Bio-efficacy of *Bacillus subtilis* against Damping off disease in Brinjal

Abstract

Bacillus sp as biocontrol agents are extensively used in management of fungal diseases of crop plants, exhibiting mycoparasitism against a wide range of plant pathogens. In the present investigation Efficacy of *Bacillus* subtilis was tested against *Pythium aphanidermatum* under glass house and field conditions. With regard to the germination and seedling growth parameters, the treatment T_2 (liquid formulation of Bacillus subtilis @10ml/L) recorded the highest germination percentage (92.59%), shoot length (59.3cm), root length (24.3cm), vigor index (7740.52) and yield (22.67mt/ha). This was followed by the treatments T_3, T_1 and T_4 in the decreasing order of merit. In the biometric observations also, 10ml/L and 20ml/L concentration of liquid formulation of *Bacillus subtilis* recorded statistically significant results. Observations on total protein content in brinjal plants treated with different concentrations of *Bacillus subtilis* revealed that treatment T_2 was found best in both field and glass house conditions yielded highest protein as 0.44mg/ml and 0.30mg/ml respectively. The least values of germination, growth parameters, protein content and yield were observed in untreated control

Key words: Biocontrol, Phytopathogenic, *Pythium aphanidermatum, Bacillus subtilis*

Introduction

Brinjal (*Solanum melongena* L.) popularly known as eggplant is an important solanaceous crop of sub tropics and tropics. The brinjal is of much importance in the warm areas of Far East, being grown extensively in India, Bangladesh, Pakistan, China and the Philippines. It is also popular in Egypt, France, Italy and United States. In India, it is one of the most common, popular and principal vegetable crop grown throughout the country except higher altitudes. Brinjal has been cultivated in India for the last 4,000 years, although it is often thought of as a Mediterranean or mid-Eastern vegetable. The global area under brinjal cultivation has been estimated at 1.6 million ha with total production of brinjal fruit of about 4.2 million tons **(FAO data, 2014)**. India accounts for about 8.7 million tons with an area of 0.53 million hectares under cultivation.

The disease which limit the production of brinjal includes Damping off, *Alternaria* blight, Late blight, *Fusarium* wilt, *Phomopsis* wilt, *Cercospora* Leaf spot, Bacterial wilt, Little leaf of brinjal etc.

Among the various diseases; Damping-off caused by *Pythium aphanidermatum* is one of the most widely spread and prevalent throughout the country causing considerable loss in India. The pathogen is soil borne in nature, also known as water mold. *Pythium aphanidermatum* overwinters in the soil as oospores, hyphae and/or sporangia, oospores can produce a germ tube and infect the plant directly, or, if the environment is favorable (that is an adequate amount of water is present), the oospore may produce sporangia, which in turn produce motile, bi-flagellate zoospores that swim to the host plant, encyst, and germinate. Various control measures which are generally used to manage the disease, are cultural practices, chemical, biological control and use of resistant varieties. Among these, use of resistant varieties is found to be most beneficial measure but development of new races of pathogen overcome resistance. Among cultural practices deep summer ploughing, destruction of diseased crop debris, crop rotation and use of soil amendments are recommended for the management of the disease. But all these cultural practices are not reliable to manage the disease in standing crop. Therefore, biological control has been adopted to minimize the disease severity.

Biological control is also likely to be more effective than disease control, that is based on synthetic chemicals. The complexity of interactions between organisms, the involvement of numerous mechanisms of disease suppression by a single microorganism, and the adaptness of most biocontrol agents to the environment in which they are used, all contribute to the belief that bio-control is more durable than synthetic chemical control **(Benbrook *et al*., 1996; Cook, 1993)**.

Among bio-control agents *Bacillus subtilis* is considered as potential biocontrol agent and plant growth promoter agent (PGPR) for many crops. Various mechanisms are involved in the biological control of

fungal pathogens by PGPRs. These mechanisms include the production of secondary metabolites such as antibiotics, siderophores, hydrolytic enzymes, phytohormones, volatile extracellular metabolites, hydrogen cyanide and competition for nutrients, promotion of plant growth and, finally, induced resistance within the plants **(Defago and Haas, 1990)**. PGPR may also promote plant growth by providing nitrogen **(Dobbelaere *et al.*, 2003)**, simplifying nutrient uptake **(Biswas *et al*., 2000**).

