

Decolourization and Detoxification of Azo dye, Malachite Green by *Pseudomonas monteilii* strain RZT1, a Bacterium Isolated from Textile Wastewater

ABSTRACT

In most parts of Bangladesh, indiscriminate discharge of industrial effluent has become a severe hazard for the agro-ecological ecosystem. Textile dyes that are released directly into the environment without proper treatment are a potential threat to living organisms. Therefore, this study was designed to isolate azo dye-degrading bacteria from textile wastewater and evaluate their ability to decompose reactive dyes into non-toxic products. The bacterial strain was identified as *Pseudomonas monteilii* strain RZT1 on the basis of 16S rDNA sequence. The bacterial strain exhibited good decolorization ability with yeast extract supplementation as cosubstrate in static conditions. The optimal condition for the decolorization of Malachite Green dye by *Pseudomonas monteilii* strain RZT1 were at pH 7.0 and 35°C. *Pseudomonas monteilii* strain RZT1 bleaching 84.8%, 75.4%, 63.4% and 45.5% of 100ppm, 200ppm, 300ppm and 400ppm dyes, respectively. We investigated the effects of dyes used in the textile industry on the seed germination of Five crops - Rice (*Oryza sativa*), Wheat (*Triticum aestivum* L.), Khesari (*Lathyrus sativus*), Mustard (*Brassica Nigra*) and Bitter Melon (*Momordica Charantia*). It was found that textile dye Malachite Green had negative effect on seed germination and seedling growth in test cultures. The harmful effects of dye on seed germination and early seedling growth parameters were increased with increase of dye concentration. However, treatment of the Malachite Green dye with isolated bacteria reduced the adverse effects of that dye on seed germination and seedling growth. Thus, it indicated the potentiality of bacterial isolate for bioremediation of textile effluents into a non-toxic form for plants.

Keywords: Decolourization, Detoxification, Azo dye, *Pseudomonas monteilii* strain RZT1, Seed germination

1. INTRODUCTION

Environmental pollution due to the release of a many types of azo dyes in industrial wastewater is a big problem these days. World every year azo dye production is estimated at one million tons, and more than 2000 azo compounds with different structures are currently used [1]. Azo dyes are used in some industries such as textiles dyestuffs, foodstuffs, cosmetics and printing paper. The textile industry is known to handle large amounts of water and various chemicals [2, 3]. High quality water is an important factor in many processes such as cleaning, rinsing, dyeing and washing [2]. Being an agricultural country, industrialization in Bangladesh is occurring in an expanding stage. Recently, the deterioration of water resources with the rapid growth of

industries (sugar, paper, tannery, textile and dyeing industries) in the country has come into the discussion. It generally disrupts the habitats of the living organisms when discharged into the environment without proper treatment [4]. The continuous irrigation of agricultural land with the effluent wastewater causes heavy metal accumulation in the grown crops [5]. When this effluent discharged into the watercourses, they restrict the light penetration and inhibit the activities of aquatic lives [6].

In addition, most areas of Bangladesh has an arid and semi-arid climate where there is water deficiency is a limiting factor for agricultural production. Thus, the treatment of such a volume of wastewater can help meet the irrigation needs of crops. The sacrifice of these colorful industrialists wastewater can be very dangerous for the receiving water resources, because these dyes in water absorb sunlight, thus reducing the intensity of light absorbed by water plants and phytoplankton, eventually decreasing photosynthesis and oxygenation of water reservoirs. These dyes are xenobiotics intrinsically and in some cases mutagenic and carcinogenic [7, 8]. The effects of these dyes have also been reported by some scientists [9, 10]. Therefore, wastewater contaminated with azo dyes appears as serious problems due to their negative impact on water ecosystems and people.

Increase competition for water and reduce freshwater resources poses a new one environmental management challenge. Industrial effluents often contain different toxic and toxic metals gas, and some organic and inorganic compounds [11]. Main major industries that cause water pollution are identified such as tanneries, chemical plants, refineries, sugar mills, textile dyeing and pulp industry [12]. Trash water in the textile industry is a complex mixture many pollutants ranging from heavy metal organochlorine waste related to dyes and dyeing processes [13]. Daily water consumption of a medium-sized textile mill production is about 8000 kg of lychee per day corresponding to about 1.6 million liters.. This water is used for irrigation purposes agricultural land causing various damages to plants and soil directly and indirectly. It also reduces soil fertility due to toxic substances. Most dye factories discharge their wastewater into the environment, after partial treatment or no treatment at all. Industrial wastewater contains various organic materials and inorganic chemical compounds. The presence of these chemical compounds might e harmful effect on the germination and growth of plants.

Effects of wastewater from the dyeing industry on plants have been studied by some workers [14-16]. However, the harmful effects of textiles wastewater from plants depends on the species, the stage of the plant's life cycle is affected by, and the types and concentrations of harmful substances in the effluent. Over the past three decades, some physics, chemical and biological bleaching methods have accepted by the paper and textile industries [17-20]. Various types microorganisms including bacteria, fungi, yeasts, actinomycetes and algae capable of breaking down azo dyes have been reported [17, 21-27]. Various physical methods can used to remove Azo dyes of wastewater. Some of these methods are effective but quite expensive because they create a significant amount of chemicals. In such situations, bioremediation can be a real hope. These methods have the advantage of ecology. So, the present study was designed for isolation of azo dye decoloration bacterial strains from textile wastewater. Since bacterial strains isolated from textile wastewater contaminated with local industrial dyes, they can easily adapt to the local environment. Therefore, these bacteria can be used to grow biological wastewater treatment system contaminated with azo dyes. In this study, an attempt was also made to recognize the effect of the textile wastewater on seed germination and seed development.

2. MATERIALS AND METHODS

2.1 Collection and Storage of the Sample:

Wastewater was collected from different textile dyeing industries in Sathia, Sirajgonj, Gazipur, Madhapdi, Narshingdhi, Bangladesh. Samples were taken from a variety of locations, such as drainage channels for stagnant textile dyeing wastewater. Samples were in the form of untreated liquid wastewater and untreated sludge. All samples were collected in sterile plastic bottles and polyethylene bags and stored at 4°C in the refrigerator for 24 h to avoid changes in their physicochemical properties.

2.2 Dyes and Culture Media:

Malachite Green dye purchased from DysinChem limit, Dhaka. All media components and chemicals used in studies of analytical quality.

The Malachite Green dye used in this study is of industrial origin.

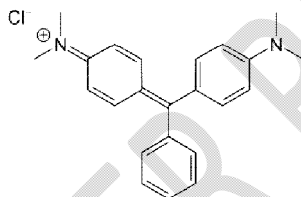


Fig 1: Malachite Green

2.3 Isolation and Screening of Dye decolorizing bacteria:

All samples (untreated textile wastewater) were used for isolation of bleaching bacteria from dyes by LuriaBertani enrichment culture technique (LB) medium modified with 20 ppm of test dye Malachite Green for microbial adaptation. Bacterial colonies showed a clear discolored area around them on LB agar were collected and cultured for 24 h in MS medium modified with 1 ml/1 TE dissolution. The growth of bacterial colonies was observed after 24 h of incubation at 35°C. Effect of dyes on the growth of bacterial strains was determined in MS medium supplemented with 20 ppm of azo Malachite Green dye.

