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BIO-EFFICIENCY OF FLUORESCENT *PSEUDOMONAS* SPP. AND *TRICHODERMA* SPP. ON MUSTARD (*BRASSICA NIGRA* (*L.*) *KOCH*) DEVELOPMENT AND DISEASE INFECTION

SUMAIRA IQBAL[1], NOUREEN REHAN[1], TAYYAB RAZA FRAZ[2] AND HAFZA ASMA SHAFIQUE[1*]

[1]*Agricultural Biotechnology and Phytopathology Laboratory, Department of Botany University of Karachi, Karachi-75270, Pakistan*
[2]*Department of Statistics, University of Karachi, Karachi--75270, Sindh, Pakistan*
**Corresponding author's email: h.a.shafique@uok.edu.pk*

ABSTRACT

Endophytic bacteria are extremely beneficial to the host plant. Bacteria live in root tissues, where they absorb nutrients like phosphate and nitrogen, defend host plants against diseases, and promote plant growth. This study centered on the efficiency of *Pseudomonas aeruginosa* and *Trichoderma* spp. in vivo by perspective its result on growth performance, infection percentage of root rotting fungi and effect on biochemical activities of mustard (*Brassica nigra*). Five strains of *Fluorescent Pseudomonas* were provided by Biological Research Centre, University of Karachi and two *Trichoderma* spp. were isolated from wild plants. In vivo, after application of five strains of *Pseudomonas* spp., *Trichoderma* spp. and topsin showed great impact on mustard plant (*Brassica nigra)* growth by increasing root length and shoot length. Significant differences in chlorophyll, carbohydrate and protein contents were found in mustard plants inoculated with *Fluorescent Pseudomonas* strains and *Trichoderma* spp., as well as fungicide, as compared to control.

Key-words: Endophytes, Biocontrol agents, Mustard plant, Agriculture damages, Phyto-pathogens, Root rotting fungi

INTRODUCTION

Biological control by competing microorganisms is an alternative non-chemical strategy for controlling plant diseases. *Pseudomonas as well as Trichoderma* species have been documented for their ability to diminish plant illnesses caused by pathogenic fungi, and their importance as potential hostile microbes has expanded dramatically (Parewa *et al*., 2014).

The capacity of *Pseudomonas fluorescens* to prevent a range of soil-borne and foliar diseases has piqued researchers' curiosity. Several plant infections have been successfully treated biologically using *Pseudomonas fluorescens* (Rolli *et al*., 205), and using PGPR strains, particularly those from the genus *Pseudomonas*, is a viable substitute for chemical pesticides in the control of plant illnesses (Chen *et al*., 2017). The fundamental mechanism is bacterial stimulation of plant host defence mechanisms, which inhibits the pathogen indirectly. *Pseudomonas* might indirectly decrease fungal infections by releasing siderophores to scavenge iron in the rhizosphere environment, which may trap remnants of insoluble iron and form stable complexes (De Zoysa *et al*., 2012). *Trichoderma* spp.'s antagonistic behaviour have proven that they parasitize a variety of foliar and soil-borne diseases. This biocontrol approach includes myco-parasitism, antibiotics, and resource competition. Furthermore, it induces defensive or symbiotic resistance in plants (Naher *et al*., 2014).

Because of its gastronomic and therapeutic capabilities, *Brassica nigra* (Black mustard) shows a significant role in worldwide agribusiness, agronomy, wellbeing, and healthiness perspectives. The *Brassica nigra* plant is moreover grown to provide lubricant for modern needs and a nutritious seed meal. A seed essentially holds oligosaccharides having a place with the raffinose family; amino acids; unsaturated fats, for example, palmitic, stearic, oleic, linoleic, linolenic, eicosenoic and erucic acids; nutrients; minerals (generally iron); hostile to dietary variables (specifically, chemical inhibitors); glucosinolates; and an extensive variety of phenols. Seeds of *Brassica nigra* (Black mustard) have been validated to exhibit anti-diabetic, anti-convulsant, anti-thrombotic, anti-bacterial, and anti-fungal properties, also immune-modulatory and incendiary properties (De Zoysa *et al*., 2012).

The goal of this study was to see how *Pseudomonas aeruginosa* and *Trichoderma* spp. performed in vivo by looking at growth performance, infection percentage of root rotting fungi, and effect on biochemical activities of mustard.

MATERIALS AND METHODS

Collection Of Endophytic *Fluorescent Pseudomonas* (Pgpr) and *Trichoderma* spp.

Five different strains of Endophytic *Fluorescent Pseudomonas* and two different species of *Trichoderma* were obtained from "Agricultural Bio-technology and Phyto-pathology Laboratory, Biological Research Centre (BRC), Botany Department, University of Karachi"

Population of Pgpr and Antagonistic Fungi

Colony forming unit per ml in suspension of Fluorescent *Pseudomonas*:

The dilution plate approach was applied to find out the population of bacteria. Liquid broth of biological antagonists were prepared. One mL solution was added to nutrient agar medium, and it was cultured for three to seven days at 28ºC. The dilution factor was used to multiply and count the bacteria growing on plates, yielding cfu/mL of bacteria (Inam-ul-Haq *et al.*, 2012).

$$\text{Colony forming unit (cfu) /mL} = \frac{\text{Number. of colonies counted on plate} \times \text{dilution}}{\text{Volume of sample}}$$

Number of spores / mL in liquid suspension of *Trichoderma* spp.