Bacillus is also identified as potential bio-control agent used as alternatives to pesticide since they achieve disease suppression without negative effects on user, consumer or the environment **(Johnsson, *et al*., 1998)**. Plant associated bacilli are recognized either as saprophytes, or as biological control agents. The genus *Bacillus* includes a variety of important species used in the fermentation industry. *Bacillus spp.* are nonpathogenic, good secretors of proteins and metabolites, and easy to cultivate. Products currently available commercially include enzymes, antibiotics and insecticides.
The present study was undertaken with the main objective for assessing the efficacy of antagonistic rhizobacteria belonging to genus *Bacillus* against *Pythium aphanidermatum*,

Materials and Methods

Collection, isolation, purification and identification of pathogen

Pythium aphanidermatum was isolated plant samples were collected from Brinjal field of vegetable farm of **C.S. Azad University of Agri. & Technology,** Kanpur and adjoining farmer's field.. Cuatural and Morphological characterizations was also done.

Isolation of antagonistic bacteria (*Bacillus* sp.)

Serial dilution technique (Johnson & Curl, 1972) was adapted for isolation of *Bacillus* sp. from rhizospheric soil samples collected from brinjal eco-system.Morphological and cultural characterization of isolated bacterial colonies were done.
For identification at species level purified cultures of bacterial bio-agents were send to ITCC, New Delhi. Based on the identification report bacterial bio-agents was identified as *Bacillus subtilis* and were used for further studies.

Pathogenicity Test

The inoculum of the isolates identified as *Pythium apahnidermatum*, based on their morphological and cultural characters was multiplied on sand maize meal medium **(Miller, 1946)** for 25 days at 25 $\pm 1^0$C. The inoculum thus obtained was mixed in sterilized soil @ 5% w/w and was filled in 8 inches diameter earthen pots, which were thoroughly cleaned with laboline detergent. In each of these pots 5 brinjal seedlings of a highly susceptible brinjal variety (J-2) were sown and kept in glass house during December 2015. For each isolate separate pots were used, and for each treatment 3 replications along with untreated control was maintained. The pots such sown were regularly observed for the appearance of the disease. Diseased seedlings from these pots were collected. These plants were used for re-isolation of the pathogen (isolate). The isolates obtained from these plants were compared with the original isolate with which these were inoculated.

In vitro screening of antagonistic bacteria

To assess the *in vitro* effect of *Bacillus subtilis* against *P. aphanidermatum* a laboratory bioassay by using Dual culture technique (Morton and Stroube, 1955) was conducted at Biocontrol Lab, Department of Plant Pathology, C. S. Azad University of Agriculture & Technology, Kanpur. The antagonist *B. subtilis* was inoculated first with the help of sterilized inoculation loop from liquid bioformulations prepared with sterilized water (@ 5ml/l, 10ml/l and 20ml/l) and one day later, 5mm disc from pathogen culture was inoculated since, *P. aphanidermatum* was fast growing. Observations were recorded when the control plates were completely covered by the mycelial growth of *P. aphanidermatum* and per cent growth inhibition was calculated using following formula -
Per cent growth inhibition = $\frac{\text{Mycelial growth in control - Mycelial growth in treatment}}{\text{Mycelial growth in control}} \times 100$

Mycelial growth in control

Bio-efficacy of *Bacillus subtilis* against *P. aphanidermatum* under *in vitro* conditions

To evaluate the bio- efficacy of antagonistic bacteria as seedling treatment against *P. aphanidermatum*, trials were conducted under green house conditions (pot trial). The disease grading was done by following the scale proposed by **Srivastava *et al*. (2002)**. Seedlings of brinjal (Var. J-2) were collected from Vegetable Farm, C.S.A.U.A.&T, Kanpur. The seedlings were kept in a beaker (100 ml capacity). Bacterial suspension was prepared in sterile distilled water with a cell density of $9X10^8$ $CFUml^{-1}$. Different concentrations of liquid formulation of antagonistic bacteria *viz*., (T_1) 5ml/kg seed (T_2)10ml/kg seed (T_3) 20ml/kg seed (T_4) Carbendazim 50% WP @ 2 g/kg and (T_5) Control (sterilized distilled water) applied to the seedling in 3 replications for 12 h and then sown in earthen pots. Pots were kept under greenhouse conditions till the end of the experiment. For rating the bio agent, the disease grading scale proposed by **Srivastava *et al*., (2002)** was followed. Brinjal plants were also observed for phytotoxic symptoms (If any) such as chlorosis, necrosis, scorching, epinasty and hyponasty on 1, 3, 5, 7 and 10 days after treatment of *Bacillus subtilis.* The data on disease incidence and other biometrics were analyzed by using standard statistical techniques.