2.4 Genomic DNA Extraction & 16S rDNA Gene Amplification:

Genomic DNA was extracted from dye decolourizing bacteria using CTAB method [28]. The PCR primers used to amplify 16S rDNA fragments were the bacteria-specific primers a forward primer 8F (5'-AGA GTT TGA TCC TGG CTC AG-3'; T_m: 61°C); and a reverse primer 806R (5'-GGA CTA CVS GGG TAT CTA AT-3'; T_m: 67.4°C). A total of 25 µl of reaction mixture consisted of – water 15µl, MgCl₂ 2.5µl, buffer 2.5, dNTPs 0.5µl, template 1µl, primer (forward 2 µl and reverse 2 µl). The PCR amplification was performed by Swift™ Minipro Thermal Cycler (Model: SWT-MIP-0.2-2, Singapore) using the following program: Denaturing at 95°C for 5 minutes, followed by 40 cycles of 40 seconds of denaturing at 95°C, 60 seconds of annealing at 65°C and 2 minutes of elongation at 72°C with a final extension at 72°C for 10 minutes. Then, the

PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products.

The amplified PCR product was purified using AccuPrep® Gel Purification Kit (Bioneer Company, Korea) according to the manufacturer's protocol. PCR amplified 16s rDNA of the isolates screened as submitted for automated sequencing (Applied Biosystems 3130) at the Center for Advanced Scientific Research (CARS) of the University of Dhaka, Bangladesh. The sequence generated from the automatic sequence of PCR amplified DNA analyzed by NCBI BLAST Program (<http://www.ncbi.nlm.nih.gov>) for discover a similar organism possible through association of similar sequences. Finally, the isolates were determined based on partial sequence alignment of 16S rDNA with sequences available in database.

2.5 Sequencing of 16S rDNA & BLAST Analysis:

The nucleotide sequence of the 16S rDNA was sequenced on both sides through the BigDye chain termination cycle sequencer (ABI) and the sequence is decoded on Dideoxy Sanger 3130XL String Genetic Analyzer (ABI). The final method is then assembled by the Cap3 program for genetic sequencing. Gene sequence was determined by looking for similarities in the database via BLASTn for 16S rDNA.

2.6 Effect of Different Parameters on Process of Dye Decolorization:

Effect of initial dye concentration on discoloration of Malachite Green dye by isolated bacteria was tested after 96 h incubation as described previously [23]. In short, to examine the effect of different dye concentrations on color change, MS medium supplemented with 100, 200, 300 and 400 ppm Malachite Green dye was adjusted to pH 7. Then, the medium was inoculated with bacterial strains incubated at 35 °C for 192 h.

2.7 Measurement of Decolorization Efficiency:

The decrease in absorbance at absorption maxima (λ_{max}) was monitored using a UV-visible spectrophotometer to evaluate decolorization activity in terms of % decolorization. Uninoculated MS medium supplemented with corresponding dyes were used as a reference. At different time interval, 2 ml of sample was taken from reaction mixture and centrifuged at 10000 rpm for 10 min for biomass separation. The concentration of dye in the supernatant was determined by monitoring the absorbance at maximum absorption wavelength (λ_{max}) at 660 nm. Bleaching dosage was calculated according to the following formula

$$\text{Dye Decolourization (\%)} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

2.8 Seed Germination Test:

In this experiment, the effect of four concentrations of Malachite Green dye were assessed on the seed germination of five crops viz. Rice (*Oryza sativa*), Wheat (*Triticum aestivum* L.), Khesari (*Lathyrus sativus*), Mustard (*Brassica Nigra*) and Bitter Melon (*Momordica Charantia*). Healthy and uniform seeds were selected. Seeds were washed with tap water and with distilled water. After being treated with 0.2% mercuric chloride for 2 minutes, seeds were rinsed with

1	Sample 1	Water	3700	2300	6.3	710	270	35	Black	Foul
2	Sample 2	Sludge	4800	2100	6.8	750	280	35	Black	Foul
3	Sample 3	Water	4300	2500	7.4	620	250	35	Black	Foul
4	Sample 4	Sludge	4100	2700	6.0	785	310	35	Black	Foul

3.2 Isolation and Identification of Dye Decolorizing Bacteria:

Isolated bacterial strains identified by morphological and biochemical tests were subjected to 16S rRNA gene sequence analysis. Analysis of 16S rRNA gene sequence revealed that the isolate was *Pseudomonas monteilii* strain RZT1 (Accession Number: OM095453) (Fig. 2).

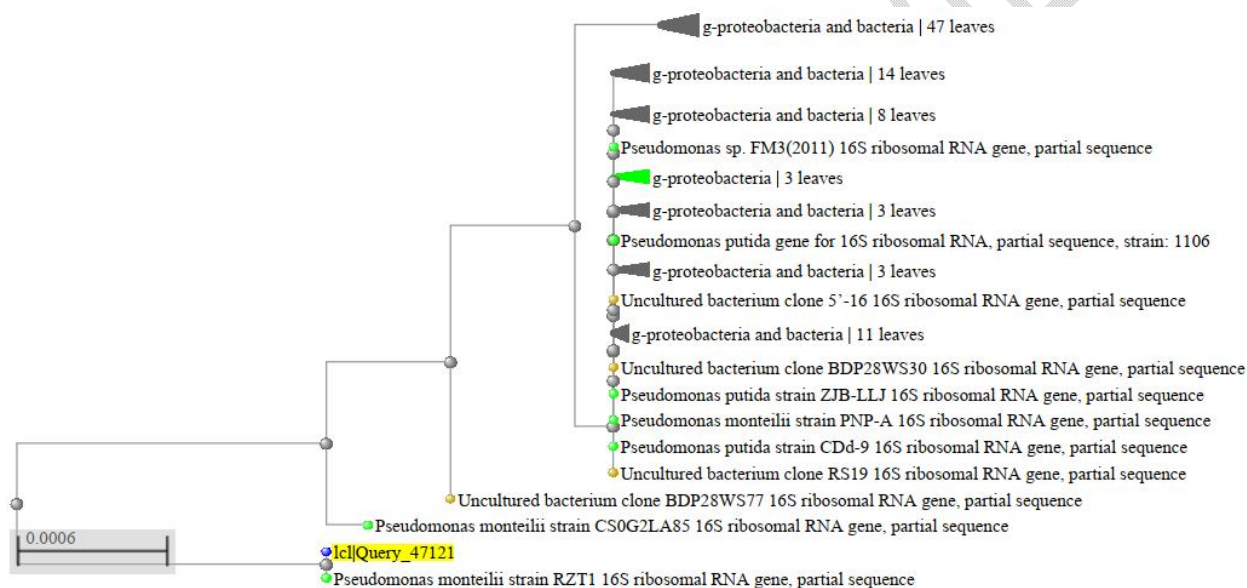


Fig 2: Phylogenetic tree of the *Pseudomonas monteilii* strain RZT1

3.3 PH and Temperature

In this study, the bacteria having potential to decolorize textile dyes were isolated from collected wastewater of fibers industrial sector. 3 morphologically different bacteria were separated from the wastewater and 1 of them was able to decolorize the Malachite Green dye. This isolates grew optimally at 28° C and pH 7 (Fig. 3 and 4).