After making liquid suspension of fungal antagonist, for population of fungal spores, haemocytometer is used. 1ml suspension was poured on glass slide (haemocytometer), place the coverslip on haemocytometer and observe the no. of spores under compound microscope. Count the no. of viable spores.

$$\text{Number of viable cells count/mL} = \frac{\text{No. of cells counted} \times 10000 \times \text{dilution factor}}{\text{No. of large square corner counted}}$$

SCREEN HOUSE EXPERIMENT

Brassica nigra was used as test plant to carry out in-vivo studies. Experiment was carried out by the use of PGPR and *Trichoderma* spp. as soil drench for controlling the fungi that cause root infection in mustard (*Brassica nigra).* There were total 9 treatments;

1. Control
2. fungicide (Topsin)
3. *Trichoderma* strain 1
4. *Trichoderma* strain 2
5. PGPR 1
6. PGPR 2
7. PGPR 3
8. PGPR4
9. PGPR5

Each treatment was replicated 4 times so there were 36 replicates in total. Pots were randomized at BRC (Biological Research Centre), University of Karachi. After 45 days of experiment, plants were uprooted and then noted root length (RL), root weight (RW), shoot length (SL), and shoot weight (SW), fresh weight and number of leaves per plant.

Root Platting Method For Fungal Infection Percenage

After 45 days of planting, plants were carefully pulled at random from the soil and their roots were severed 3 cm above the earth; they were tagged and saved for subsequent study. The roots were cleaned with distilled water before being surface sterilized for 2 minutes in 70% ethanol and 2 minutes in 1% NaOCl. Applying the poured plate method, four roots were planted in each Petri dish. PDA was utilized as fungus culture media. At 28°C, the Petri dishes were incubated, for 48 - 72 hrs. & was then examined (Orole *et al*., 2011).

The infection percentage of fungal colonies on roots was estimated by formula:

$$\text{Infection percentage} = \frac{\text{Total number of plant roots infected by a pathogen}}{\text{Total no. of plant roots}} \times 100$$

BIOCHEMICAL PARAMETERS

Chlorophyll and carotenoid

Chlorophyll and carotenoid were extracted from leaves of mustard plant with 80% aqueous acetone and estimated by Lichtenthaler and Wellburn method (1983).

Carbohydrate

Carbohydrates content was analyzed from treated and control plant leaves samples with Anthrone reagent using the method of Yemm and Willis (1954).

Protein

The method of Lowry *et al*. (1951) is the most widely used procedure for the quantitative determination of protein.

EXPERIMENTAL RESULTS

Colony forming units/ml of bacteria:

Number of colonies in 5 different bacterial strains were calculated by dilution plate method. 0.5mL of bacterial suspension were transferred onto agar plates. No. of bacterial colonies observed in PGPR-1 were 0.000320 cfu/ml, in PGPR-2 0.000333 cfu/ml, in PGPR-3 0.000274 cfu/ mL, in PGPR-4 0.000308 cfu/mL and in PGPR-5 0.000329 cfu/mL **(Table 1).**

Number of spores per /mL of fungal suspension:

Number of spores/mL of fungal suspension was calculated by transferring 1mL of fungal suspension in haemocytometer and then at 40x, counted number of spores in *Trichoderma* spp. The number of spores present in T-1 were 14176000cfu/ mL and number of spores present in T-2 were 18520000cfu /mL. **(Table 1)**

EFFECT OF *FLUORESCENT PSEUDOMONAS* AND *TRICHODERMA* SPP. ON GROWTH PARAMETERS OF BLACK MUSTARD PLANT

At the time of seed sowing, 5 different strains of *fluorescent pseudomonas*, 2 different species of *Trichoderma* and topsin was drenched in soil along with control in which water was given. Maximum increase in shoot length as well as little increase in root length was observed as compared to root and shoot weight. Significant increase in shoot length of PGPR-3 plants was observed that was 46.1375cm, then in PGPR-4 36.0437 shoot length was observed, in PGPR-5 34.8225cm, and in T-1 shoot length was 34.206cm was noted. Whereas maximum root length was observed in T-1 which was 16.7375cm, in topsin 12.906cm, PGPR-2 11.925cm, PGPR-3 11.2875cm and in PGPR-4 root length was observed 10.7375cm. Very small amount of increase in shoot length was observed in PGPR-3 1.8056g, in PGPR-2 1.6793g and in T-1 shoot weight observed was 1.5037g. Maximum root weight was observed in topsin 0.3325g, in PGPR-1 0.3325g and in PGPR-3 0.1725g. **(Plate 1-4; Fig. 1, 2, 3, 4)**

INFECTION PERCENTAGE OF ROOT ROTTING FUNGI BY USING ROOT PLATING METHOD

The infection percentage of root infecting fungi on roots of black mustard was observed by using root plating technique. In different treatments that were 5 strains of *Fluorescent Pseudomonas*, *Trichoderma* spp. and topsin, infection percentage % were different like in control, 75% *Fusarium solani* and 25% *Fusarium oxysporum* caused infection on roots, in topsin 50% *Macrophomina phaseolina* and 25% *Rhizoctonia solani* was observed, in PGPR-1 mycelium of 50% *Fusarium solani* and 25% *Fusarium oxysporum* was observed, in PGPR-2 roots were infected with 25% *Fusarium solani* and 25% *Rhizoctonia solani*, in PGPR-3 25% *Fusarium oxysporum* and 25% *Rhizoctonia solani* were observed, in PGPR-4 25% *Macrophomina phaseolina* and 25% *Fusarium solani* were present, in PGPR-5 25% *Rhizoctonia solani* was observed, in T-1 25% *Macrophomina phaseolina*, 25% *Fusarium solani* and 25% *Fusarium oxysporum* were observed and in T-2 roots were infected with only 25% *Fusarium oxysporum*. **(Fig. 3)**

BIOCHEMICAL TEST OF MUSTARD PLANT

Chlorophyll

High content of chlorophyll "a" was observed in PGPR-1, T-1, PGPR-4 and PGPR-5 which were 0.00843mg/g, 0.00417mg/g and 0.00396mg/g and 0.00367mg/g. Maximum amount of chlorophyll "b" was present in PGPR-2, PGPR-1 and PGPR-5 and PGPR-4 which was 0.00416mg/g, 0.00393mg/g and 0.00206mg/g and 0.00147mg/g. In this study maximum amount of total chlorophyll "a" and "b" was observed in PGPR-1, PGPR-2, PGPR-5 and PGPR-4 which were 0.01236mg/g, 0.00622mg/g, 0.00573mg/g and 0.00543mg/g. **(Fig. 4,5,6)**

Table 1. Colony forming unit (CFU) /mL of F*luorescent Pseudomonas* and number of spores in *Trichoderma* spp.