Bio-efficacy of *Bacillus subtilis* against *Pythium aphanidermatum* under field conditions

A field experiment was conducted at **Research farm of C.S.A.U.A&T, Kanpur** to find out the effect of *Bacillus subtilis* as seedling treatment against *P. aphanidermatum* causing damping off on Brinjal crop in plot size 6.75 X 3.60 mts. (24.3 sq.m. per treatment) in Randomized Block Design (RBD) with five treatments and three replications. The Brinjal seedlings (var. J-2) treated with liquid formulation of *Bacillus subtilis (9 X10^8 cfu/ml)* @ 5 ml/kg seed, 10 ml/kg seed, 20ml/kg seed were sown in line in hot spot area (sick soil). The treated seedlings were kept for 24 hrs in shade and then sown. Carbendazim (Bavistin 50% WP) @ 2g/kg of seed was treated and used for comparison and an untreated control was also maintained. The crop was maintained with judicial irrigation and all the agronomic practices and fertilizer. Effect of *Bacillus subtilis* on root length, shoot length, vigour index, yield/treatment and total leaf protein of brinjal crop was estimated. Seedling vigour index was calculated by using the formula as described by **Baki and Anderson (1973).**

Vigour index = (Mean root length + Mean shoot length) X Germination (%)

Estimation of total Protein of brinjal plants treated with different concentration of *Bacillus subtilis* through Lowry Method

Total protein extraction

Total leaf protein was extracted using method developed by Goggin et al., (2011). 0.5g leaves of treated brinjal plants were frozen by liquid nitrogen, grinding to a fine powder using mortar and pestle then transferred to a fresh centrifuge tube. Two ml of extraction buffer (Tris-HCl 1M, pH 8, EDTA, 0.25), SDS, 10%, glycerol, 50%) was added and mixed well. The content of the tubes were centrifuge at 12000 rpm for 20 min at 4°C. After centrifugation process supernatant was discarded. Mixed the pellets with 1ml of sample buffer (80% Acetone, 0.07% β- mercaptoethanol and 2mM EDTA) and centrifuged at 12000 rpm for 5 minutes. The process was repeated until all chlorophyll removed. Mixed clear pellet with milli Q water and stored at -20°C. Protein concentration of all the samples was determined using Lowry assay (*Lowry* et al.,1951).

(ii) Protein Quantification

For quantification of protein content 1mg/ml of BSA standard was used. Different dilutions of the standard were made. To each tube of standard and sample 2ml of complex forming reagent was added and kept for 10 minutes at room temperature. After 10 minutes of incubation period, 0.2ml of Folin-Ciocalteu reagent solution was added to each tube and incubated for 20-30 minutes at room temperature in dark. After incubation period sample absorbance was taken at 660nm by using spectrophotometer (Bio-Rad). Calibration curve was constructed by plotting absorbance reading on Y

axis against standard protein concentration (mg/ml) on X axis. Sample concentration was calculated using standard graph as a reference.

Results

To obtained pure cultures of *P. aphanidermatum* from infected brinjal plants, isolations were made on PDA. Pure culture of fungal pathogen were maintained on Potato Dextrose Agar Medium using hyphal bit method (Trivedi and Gurha, 2007). The pathogen was identified on the basis of cultural and morphological characters. Based on the cultural and morphological characters on PDA, isolates was identified as *P. aphanidermatum*

Fig. 1: *P. aphanidermatum* (A) Growth on PDA medium (B) Microscopic observation at 40x

The culture was found to be *Pythium aphanidermatum* by the characteristics such as colony color, sporangia consisting of a terminal complex of swollen hyphal branches of varying length; oogonia terminal, globus smooth with 24-29 µm diameter. Antheridia terminal and intercalary of broadly sac shaped**.**

