2006; Kumar S, Kaushik N, *et al* 2011; Li HY *et al* 2010; Narisawa K, *et al* 2002). However, selection and identification of effective antagonistic organisms is the first and foremost step in biological control (Campanile G 2007 ; Kamalakannan A, 2004) In view of the above mentioned facts, the present study was undertaken to characterize endophytic fungi isolated from rhizome of *Acorus calamus* Linn. , and assess fungal antagonism against *C. acutatum and C. gloeosporioides* along with plant growth promoting traits in chilli crop at seedling stage.

Materials and Methods:-

Fungal pathogens:-

Chilli fungal pathogens cultures of *C. acutatum* (MTCC2214) *and C. gloeosporioides* (MTCC 3439) were purchased from MTCC Chandigarh, India. The fungal culture was grown on PDA plates and incubated at 28˚c for 4-6 days. Stock cultures were maintained on PDA slants.

Isolation of endophytic fungi:-

The endophytic fungus was isolated from the rhizome. Rhizomes were washed in running tap water and its scales were removed using a sterile blade. It was then washed with 70% ethanol for 1min, followed by 0.5% aqueous solution of sodium hypo chloride for 3 minute. It was again washed with 70% ethanol for 1-4 min and rinsed with sterile distilled water. It was aseptically cut with sterile blade and inner tissue was excised. The excised tissue pieces were inoculated to potato dextrose agar (PDA) containing 1 mM Gentamicin (to avoid bacterial growth) inoculated for 6-25 days at 25 ± 1^0C. Pure cultures were then transferred to PDA plates free from antibiotics and cultivated for 20 days at 28^0C (Petrini O 1984).

Screening of Endophytic Fungi for Antifungal Activity:-

The isolated strains were screened for their antagonistic activity against *C. acutatum* and *C.gloeosporioides* by dual culture method (Campanile G 2007). Mycelial growth (6 mm plugs with the mycelium) removed under aseptic conditions from 4 to 5 day-old pure culture of each isolate of endophytic fungi and pathogenic fungi was transferred to Petri dish (9 cm^2) containing 20 ml of PDA and was kept 4 cm apart from each other. The plates were incubated at 28˚C for 8 days.The treatments were replicated in triplicates. The growth of the pathogen and the endophyte was observed constantly and radial growth was recorded by measuring the mean colony diameter on 8th day of inoculation. The percent of inhibition of the test phyto pathogenic fungi was calculated using the formula

$$(R1 - R2/R1) \times 100$$

Where R1 was the radial growth of pathogen without endophytic fungi, R2 represent the radial growth of pathogen inoculated with endophytic fungi (Ghildial A, & Pandey A 2008).

Identification of Endophytic Fungi:-

The fungal cultures were identified based on colony morphology, conidial, shape–size and growth rate (Photita W *et al* 2005).Extraction of genomic DNA from the culture of strain was performed according to the extraction method described by Raeder and Broda P (1985).fungal identification was carried out by 18S rRNA partial gene sequencing by Macrogen inc seoul,Soth Korea usin ITS primers, forward (ITS4-5'-TCCGTAGGTGAACCTGCGG-3') and reverse (ITS4-5'TCCTCCGCTTATTGATATGC-3') Fungal identification was carried out by an analysis of similarity by means of the BLASTn tool suite from the BLAST software package (Astchul SF *et al* 1990) to compare its ITS1-5.8S- ITS2 region to sequences deposited in Gen Bank. To perform the phylogenetic analysis, ITS

sequence of fungal strain was aligned with characterized sequences retrieved from GenBank by means of the tools available in the MEGA 4.0 software package (Tamura K, *et al* 2007). Tree reconstructions were obtained by the Neighbor-Joining method 1000 bootstrap replicates were performed to assess the statistical support for each branch in the tree.

Qualitative Extracellular Enzyme Assay:-
The isolates of endophytic fungi were tested for their ability to produce the hydrolytic enzymes i.e., amylase, protease and lipase (Hankin L, & Anagnostakis SL 1975; Kumaresan V, & Suryanarayanan TS 2002)

Plant growth promoting traits:-
Indole acetic acid (IAA):-
IAA was quantified by the method of Patten and Glick (2002). Fungal isolate was cultured in flasks containing 10 ml of PDA broth supplemented with tryptophan (L-trp) 0.2 mM and incubated at room temperature (25 to 28°C) for 72 h. The cultures were then centrifuged for 15 min at 10 000 rpm. Each 2 ml of the supernatant was mixed with 2 ml of Salkowski reagent (150 ml H_2SO_4, 250 ml distilled water, 7.5 ml $FeCl_3.6H_2O$ 0.5 M) and incubated at room temperature for 30 min. The presence of IAA was determined by the development of pink color and the IAA concentration was measured spectroscopically at 520 nm and quantified in an IAA standard curve.