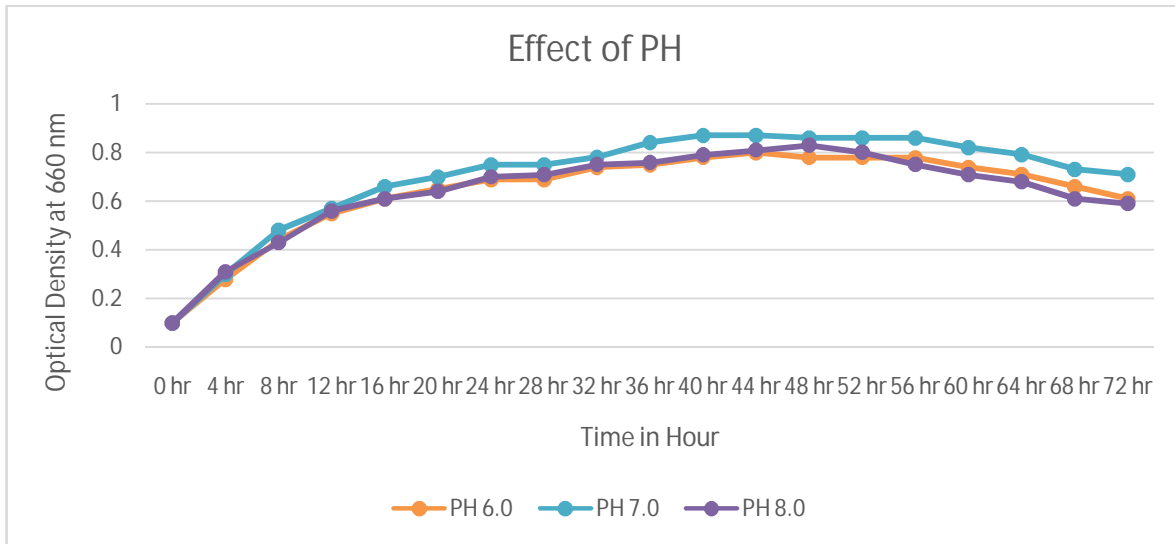


Fig. 3: Effect of PH on bacterial growth

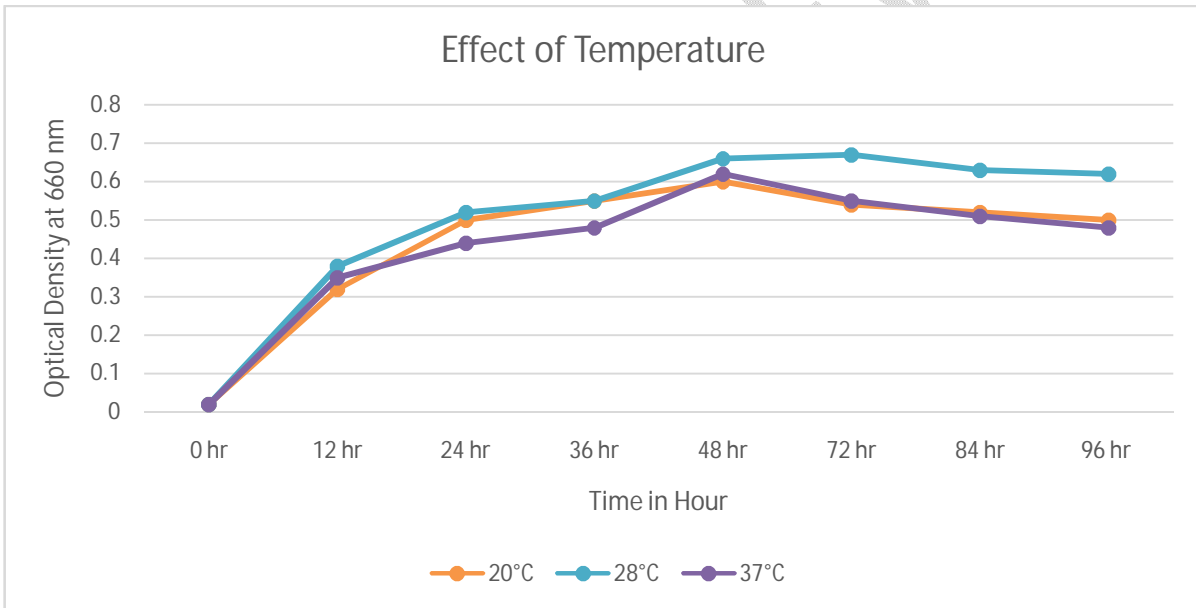


Fig. 4: Effect of Temperature on bacterial growth

3.4 Effect of Textile Dye Concentration on Decolorization:

Effect of initial dye concentration on dye discoloration capability of the bacterial isolate was measured. 0.5% yeast extract was used as a co-substrate. For that decolourization rate rapidly increase. Percentage of decreased the depigmenting activity as an initial dye concentration increased (Fig. 5). 84.8%, 75.4%, 63.4% and 45.5% decolourization occur after 192 hours of incubation period in 100, 200, 300 and 400 ppm dye concentration.