SNO. #	*PSEUDOMONAS SPP. and TRICHODERMA* SPP.	COLONY FORMING UNIT (CFU)/mL
1	PGPR-1	0.000641
2	PGPR-2	0.000666
3	PGPR-3	0.000054
4	PGPR-4	0.000606
5	PGPR-5	0.000479
6	T-1	14176000
7	T-2	18520000

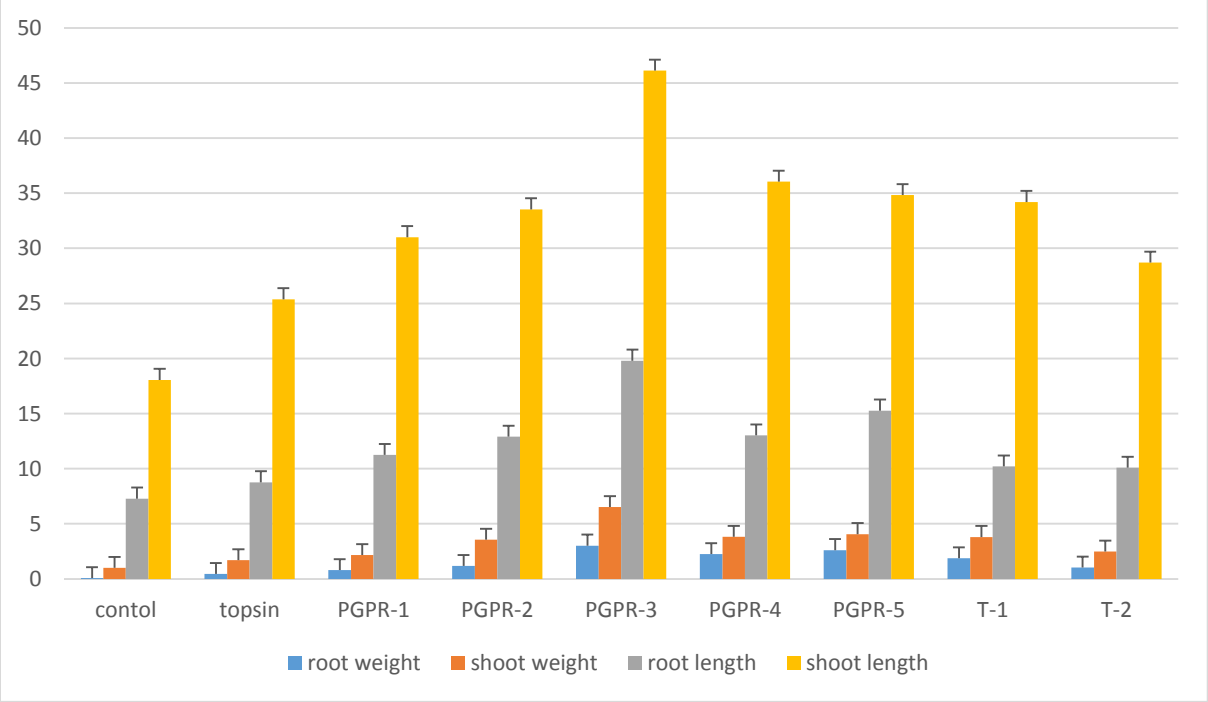

Fig. 1: Effect of *Fluorescent Pseudomonas* spp., *Trichoderma* spp. as soil drench on growth parameters of Black mustard plant. Bar graph represents means of four replicates of each treatment. Error bars represents standard deviation. Statistical difference determined by analysis of variance followed by the same letter in each bar are not significantly different ($p<0.05$) according to Duncan's multiple range test.