Production of ammonia:-
Fungal isolate were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48- 72h at 37^0C and 28^0C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color was a positive for ammonia production (Cappuccino, J. G. and N. Sherman.1992).

Qualitative and Quantitative analysis of Phosphate Solubilization:-
The isolates were screened for phosphate solubilization as per methodology described by Gupta (1994).On modified Pikovskaya agar with insoluble tricalcium phosphate (TCA); a loop full of each culture was placed on the centre of agar plates and incubated at 30 ± 0.1^0C for 5 days. The solubilization zone was determined by subtracting the diameter of fungal colony from the diameter of total zone.

Quantitative estimation of inorganic phosphate solubilisation was done as per methodology described by Nautiyal (2001) and Jackson. (1973).Fungal isolate was grown in National Botanical Tricalcium phosphate (TCA). The flask containing 50ml medium was inoculated with 500µl fungal culture in Tricalcium and incubated at 30 ± 0.1^0C at 180 rpm for 5 days in incubator shaker. Simultaneously, uninoculated control was also kept under similar conditions. The culture was harvested by centrifugation at 10,000 rpm for 10 min. The phosphate in supernatant was estimated by vanado-molybdate-yellow color method. To 0.5 ml aliquot of the supernatant, 2.5 ml Barton's reagent was added and volume was made to 50ml with deionized water. The absorbance of the resultant color was read after 10 min at 430 nm in UV/Visible Spectrophotometer. The total soluble phosphorus was calculated from the regression equation of standard curve. The values of soluble phosphate liberated were expressed as µg ml-1 over control. The pH of culture supernatants were also measured using a pH Meter.

Seed treatment and nursery experiment:-
Plastic pot trays containing 98 cavities filled with well sterilized coco peat was used to produce the chilli seedlings. Chilli seeds were treated with 7 days old fungal culture broth (1ml) of the selected isolate for 30 min and were shade-dried at 28^0C for 1 h. The treated seeds (100) were sown in pots containing coco peat in a green house. Seed receiving only sterile distil water served as untreated controls. Seedlings were allowed to grow for 30 days at 25 ± 28^o C under natural light. Observations were recorded on germination percentage in the beginning, root length; shoot length and fresh weight of the seedlings every two weeks interval of sowing by removing 10 seedlings from each replication.

Results:-

Isolation and Screening of Endophytic Fungi against Chilli Fungal Pathogens:-
A total 17(AVCE 1-17) morphologically distinct fungal colonies were isolated from rhizome of *Acorus calamus.* All 17 AVGE isolates were screened for fungal antagonism against *Colletotrichum sp* using dual culture method. Six endophytic fungal strains showed significant antagonism against *C. acutatum and C. gloeosporioides* causing

anthracnose in chilli. Isolate AVGE5 which showed significantly higher antagonistic activity (56%) was selected for this study (Fig1).

Colletotrichum sp Antagonism by AVGE5

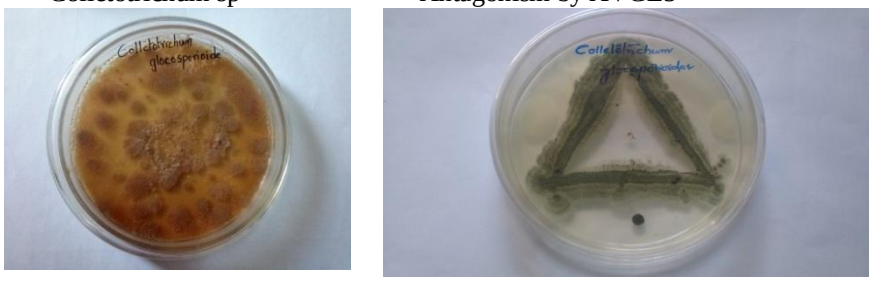


Fig1AVGE5 showing antagonism against Colletotrichum sp in dual culture:-

Identification of Endophytic Fungi:-

Morphological nature of mycelium, conidiophores and conidial characters were identified under light microscope. Based on the morphology of mycelium, nature, structure and arrangement of conidia and conidiophores AVGE 5 was tentatively identified as genus *Pencillium sp* (fig 2)