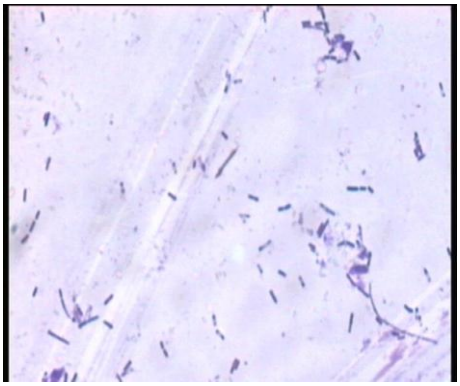
Based on the disease severity the isolate was found to be highly pathogenic with 97.5 percent damping-off incidence on brinjal . Further studies were conducted with this isolate of *P. aphanidermatum.*

Fig. 2 : Pathogenicity test of *Pythium aphanidermatum* on Brinjal seedlings (A) Untreated control (B & C) Diseased seedlings

Using serial dilution isolation techniques 50 distinct colonies were subculture on Nutrient Agar on the basis of colony morphology. The isolates which were found to be Gram-positive, rod-shaped,

endospore-forming, motile when observed through sterio-binocular were considered as *Bacillus subtilis*. Moreover, they did not grow under strictly anaerobic conditions.The solitary colonies of the isolate on Nutrient agar after 3 days of incubation were small, round, opaque, spreading or non-spreading dull, circular with an irregular circumference and cream-colored. No diffusible pigment was produced. It was thus preliminarily characterized as members of the genus *Bacillus* which was further identified as *Bacillus subtilis.*

A



B

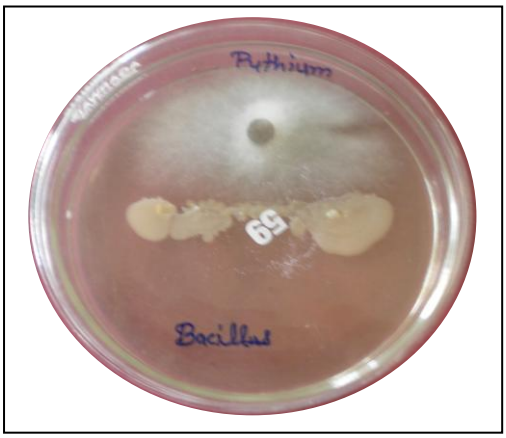
Fig. 3 Bacillus subtilis (A) Growth on Nutrient agar (B) Microscopic observation at 40x

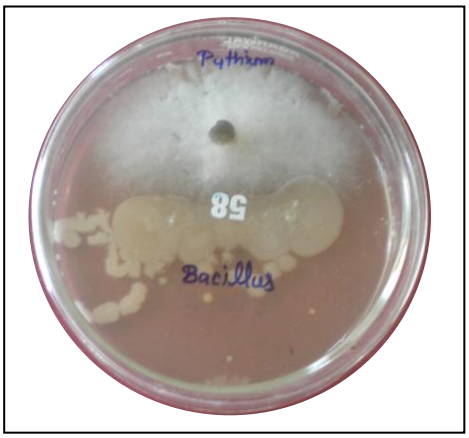
The results of *in vitro* studies revealed that *Bacillus subtilis* significantly reduced the mycelial growth of *P. aphanidermatum* with 71.1 per cent reduction @10 ml/l (T_2) over control. Treatment T_3 and T_1 also reduced the growth of the pathogen as 66.6 and 61.1% respectively over control. The chemical treatment reduced the mycelial growth of the pathogen by 44.4 per cent (Table 1).