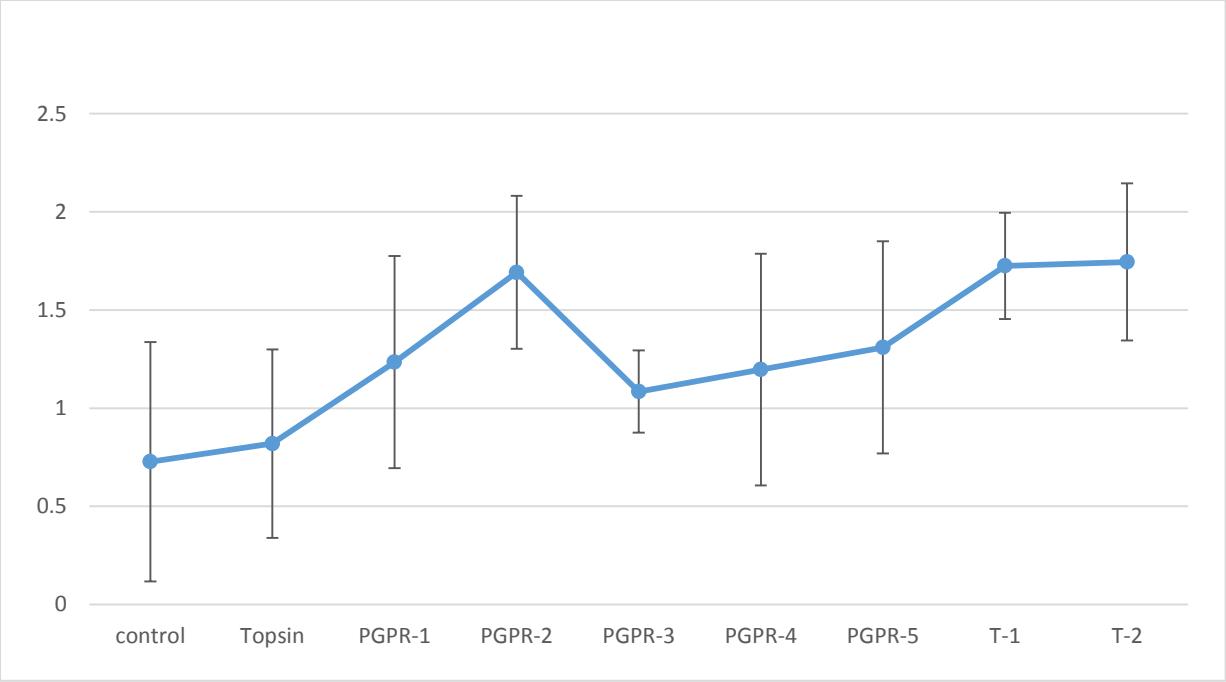

Fig. 2. Effect of endophytic *Fluorescent Pseudomonas* spp., *Trichoderma* spp. and topsin as soil drench on the leaves fresh weight. Bar graph represents means of four replicates of each treatment. Error bars represents standard deviation. Statistical difference determined by analysis of variance followed by the same letter in each bar are not significantly different ($p<0.05$) according to Duncan's multiple range test.

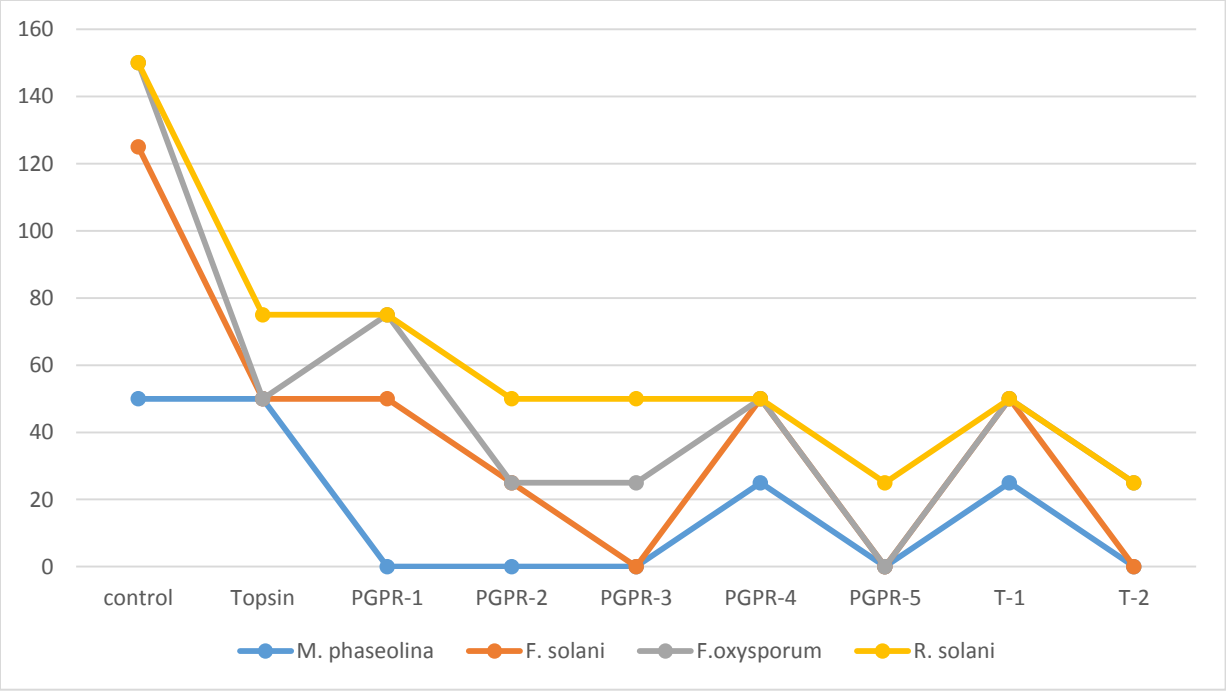

Fig. 3. Effect of *Fluorescent Pseudomonas* spp., *Trichoderma* spp. and topsin as soil drench on the infection percentage % of roots of black mustard plant.