Fig.2 Morphological characters of AVGE under Olympus Light microscope

When amplified the ITS region of r RNA of *P.citrinum* AVGE 5 the primary ITS 1 and ITS 4 produced expected size of PCR product showing band at 1090 bp. A Sequence similarity search showed that18S r RNA sequence of AVGE5 showed 99% similarity with their respective corresponding gene sequence of *P.citrinum*KUC3084 (HM469428.1),and *P.citrinum* MSSRF-IS 1(HQ232482.1) in data base. Phylogenetic analysis was carried out with neighbor joining method revealed that it is closely related *P. citrinum*. The sequence was submitted in GEN BANK NCBI *P.citrinum* AVGE5 with accession number **KM 389208** (Fig3)

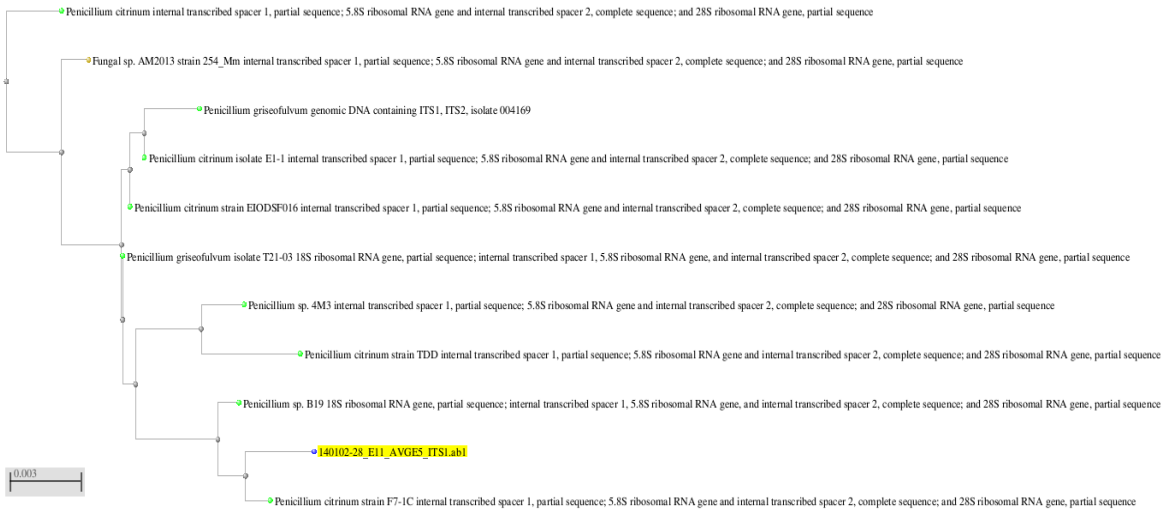


Fig 3 Phylogenetic analysis of *P.citrinum* AVGE5, isolate based on 18S rRNA base sequence

Qualitative and Quantitative analysis of IAA, Ammonia and phosphate solubilization:-

In the qualitative analysis of PGP traits,*P.citrinum* AVGE 5 showed positive response to phosphate solubilisation, IAA and Ammonia production In Quantitative analysis,. *P.citrinum* AVGE5 showed significantly high levels of IAA (116µg/ml) and Ammonia (15.75 µg/ml). *P.citrinum* AVGE 5 was also capable of solubilizing inorganic phosphate with solubilisation index of 975 ppm another important trait in PGP (Table1)

Hydrolytic enzymes of *P. citrinum* AVGE5:-

Plate assay for the production of hydrolytic enzymes indicated that *P. citrinum* AVGE5 was positive to Amylase, lipase, protease production. Clear zone around the fungal colony indicating the positive amylase production and undigested gelatin precipitated with ammonium sulphate and the digested area around the colonies appeared clear and thus indicating the positive protease activity by AVGE5. After incubation a clear zone around the colony indicated lipase positive activity by the fungi (Table 1). The result obtained revealed that *P.citrinum* AVGE5 possessed excellent enzymatic activity.