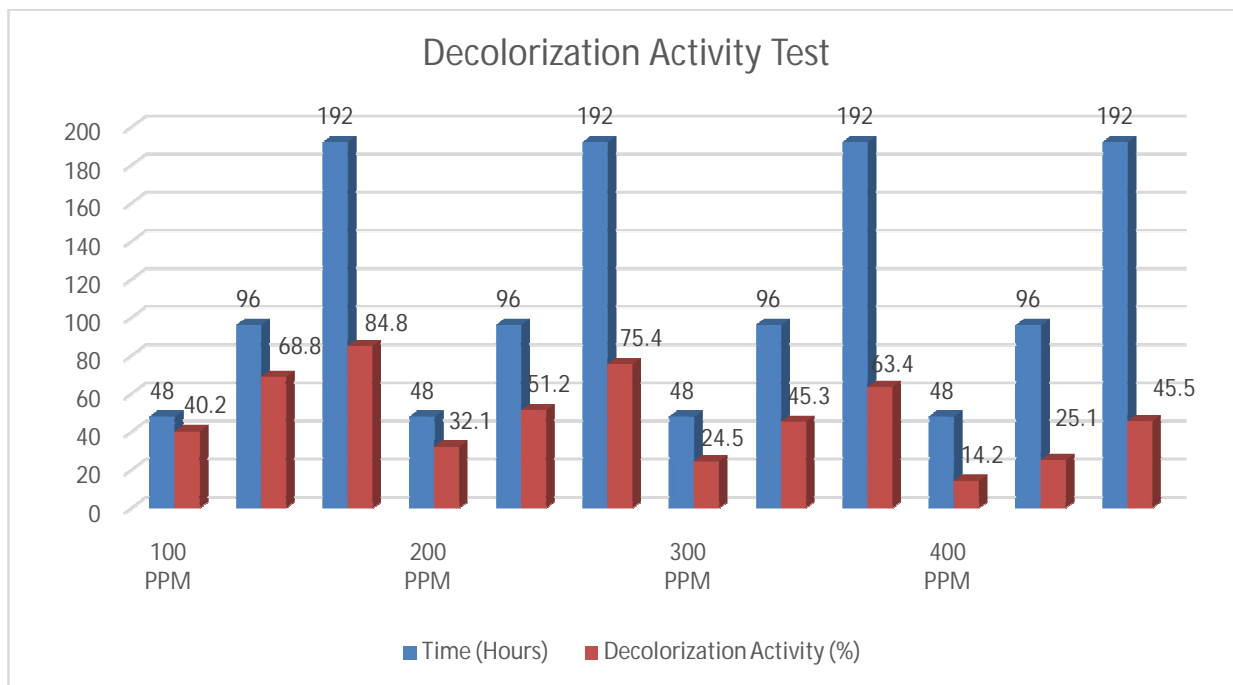


Fig. 5: Effect of Textile Dye Concentration on Decolorization Activity of malachite green

3.5 Effect of Textile Dye on Seed Germination:

We investigated the effect of the dye Malachite Green on the seed germination of five crops viz Rice (*Oryza sativa*), Wheat (*Triticum aestivum L.*), Khesari (*Lathyrus sativus*), Mustard (*Brassica Nigra*) and Bitter Melon (*Momordica Charantia*). It was found that the germination rate of seeds decreased with increasing dye concentration (Fig. 11, 12, 13, 14, 15 and 16), indicating that the germination rate was dependent on the concentration. The highest seed germination rate was found in control (irrigated with water), the lowest germination rate was observed in seeds irrigated with 400 ppm dye. The germination rate of Rice, Wheat, Mustard, Khesari, and Bitter Melon at 400 ppm dye concentration was 20%, 20%, 20%, 30% and 10% respectively. In this experiment, the seed germination effect of a 200 ppm Malachite Green dye treated (bleached) or untreated with the isolated bacteria was investigated to determine the feasibility of isolated bacteria for crop release. The germination rate of Rice, Wheat, Mustard, Khesari, and Bitter Melon was 40%, 40%, 40%, 40% and 20% respectively when irrigated with 200 ppm dye, and 68%, 65, 55%, 57% and 35% respectively when irrigated with 200 ppm dye treated with bacterial isolate *Pseudomonas monteilii* strain RZT1. The germination rate of seeds soaked with untreated dye was significantly lower than that of seeds soaked with treated dye and distilled water (control) indicating that the isolate *Pseudomonas monteilii* strain RZT1 was able to reduce the toxic effects of the dye Malachite Green (Fig. 11, 12, 13, 14, 15, and 18).

3.6 Effect of Textile Dye on Root, Shoot, Seedling and Radical Length of 5 Crops:

Inhibition of plant growth by toxic pollutants is a global agricultural problem. This study found that the root, shoot and radical lengths of the 5 studied crops gradually decreased with increasing dye concentration, demonstrating the toxic effect of the Malachite Green dye on these crops growth (Fig. 6, 7, 8, 9, 10 and 17). Highest and lowest growth of these crops was

observed at control and 400 ppm dye concentrations respectively. Maximum root length was observed on control; 10.18, 12.11, 11.30, 11.8 and 13.1 cm for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. While minimum root length was obtained at 400 ppm dye concentration; 1.5, 2.1, 3.1, 2.5 and 1.5 cm for Rice, Wheat, Mustard, Khesari, and Bitter Melon. Similarly, maximum shoot length was recorded on control; 15.01, 17.5, 15.2, 14.2 and 15.9 cm for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. Minimum shoot was obtained from 400 ppm dye; 3.1, 2.8, 4.2, 3.1 and 2.8 cm for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. Likewise, maximum radical length was observed on control. There was no significant difference between seedling growth parameters for the control and the lower dye concentration (50 ppm) treatment. But at higher concentration of dye (200 ppm), the growth parameters of seedling was affected negatively which was recovered by the treatment of dye with the bacterial isolate (Fig. 6, 7, 8, 9, 10 and 17).

3.7 Effects of Textile Dye on Seedling Fresh Weight

The raw weight of seedlings decreased with the increase of wastewater concentration (Table 2). The best fresh weight of seedlings of control Rice, Wheat, Mustard, Khesari, and Bitter Melon was 1.1, 1.2, 1.1, 1.2 and 1.4 gm respectively. Contrary, the lowest fresh weight of seedling of Rice, Wheat, Mustard, Khesari, and Bitter Melon was recorded at 400 ppm dye concentration which was 0.40, 0.31, 0.31, 0.45, 0.51 gm respectively (Table 2).

3.8 Phytotoxicity of Textile Effluent on Seedling Growth:

The increase in wastewater concentration significantly impaired the growth of seedlings (Table 2). The application of wastewater at a higher concentration suppressed the total dry matter production, globules and root length of seedlings. There are no phytotoxicity observed in control but maximum phytotoxicity was observed at 400 ppm dye which were 79.26% 71.12%, 75.6%, 63.2% and 64.6% for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. In rice 48.78% phytotoxicity was observed at 200 ppm dye concentration while that was 24.39% at 200 ppm treated dye.

3.9 Seedling Vigor Index and Germination Index:

The highest seedling vigor index was observed in control which were 2519, 2961, 2650, 2600 and 2900 for Rice, Wheat, Mustard, Khesari, and Bitter Melon. Contrary, the lowest seedling vigor was observed at 400 ppm dye which were 92, 98, 548, 608 and 126 for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. In rice, the vigor index was recorded 492 at 200 ppm dye concentration, but the vigor index was increased up to 1150 after treatment of 200 ppm dye with the bacterial isolate. For the germination index, similar characteristics was recorded as shown in Table 2.