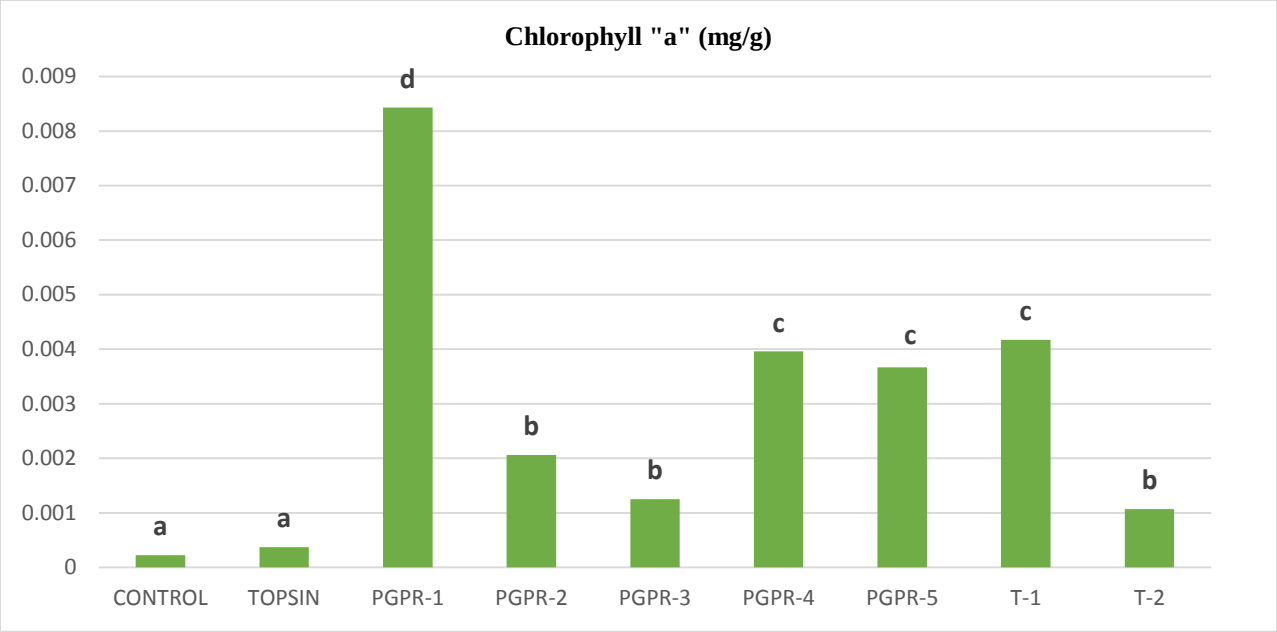

Fig. 4. Effect of endophytic *Fluorescent Pseudomonas* spp., *Trichoderma* spp. and topsin as soil drench on the chlorophyll "a" content. Bar graph represents means of four replicates of each treatment. Statistical difference determined by analysis of variance followed by the same letter in each bar are not significantly different ($p<0.05$) according to Duncan's multiple range test.

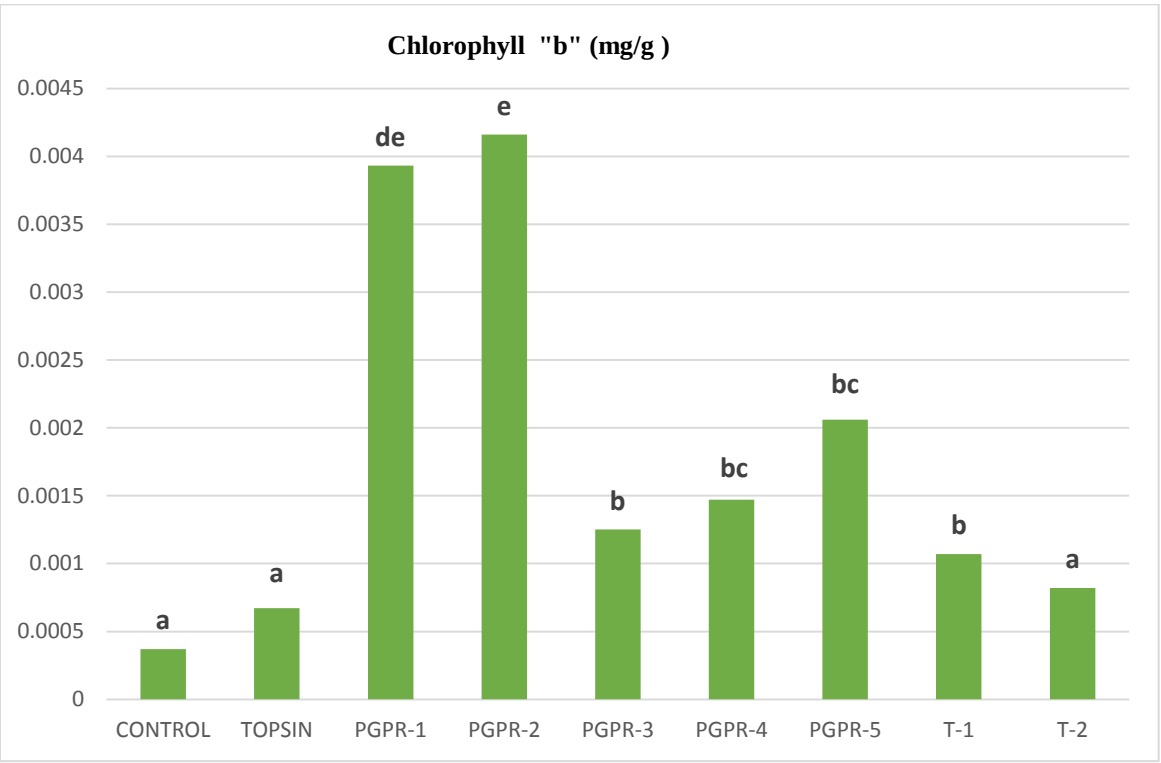

Fig. 5. Effect of endophytic *Fluorescent Pseudomonas* spp., *Trichoderma* spp. and topsin as soil drench on the chlorophyll "b" content. Bar graph represents means of four replicates of each treatment. Statistical difference determined by analysis of variance followed by the same letter in each bar are not significantly different ($p<0.05$) according to Duncan's multiple range test.