Table: 1 Plant growth promotion traits and hydrolytic enzyme production of *P.citrinum* AVGE5

PGPR traits	*P.citinum* (AVGE5)
IAA	116µg/ml
Ammonia	15.75µg/ml
Phosphate solubilisation	975ppm
Amylase	Positive
Protease	Positive
Lipase	Positive

Effect of *P. citrinum* AVGE5 on growth of chilli seedlings:-

P. citrinum AVGE5 was characterized for its plant growth promotion ability on seedlings of chilli (*Capsicum frutescence LCA 334*). In vitro growth parameters root length, shoot length, leaf surface area determined for 100seedlings at an interval of 2 weeks after sowing. Inoculation of pot trays containing chilli seeds with *P. citrinum* AVGE5 lead to a significant increase in growth parameters of chilli seedlings (Table 2) *P. citrinum* AVGE5 increased root length, shoot length, fresh weight and seedling vigor.

Table 2 Effect of *P.citrinum* AVGE5 on growth of chilli seedlings

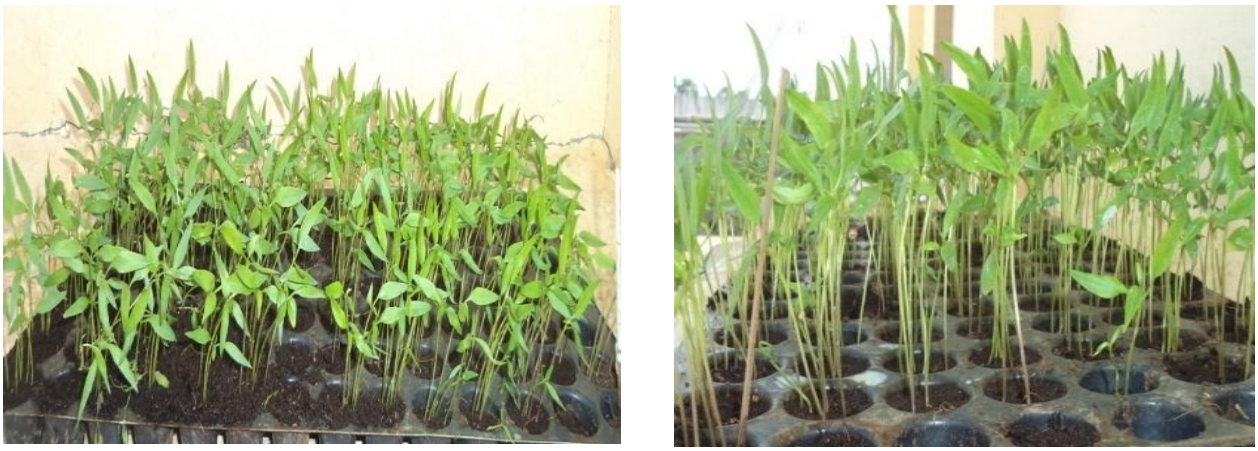
Time period	Name	Root length (cm)	Shoot length (cm)	Fresh weight (gram)	Seedlings vigor index#
2nd week	Control	2.3±0.15	3.64±0.15	0.06±0.02	594
	P.citrinum (AVGE5)	6.23±0.02 (170.86%)	6.61±0.02 (81.59%)	2.58±0.02 (4200%)	1272
4th week	Control	4.86±0.15	4.86±0.01	0.09±0.01	972
	P.citrinum (AVGE5)	9.24±0.15 (90.12%)	9.66±0.04 (98.76%)	3.99±0.04 (4333%)	1873
6th week	Control	6.55±0.15	6.82±0.02	0.16±0.01	1337
	P.citrinum (AVGE5)	15.24±0.01 (132.67%)	17.56±0.15 (157.47%)	4.17±0.15 (2506%)	3254
8th week	Control	10.69±0.25	10.93±0.02	0.20±0.02	2162
	P.citrinum (AVGE5)	17.51±0.20 (63.79%)	21.88±0.17 (100.18%)	4.88±0.03 (2340%)	3700

**Values are mean of three replicates. ± SE*

#Seedlings viour index= seedling length (cm) × germination percentage.

Vigor index calculated after every 2 weeks.