Bacillus subtilis **(5ml/l) +*P.aphanidermatum***

Bacillus subtilis **(10ml/l) + *P.aphanidermatum***

Bacillus subtilis **(20ml/l) + *P. aphanidermatum***





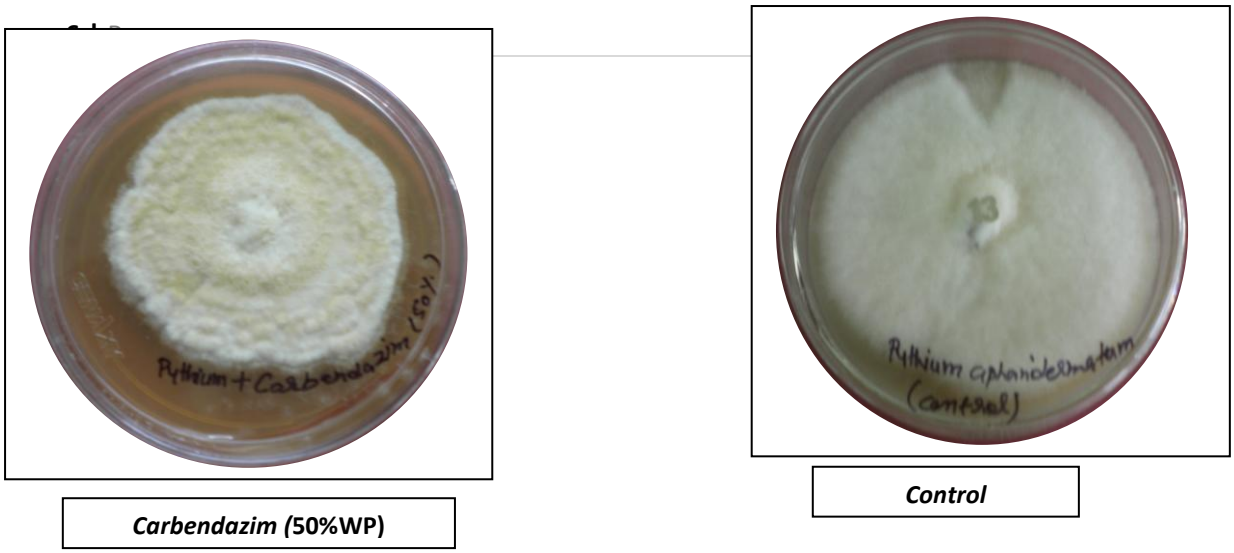


Fig. 4 Antagonism of ***Bacillus subtilis*** **against** ***P. aphanidermatum*** **(Dual Culture)**

Table1. ***In vitro*** **efficacy of** ***Bacillus subtilis*** **against** ***P. aphanidermatum***

S. No.	Treatment details	Radial growth of *P. aphanidermatum* (mm)*	Per cent growth reduction
T_1	*B. subtilis* @5ml/l seed	35.0	61.1
T_2	*B. subtilis* @10ml/l	26.0	71.1
T_3	*B. subtilis* @20ml/l	30.0	66.6
T_4	Carbendazim 50% WP @0.1 % conc.	50.0	44.4
T_5	Untreated	90.00	-
	SE	**0.23**	
	CD (p=0.05)	**0.50**	

* Mean of three replications

The results presented in table 2 revealed the efficacy of *B. subtilis under glass house condidtions*. Among the various dosage levels treatment T_2 (Seedling treatment with *B. subtilis* @10ml/kg seed) recorded the maximum germination (83.33%) and significantly reduced the pre emergence and post emergence damping off disease on brinjal to the minimum (16.6 and 20.0 per cent pre and post

emergence damping off respectively). Seedling treatment with *B. subtilis* @20 ml/kg of seed also recorded significant results with that of T_2. Based on the results treatment T_2 was rated as "Highly Efficient (HE)". The maximum of 41.6 and 71.4 per cent pre and post emergence damping off was observed in untreated control.

Table 2. Bio efficacy of *Bacillus subtilis* for the management of *P. aphanidermatum* causing damping off on Brinjal (Pot tria)

Tr. No	Treatment details	Germination (%)	Damping off incidence (%)*		Disease control (%)		Rating of the bio-efficacy of *B. subtilis*
			Pre emergence	Post emergence	Pre emergence	Post emergence	
T_1	Seed treatment with *B. subtilis* @5ml/kg seed	68.3	31.6	46.3	24.0	35.1	Moderately Efficient (ME)
T_2	Seed treatment with *B. subtilis* @10ml/kg seed	83.3	16.6	20.0	**60.0**	**71.9**	Highly Efficient (HE)
T_3	Seed treatment with *B. subtilis* @20ml/kg seed	75.0	25.0	33.3	39.9	53.3	Efficient (E)
T_4	Seed treatment of Carbendazim 50% WP @ 2g/kg seed	66.6	33.3	50.0	19.9	29.9	--
T_5	Untreated Control	58.3	41.6	71.4	--	--	--
	SE	**0.16**	**0.15**	**0.14**			
	CD (p=0.05)	**0.34**	**0.33**	**0.34**	--	--	--