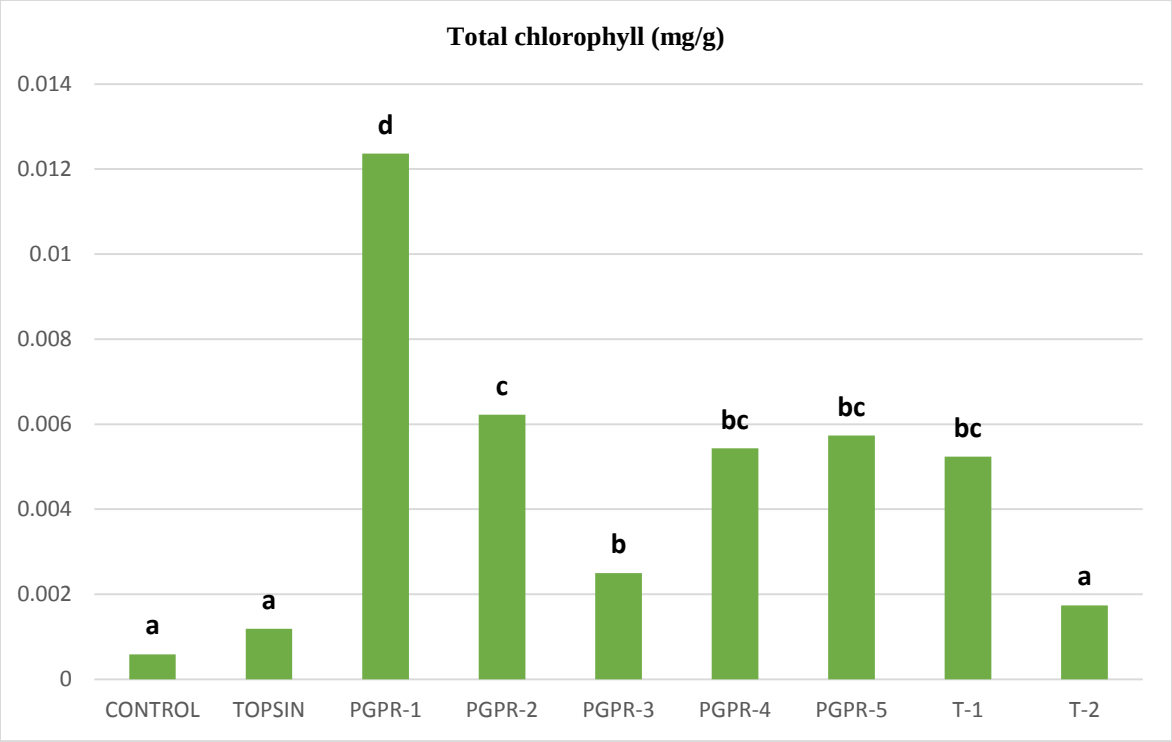

Fig. 6. Effect of endophytic *Fluorescent Pseudomonas* spp., *Trichoderma* spp. and topsin as soil drench on total chlorophyll content in mustard plant. Bar graph represents means of four replicates of each treatment. Statistical difference determined by analysis of variance followed by the same letter in each bar are not significantly different ($p<0.05$) according to Duncan's multiple range test.

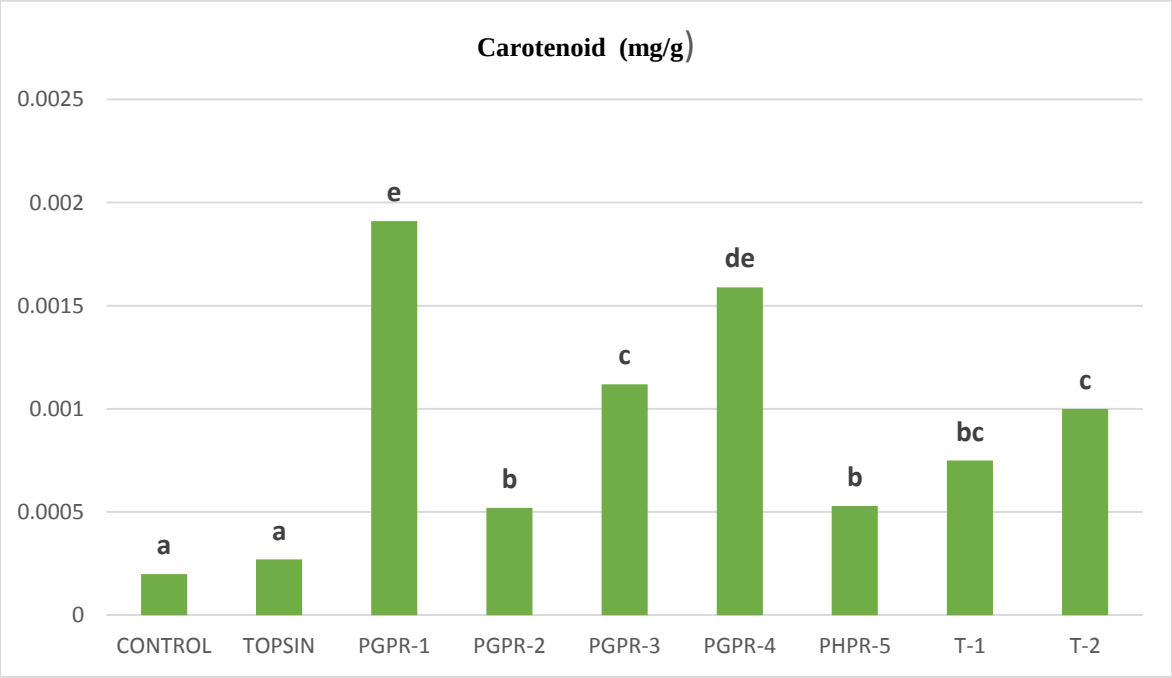

Fig. 7. Effect of endophytic *Fluorescent Pseudomonas* spp., *Trichoderma* spp. and topsin as soil drench on the carotenoid content. Bar graph represents means of four replicates of each treatment. Statistical difference determined by analysis of variance followed by the same letter in each bar are not significantly different ($p<0.05$) according to Duncan's multiple range test.