Table-2: For the germination index, similar characteristics was recorded

Name of the Plant	Concentration of the dye	Fresh Weight	Vigor Index	Germination Index
<i>Oryza sativa</i>	Control	1.1	2519	1.34
	50 PPM	0.89	1808	0.89
	100 PPM	0.56	1311	0.78
	200 PPM	0.49	492	0.45
	400 PPM	0.40	92	0.23
	Treated Dye	0.75	1150	0.72
<i>Triticum aestivum</i>	Control	1.2	2961	1.23
	50 PPM	0.75	2020	0.78
	100 PPM	0.62	1027	0.56
	200 PPM	0.41	626	0.45
	400 PPM	0.31	98	0.23
	Treated Dye	0.69	1021	0.68
<i>Brassica Nigra</i>	Control	1.1	2650	1.23
	50 PPM	0.98	1784	1.00
	100 PPM	0.75	970	0.67
	200 PPM	0.45	548	0.45
	400 PPM	0.31	146	0.23
	Treated Dye	0.88	715	0.69
<i>Lathyrus Sativus</i>	Control	1.2	2600	1.45
	50 PPM	1.0	1631	0.89
	100 PPM	0.85	1140	0.78
	200 PPM	0.55	608	0.45
	400 PPM	0.45	168	0.34
	Treated Dye	0.78	912	0.75
<i>Momordica charantia</i>	Control	1.4	2900	1.23
	50 PPM	1.1	984	0.56
	100 PPM	0.93	172	0.34
	200 PPM	0.75	126	0.23
	400 PPM	0.51	43	0.12
	Treated Dye	0.83	168	0.35