** Value in parenthesis is % increase of growth compared to control.



Control seedlings AVGE5 treated seedlings

Fig: 4 Green house studies of chilli seedlings treated with *P.citrinum* (AVGE5)

Discussion:-

Using plant products for the management of plant diseases has a special significance in the context of environmental pollution, accumulation of toxic substances in the produce and development of resistance by plant pathogens.In the present study, *P.citrinum* AVGE 5 showed inhibitory activity against both the test fungal pathogens. However, the antifungal activity demonstrated by endophytic fungi, *P.citrinum* was due to certain diffusible metabolites as revealed by dual culture method. The extracellular metabolites produced by endophytic fungi *P.citrinum* AVGE5 are responsible to inhibit the pathogens. Fungal protease also plays a significant role in cell wall lysis that occurs during pathogen–host interactions (Haran S *et al* 1996). *P.citrinum* AVGE 5 produced higher quantities of IAA in culture broth and also induced their production within the treated chilli plants. The increase in IAA levels is one of the direct mechanisms by which bio control agents promote shoot and root growth and leaf area in chilli plants. The role of IAA in lateral and adventitious roots initiation and emergence, as well as in shoot development by influencing cell division, expansion and differentiation (Saikkonen, K., *et al* 2011).They also colonize the plant roots, provide protection against certain soil borne fungal pathogens as well as stimulate growth and crop yield by hormonal stimulation through induction of host resistance by elicitation (Neito, K.F. & W. T. Frankenberger.1989). *P.citrinum* AVGE5 has been reported to produce high levels of IAA and ammonia. These property were responsible for increased growth of chilli seedlings (table 2, fig4)

Among different microbial groups, fungi have been reported to be more efficient phosphate solubilizer in comparison to bacteria. (F. Ahmad, et al 2008). It is a well-established fact that improved phosphorous nutrition influences overall plant growth and root development (D.L. Jones & P.R. Darrah, 1994).The plant growth promoting ability of fungi may be due to their capacity to produce higher amounts of growth promoting metabolites. *P.citrinum* AVGE3 Inorganic phosphate solubilization was reported to be high (975 ppm) when compared to the earlier studies. *P.citrinum* AVGE5 tested positive for the production of HCN as a secondary metabolite and also exhibited strong production of ammonia, which is taken up by plants as a source of nitrogen for their growth (A. C. Gour, et al 1990). Fungal endophytes thus facilitate their host plants to survive under stress condition by secreting favorable secondary metabolites

The present study has clearly demonstrated that *P.citrinum* AVGE5 can be employed for basal application in pot trays to raise quality chilli seedlings to induce systemic resistance against Anthracnose disease caused by *Colletotrichum* species. In India farmers procure chilli seedlings, commonly raised in pot trays using coco peat as the growing medium, from commercial nurseries and there are a lot of chances of spread of the anthracnose disease through transplants. Indeed *Colletotrichum* sp are seed or soil borne and use of pathogen free chilli transplants will help chilli growers to prevent crop losses caused by early as well as anthracnose. This study therefore assumes significance in production of diseases free quality chilli transplants .Based on this study and other data a seed coating formulation , has been developed and its utility in enhance seed germination ,seedling growth and vigor with resistance to seed borne fungal pathogens. Management and control of the anthracnose disease are still under extensive research (Yoon, J.B *et al* 2004).Among disease control management, the use of resistant cultivars is the cheapest, easiest, safest and most effective means of controlling the disease. This is not only to eliminate losses from

the disease but also decrease the cost of chemical and mechanical control, as well as reduce contamination of the environment from the use of toxic chemicals. In the present study potentiality of *P.citrinum* AVGE5 as bio control agent was exploited for the effective management of fruit rot of chilli in green house

Bioagents of late have been known to induce systemic resistance against several plant diseases (Ramamoorthy et al. 2001; Radjacommare et al. 2002). The potentiality of *P.citrinum* AVGE5 bio control agent under in vivo conditions from the point of view whether there is any varietal or location variations in harboring and its expression against the tested pathogens and on the occurrence of diseases in nature in chilli fields where the endophyte commonly occurs need to be explored in future.

Fungi toxicity of plant products was considered to be the safe means of plant disease control. Furthermore, the combined studies with plants and bio control agents need to be tested for a better protection of the chilli crop. Thus plant products and bio agents can be well exploited in the future and their active principles can also be isolated and formulated for the effective management of various plant diseases.

Acknowledgement:-
I am thankful to Acharya Nagarjuna University, Guntur, and A.P. for granting University Research Fellowship to do my research work.

Conflict of interest: No conflict of interest.

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