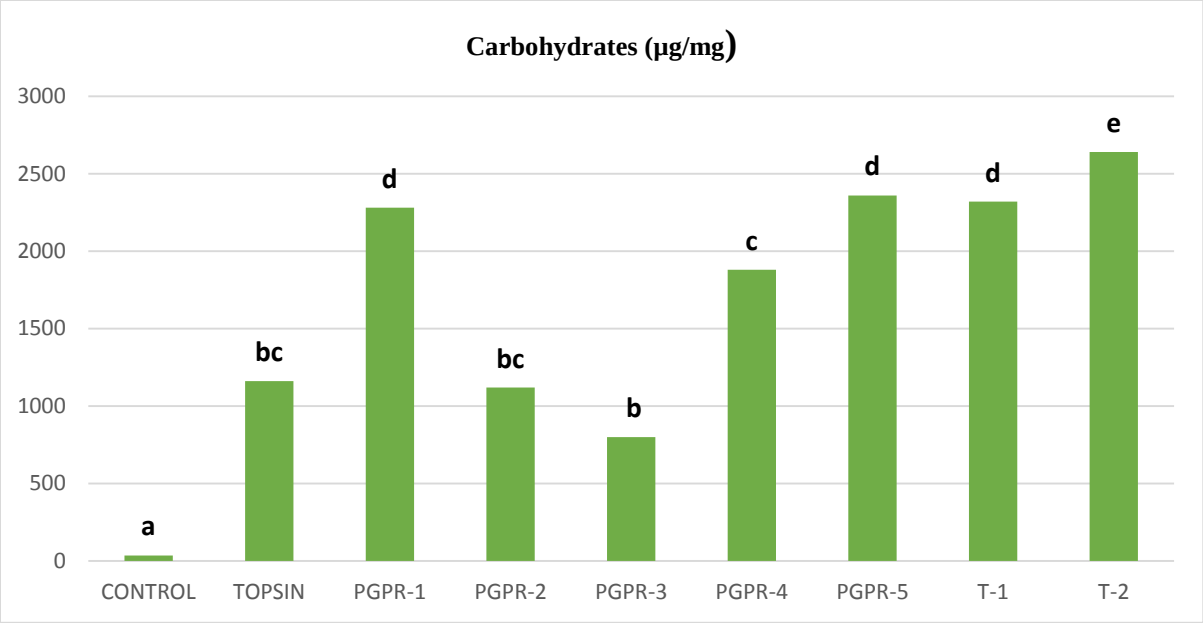

Fig. 8. Effect of endophytic *Fluorescent Pseudomonas* spp., *Trichoderma* spp. and topsin as soil drench on the carbohydrates content. Bar graph represents means of four replicates of each treatment. Statistical difference determined by analysis of variance followed by the same letter in each bar are not significantly different ($p<0.05$) according to Duncan's multiple range test.

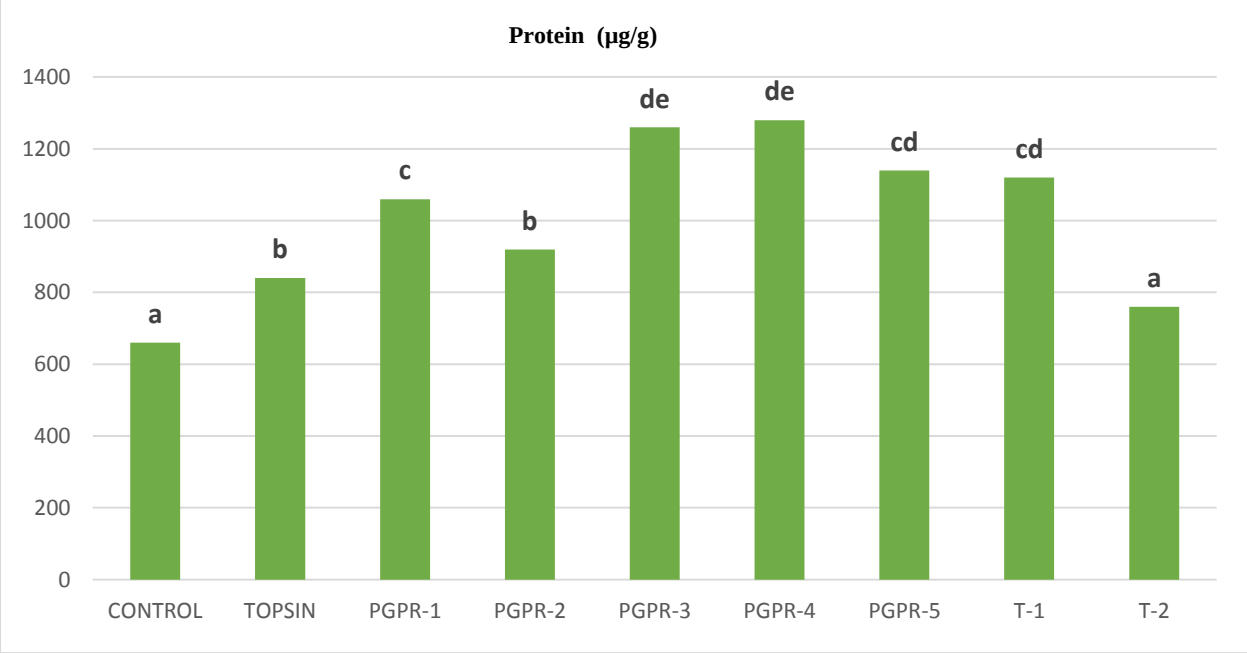

Fig. 9. Effect of *Fluorescent Pseudomonas* spp., *Trichoderma* spp. and topsin as soil drench on the protein content. Bar graph represents means of four replicates of each treatment. Statistical difference determined by analysis of variance followed by the same letter in each bar are not significantly different ($p<0.05$) according to Duncan's multiple range test.