Seed treatment with *B. subtilis* @ 5ml/kg seed (T1)

Seed treatment with *B. subtilis* @10ml/kg seed (T2)

Seed treatment with *B. subtilis* @20 ml/kg seed (T3)

Seed treatment of Carbendazim 50% WP @2g/kg seed (T4)

Fig. 4 Effect of different concentration of *Bacillus subtilis* against *P. aphanidermatum* (Pot experiments)

The results of the experiment conducted under field conditions revealed that all the treatments with *Bacillus subtilis* significantly reduced the pre emergence and post emergence damping off disease on brinjal when compared to control **(Table 3)**. Among the various treatments, Seed treatment with liquid formulation of *Bacillus subtilis*@ 10ml/kg seed recorded the least disease incidence of 7.40 and 8.00 per cent pre and post emergence damping off respectively followed by the dosage levels with 20ml/kg seed and 5ml/kg of seeds in the decreasing order of merit as against pre and post emergence damping off of 33.3 and 50.0 per cent respectively in control. Further, the dosage levels of 10ml and 20ml were significantly superior in their effect in reducing the pre emergence as well as post emergence damping off. Hence, the dosage level with 10ml/kg of seeds itself is enough to manage the damping off disease successfully **(Table 3)..**

Table 3. Bio efficacy of *Bacillus subtilis* for the management of *P. aphanidermatum* causing damping off on Brinjal under field conditions. (Rabi Season)

Tr. No	Treatment details	Damping off incidence (%)*		Disease control (%)	
		Pre emergence	Post emergence	Pre emergence	Post emergence
T1	Seed treatment with *B. subtilis* @ 5ml/kg seed	18.5	22.7	44.4	55.0
T2	Seed treatment with *B. subtilis* @ 10ml/kg seed	7.40	8.00	**77.7**	**84.0**
T3	Seed treatment with *B. subtilis* @ 20ml/kg seed	11.1	12.5	66.6	75.0
T4	Seed treatment of Carbendazim 50% WP @ 2g/kg seed	25.9	35.0	22.2	30.0
T5	Untreated Control	33.3	50.0	-	-

	SE **CD (p=0.05)**	**0.18** **0.43**	**0.22** **0.52**	-	-

Table 4. Effect of seed treatment with *Bacillus subtilis* on the biometrics of Brinjal crop under field conditions (Rabi Season)

Tr. No	Treatment details	Germination (%)*	Shoot length (cm)*	Root length (cm)*	Vigour Index	Yield Mt/ha*
T1	Seed treatment with *B. subtilis* @5ml/kg seed	81.48	54.0	21.3	6135.44	15.65
T2	Seed treatment with *B. subtilis* @10ml/kg seed	**92.59**	**59.3**	**24.3**	**7740.52**	**22.67**
T3	Seed treatment with *B. subtilis* @20ml/kg seed	88.88	55.3	21.6	6834.87	20.10
T4	Seed treatment of Carbendazim 50% WP @ 2g/kg	74.07	51.0	15.6	4933.06	12.85
T5	Untreated Control	66.66	50.3	13.3	4239.57	8.09
	SE **CD (p=0.05)**	**0.21** **0.49**	**0.20** **0.48**	**0.20** **0.46**	-	**-**

Table 5. Estimation of protein in Brinjal plants treated with different concentration of *Bacillus subtilis* .

Tr. No.	Treatment details	Protein concentration (mg/ml)	
		Field trial	Pot Trial
T_1	Seed treatment with *B. subtilis* @5ml/kg seed	0.34	0.12
T_2	Seed treatment with *B. subtilis* @10ml/kg seed	**0.44**	**0.30**
T_3	Seed treatment with *B. subtilis* @20ml/kg seed	0.35	0.26
T_4	Seed treatment of Carbendazim 50% WP @ 2g/kg seed	0.30	0.07