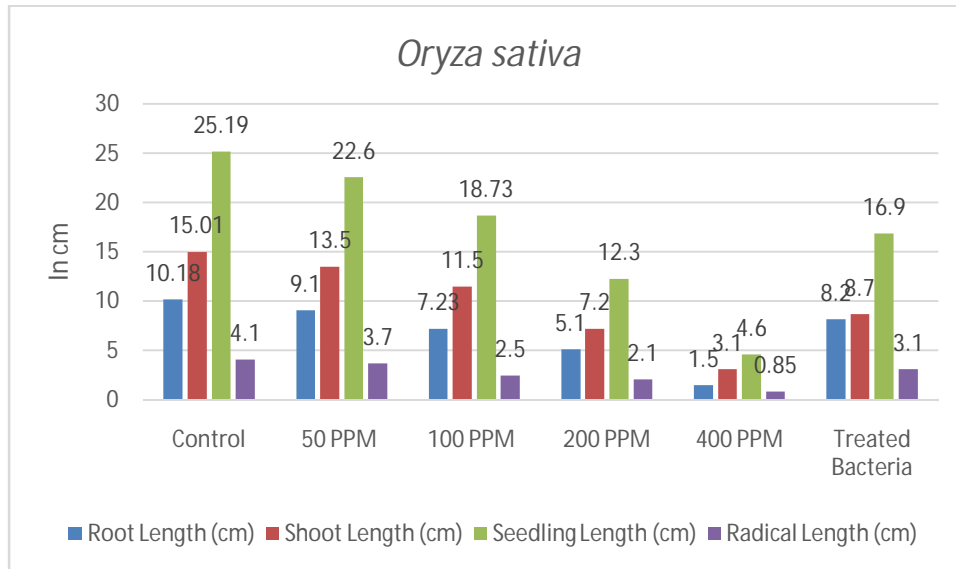


Fig. 6: Root, Shoot, Seedling and Radical length of *Oryza sativa*

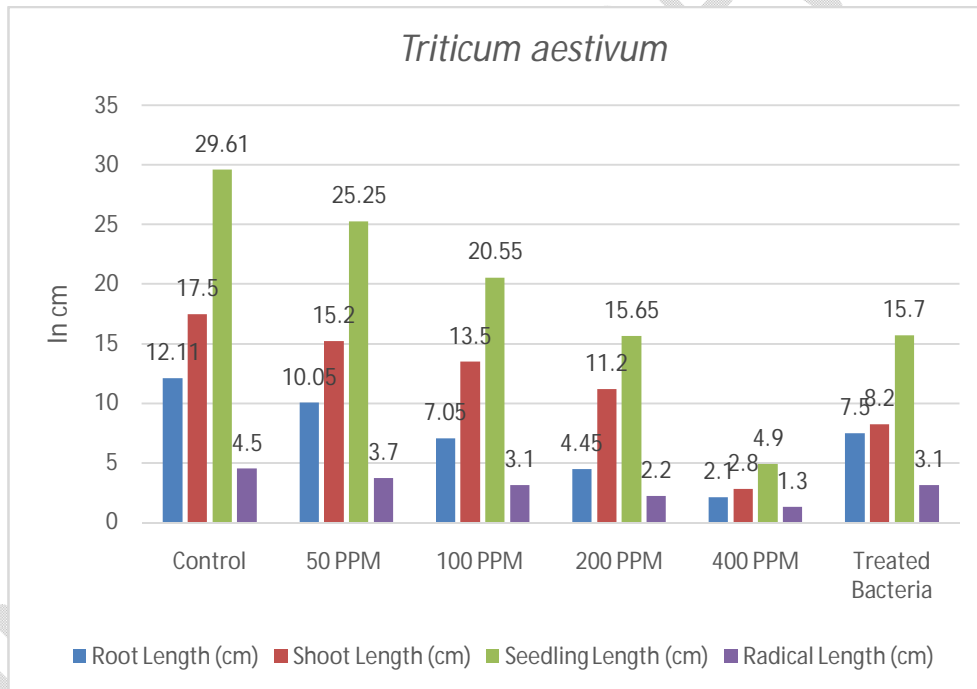


Fig. 7: Root, Shoot, Seedling and Radical length of *Triticum aestivum*

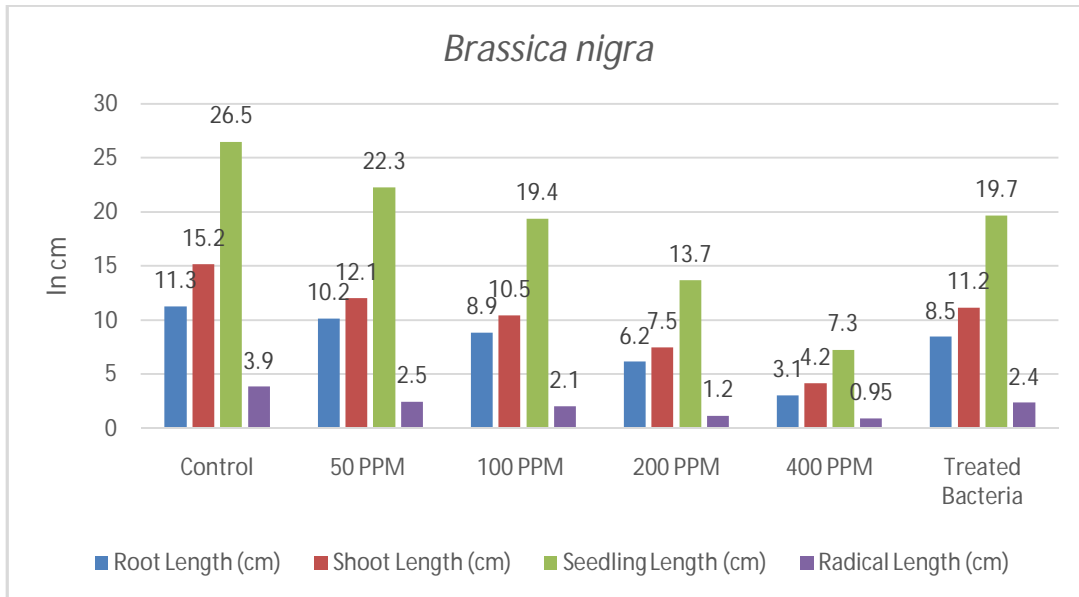


Fig. 8: Root, Shoot, Seedling and Radical length of *Brassica nigra*

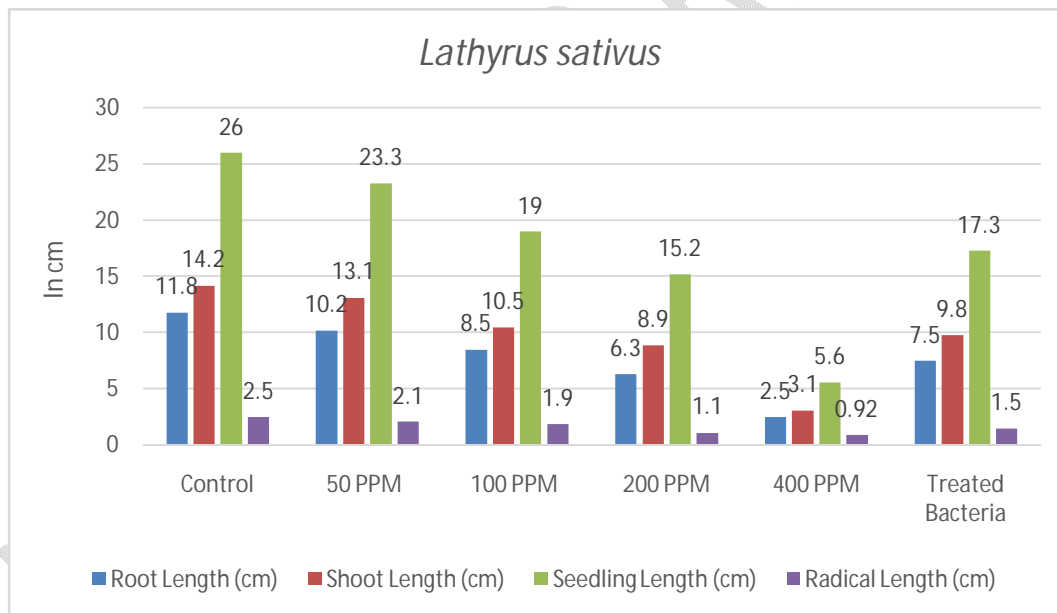


Fig. 9: Root, Shoot, Seedling and Radical length of *Lathyrus sativus*

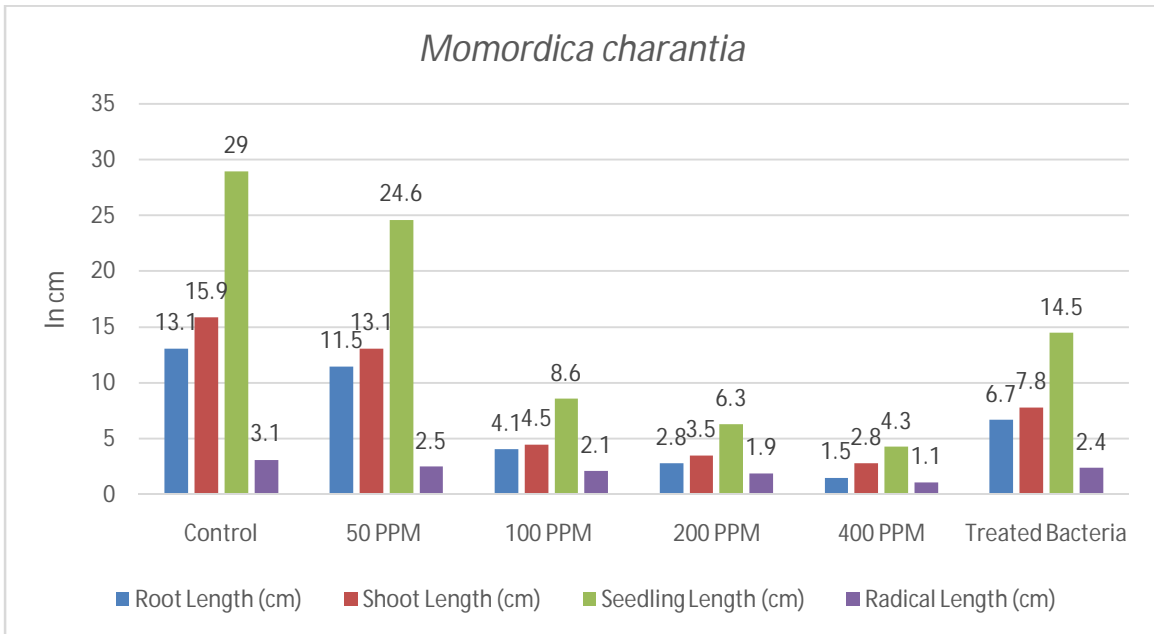


Fig. 10: Root, Shoot, Seedling and Radical length of *Momordica charantia*

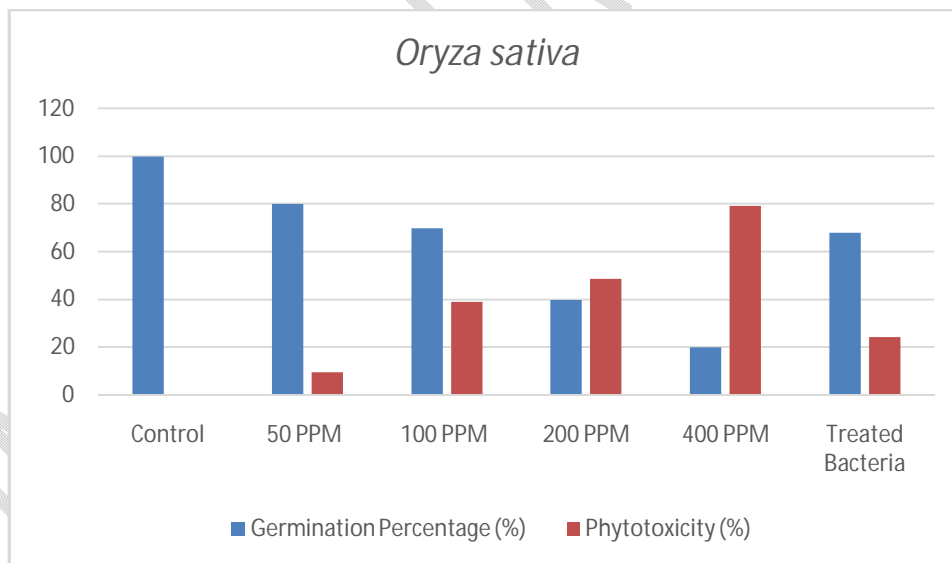


Fig. 11: Germination percentage and phytotoxicity of *Oryza sativa*

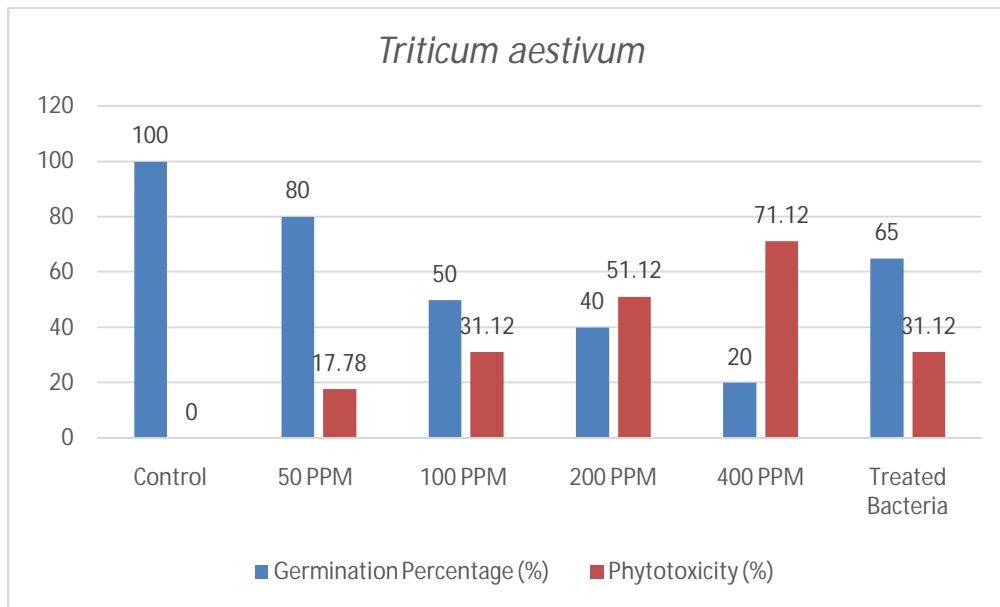


Fig. 12: Germination percentage and phytotoxicity of *Triticum aestivum*

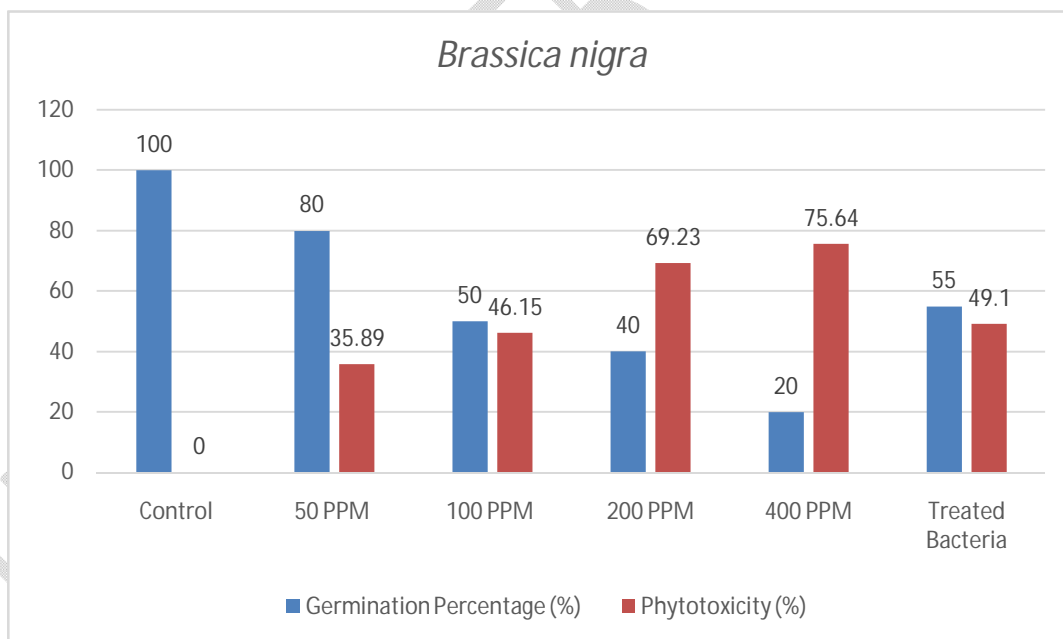


Fig. 13: Germination percentage and phytotoxicity of *Brassica nigra*

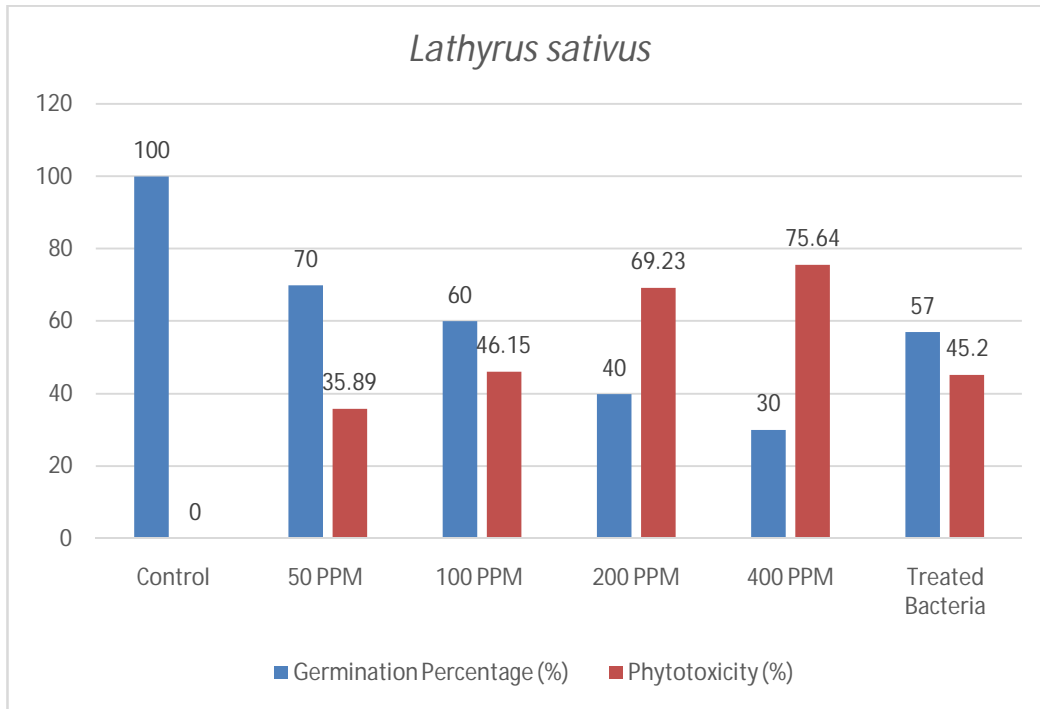


Fig. 14: Germination percentage and phytotoxicity of *Lathyrus sativus*

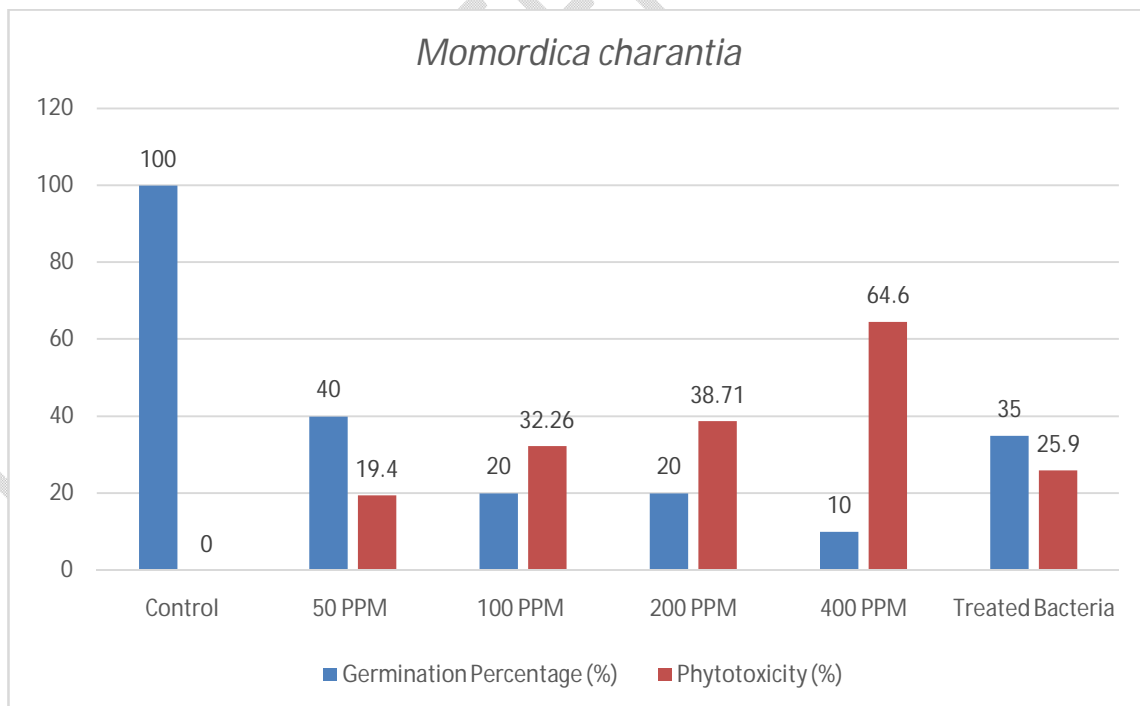


Fig. 15: Germination percentage and phytotoxicity of *Momordica charantia*



Fig 16: Effect of textile dye on seed germination on *Momordica charantia*



Fig. 17: Effect of textile dye on seedling length on *Lathyrus sativus*



Fig. 18: Effect of treated and untreated dye on seed germination of *Triticum aestivum*



Fig. 19: Effect of treated and untreated dye on seedling growth of *Momordica charantia*

4. DISCUSSION

In this study, azo dye decolourizing bacteria *Pseudomonas monteilii* strain RZT1 was isolated and characterized. This bacterial strain was selected after being grown in an enrichment medium supplemented with dye as the sole carbon source as well as in mineral salt medium which confirm the ability of the isolated bacterial species to survive in the presence of the dye. Biodegradation without any extra carbon sources is very difficult. So, optimization experiments were initiated by supplementing the mineral salt medium containing dyes with 0.5% of yeast extract. Metabolism of yeast extract is considered essential for the regeneration of NADH, which is the electron donor for the azo bond reduction [33]. Azo reductase is reported to be the key enzyme for azo dye degradation.