Plate 1. Effect of *Fluorescent Pseudomonas* PGPR-1 and PGPR-2 on the growth of Black mustard plant.

Plate 2. Effect of *Fluorescent Pseudomonas* PGPR-3 and PGPR-4 on the growth of Black mustard plant.

Plate 3. Effect of *Fluorescent Pseudomonas* PGPR-5 on the growth of Black mustard plant.

Plate 4. Effect of *Trichoderma* strains T-1 and T-2 on the growth of Black mustard.

Carotenoid

Greater amount of carotenoid was observed in PGPR-1, PGPR-3 and T-2 which was 0.00191mg/g, 0.00478mg/g, 0.00443mg/g and 0.00157mg/g **(Fig. 7).**

Carbohydrates

Maximum amount of carbohydrates was observed in T-2 which was 2640µg/ml, in PGPR-5 2360µg/ml, T-1 2320µg/ml, PGPR-1 which was 2280µg/ml and PGPR-4 1880µg/ml. **(Fig. 8)**

Protein

Significant amount of protein was observed in mustard plants treated with PGPR-4 1280µg/ml then in PGPR-3 1260µg/mL, PGPR-5 1140µg/mL, T-1 which was 1120µg/mL and in PGPR-1 1060µg/mL. **(Fig. 9)**

DISCUSSION

Plant Growth Promoting *Rhizobacteria*, mainly *Pseudomonas fluorescens*, are recognized as a vital micro-organism capable of promoting plant development and providing excellent disease control (Mathivanan *et al*., 2017). Because metabolites like siderophores, antibiotic, volatile organic compounds, HCN, enzyme and Phyto-hormones are synthesized, their potential as biocontrol agents has piqued researchers' interest. *Trichoderma* spp. are effective biocontrol agents for phytopathogenic fungi. They can influence resources and space indirectly by changing the environment or boosting plant growth, plant defence mechanisms, and antibiosis (Sharrma *et al*., 2008).

Plants treated to five strains of *Fluorescent Pseudomonas* and *Trichoderma* spp. had considerably increased root and shoot length. The longest root lengths were found in PGPR-3, PGPR-5, and PGPR-4. The maximum shoot length was measured in the PGPR-3, PGPR-4, and PGPR-5 strains. Maximum shoot and root weight was recorded in the PGPR-3, PGPR-5, and PGPR-5. The highest quantity of fresh leaf weight was found in T-2, T-1, and PGPR-2 in this investigation. *Pseudomonas fluorescens* has been recognized as a vital bacterium with the capability to stimulate plants development and strong disease control skills (Saharan *et al*., 2011). Siderophore, hydrocyanic acid (HCN), phytohormones, and other related activities such as phosphate solubilization, iron competition in the soil, and root colonization are all produced by these bacteria and have an impact on plant development (Bhatia *et al*., 2005). Endophytic bacteria promote plant development, solubilize phosphate, & give assailable nitrogen to plants (Rosenblueth and Martinez-Romero, 2006). Furthermore, the synthesis of phyto-hormones and enzymes convoluted in growth- regulator metabolism may be the origin of their plant -growth promoting activity (Trotel *et al*., 2008). Rhizobacteria that support plant growth either directly by moderating plant -hormone levels and indirectly by reducing the effects of numerous pathogens on plants progression in form of bio-control agent, roots colonizers and environmental defenders (Gupta *et al*., 2018).

Chlorophyll, a green substance found in plant cells, is essential to photosynthesis. It absorbs solar energy and uses it to produce carbohydrates from CO2 and water. Plants have two types of chlorophyll, chlorophyll "a" and chlorophyll "b," which both function as photoreceptors during photosynthesis (Karlidag *et al*., 2013). Chlorophyll has been proven to play an important function in the synthesis of ATP and the maintenance of critical plant components (Kochot *et al*., 1998). Chlorophyll molecules are essential for charge separation and electron transport in reaction centres, as well as solar energy gathering in photosynthetic antenna systems. The highest levels of chlorophyll "a" were found in PGPR-1, T-1, and PGPR-4, while the highest levels of chlorophyll "b" were found in PGPR-2, PGPR-1, and PGPR-5. Total chlorophyll "a" and "b" levels were highest in PGPR-1, PGPR-2, and PGPR-5. Strawberry plants' chlorophyll content increased considerably after PGPR inoculation (Karlidag *et al*., 2013).

Carotenoid is an auxiliary pigment that aids in photosynthesis in plants. Carotenoid concentrations were highest in PGPR-1, PGPR-4, PGPR-3, and T-2.Using multiple PGPR strain treatments, Singh *et al*., 2010 discovered that co-inoculation of *Azospirillum* + *Pseudomonas* under both normal and stressful conditions yielded the highest carotenoid levels. The crop grown in conjunction with plant-development rhizobacteria had the greatest carotenoid content (Mathivanan *et al*., 2017). Carbohydrate concentrations were highest in the PGPRs T-2, PGPR-5, PGPR-1, and T-1. Because increasing Zn levels increase the soluble carbohydrate content, Zn treatment appears to be connected with an increase in carbohydrate content. To increase the amount of proteins and carbohydrates, Sulphur and the minerals Zn, Mn, and Mo are added to PGPR (Singh *et al*., 2010). According to this study, these 5 fluorescent *Pseudomonas* and *Trichoderma* strains were effective against *Macrophomina phaseolina*, *Fusarium oxysporum*, and *Rhizoctonia solani*, but less effective against *Fusarium solani.* Plant growth parameters and physiological parameters were also increased. As a result, we can speculate that these strains will be exploited as a possible plant biocontrol agent.

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