T_5	Untreated Control	0.18	0.06

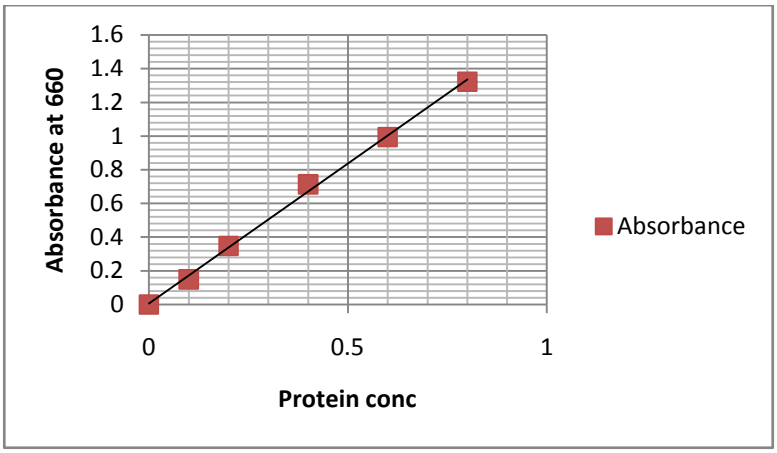


Fig. 5 BSA standard curve

Discussion

Among the various diseases; Damping-off caused by *Pythium aphanidermatum* is one of the most widely spread and prevalent throughout the country causing considerable loss in India.

It is extremely difficult to control soil-borne fungi via conventional strategies such as the use of synthetic fungicides, etc. Since their spores are able to survive for many years in the soil, biological control strategies for this pathogen should, therefore, be carefully selected and handled in an eco-friendly way instead of using chemical fungicides. The application of microorganisms as biocontrol agents is important, since they may increase beneficial microbial activity which extends for a long period of time. A group of bio-fertilizers, in dried powder formulations, also known as plant growth-promoting rhizobacteria (PGPR), have been applied to seeds and soil successfully for a number of years **(Kloepper et. al., 1990).** Root-colonizing plant-beneficial bacteria and fungi may provide good protection for plants against root-attacking pathogens **(Defago and Haas, 1990).** In the last decade, many strains of plant growth-promoting rhizobacteria were reported to induce systemic resistance against a broad spectrum of soil-borne and foliar pathogens . The plant and bacterial interactions in the rhizophere are important for plant health and resistance to disease **(Kloepper et. al., 1990)**..

As per the screening test, *Bacillus subtilis* has shown the best antagonistic behaviour against *Pythium aphanidermatum* in comparison of carbendazim. Similar results were also reported by **Smith & Saddler, 2001, Jayaraj and Radhakrishnan 2008, Kabdal *et al*., 2010**. *Bacillus subtilis* proved to be a dominant biological control agent against *Pythium aphanidermatum.*

Bacillus subtilis treated plants also showed the highest germination percentage, plant height, root length, and yield per treatment as well as total protein. Similar results on increased plant growth due to application of antagonistic bacteria was reported by **Broadbent *et al.*, 1977 and Turner and Backman,1986**. The increase in bio-matter production may be due to the production of plant growth promoters or through indirect stimulation of nutrient uptake and by producing antibiotics to protect plants from deleterious rhizosphere organisms. Therefore, the antagonist *Bacillus subtilis* is chosen to be the most promising bio-control agent against for *Pythium aphanidermatum* for management of damping off disease. On the basis of present study *Bacillus subtilis*, might be exploited for sustainable disease management programs to save environmental risk.

From the findings of the present investigation it can be concluded that *Bacillus subtilis*, @10ml/l showed promising performance against *Pythium aphanidermatum* among all the treatments.

The inhibitory effect of *Bacillus subtilis* on plant pathogenic fungi has been frequently reported in laboratory, greenhouse, and field studies by various workers **(Pusey *et al*., 1984, Cubeta *et al*., 1985,**

Bettiol *et al*., 1990). Besides the anti-fungal effects, some compounds produced by *B. subtilis* may also act as plant growth promoters **(Compant et al., 2005)**. Sometimes *B. subtilis* secretes some toxins and enzymes injurious to pathogenic organisms. Moreover, it can directly parasitize other soil borne pathogens. This mycoparasitism might be the reason of controlling damping off pathogen in brinjal. This bio-control agent could be used as an eco-friendly approach and may be advised to the farmer for profitable organic farming.

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