At lower dye concentration, bacteria showed maximum decolourization activity. 84.8%, 75.4%, 63.4% and 45.5% decolourization occurred after 192 hours of incubation period in 100, 200, 300 and 400ppm dye concentration. Decrease in decolourization ability at high dye concentration might be due to the toxicity of the dye [22]. Azo dyes generally contain one or more sulphonic-acid groups on aromatic rings, which might act as detergents to inhibit the growth of microorganisms [22]. Another reason of the toxicity at higher concentration may be due to the presence of heavy metals (metal-complex dyes) and the presence of non-hydrolyzed reactive groups which may retard the bacterial growth (reactive dyes) [34]. It is evident from our results that plants exhibited a stimulation in Germination percentage, radical length, various attributes of root development, Fresh weight, phytotoxicity, Vigor Index, germination Index at lower concentrations. In contrary, a substantial decrease was observed in these parameters at higher concentrations of textile dyes. Our results are in agreement with some earlier reports which have also demonstrated a same response of plants when irrigated with effluent [35-39]. Suppression of germination can be caused by reduced water intake by seeds with high concentrations of wastewater, which ultimately affects energy-forming compounds [40] total solids and heavy metals [41, 42].

The study showed clear inhibition of different parameters of seedlings. The germinated seeds did not get enough oxygen for the toxicity of effluent solution and the radical continuously remained in direct contact with the effluent might be responsible for affecting cell multiplication or the growth [40]. The lower effluent concentration might promote the growth because of containing plant nutrients [43]. This result was in line with the findings of [44] the other who obtained a decreasing length of radical (23.43–0.90 cm) and plumule (16.40–2.20 cm) of five paddy cultivars [45] studied the toxicity level of distillery effluent on the early growth of rice and maize, and found a decreasing rate of root and shoot lengths with increasing effluent concentration. Many researchers observed inhibitory effects of various effluents on radical and plumule length of different plant species [32, 46-49].

The germination percentage of this study was supported by the findings of [44] who found a decreasing rate of rice seed germination ranging between 100.00–41.50% among five cultivars. Previous researchers also found the same trend of germination percentage with the increasing levels of effluent [44, 50]. This result on fresh weight was in conformity with previous reports [49, 51, 52]. The promotion of sapling growth by lower concentration of effluents might be due to creating a favorable environmental condition for the germination utilizing the nutrients present in the effluent [53, 54]. Due to the high pH, EC, TDS and metallic contents of loom-dye effluent, high level of phytotoxicity might be occurred. Similar toxic effects of industrial effluent were observed by [4, 49]. Previous reports also showed higher vigor index with a lower effluent concentration [49, 50].

In this experiment, it was found that the seed germination of Rice, Wheat, Mustard, Khesari, and Bitter Melon were affected by the textile dyes. But after treatment of the dyes with bacteria, the germination of treated seeds was comparable to that of control seeds indicating that the used bacteria were able to detoxify the toxic dye.

5. CONCLUSION

It was found that physio-chemical characteristics of the textile dye has negative impact on germination and seedling growth of economical crops of Bangladesh. But, decolourization and neutralization of textile dye by the treatment of the bacterial isolate *Pseudomonas monteilii* strain RZT1 caused significant reduction of the deleterious effects of textile dye on the germination percentage and seedling growth parameters. Taken together, it can be concluded that the isolate *Pseudomonas monteilii* strain RZT1 could be used as novel bacteria for decolourization and detoxification of textile effluents in industrial treatment plant to ensure sustainable environment and development.

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