Plant Archives Vol. 20, Supplement 2, 2020 pp. 3378-3384 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

PHYTOTOXICITY AND EFFECT ON *ALLIUM CEPA* OF THE PERMEATE OBTAINED AFTER THE PHYSICO-BIOLOGICAL TREATMENT OF CONGO RED BY PHYSICO-BIOLOGICAL TREATMENT

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Abstract

The release of chemical, synthetic dyes like, congo red, into the environment is a major concern due to their associated toxicity, mutagenicity and carcinogenicity. In the present study phytotoxicity, effect on pH, carbon, phosphates, potassium and ammoniacal nitrogen on soil and *Allium cepa* study has been conducted of the permeate obtained after the adsorption study, after first and third cycle usage of the adsorbent (wheat bran), along with the treatment with *Scizophyllum*-S4, a white rot fungus for the assessment of the potential usage of reusage and sustainability of the permeate obtained. The study has revealed 90.6% increase in tolerance index on treatment with *Scizophyllum*-S4 of the dye adsorbed bran, 88.37% increase in radical and 41.4% plumule growth, but with an increase in chlorophyll a and b by 6.6 and 34.84%, respectively after 1st cycle of treatment of the dye on wheat bran rather than on organism treatment, with a variation in soil with a very high content around 392 kg/Ha of potassium as comparable with that of the control. On *Allium cepa* studies it has depicted that there are affects in the chromosomes of congo red even after being treated either by physical method of adsorption or after a combination of both physical and biological treatment. Thus, showing that the permeate obtained after the treatment can potentially reduce the pollution rate and better be used for ornamental plant growth rather than proper vegetations.
Keywords : Phytotoxicity, *Allium cepa*, congo red, wheat bran, reusability.

Introduction

Congo Red, a salt of sodium benzidinediazo-bis-1-naphthylamine-4-sulfonate, chemically a di-azo dye, which is red in alkaline and blue in an acidic solution. It is used as a histological stain and as an indicator (Samanta *et al.*, 2013; Vyas, 2017, 2019). This dye has also an immense application in the textile industries (Sudhakar *et al.*, 2015; Singh *et al.*, 2016; Bansal and Sen Gupta, 2017; Singh *et al.*, 2019). The major problem with this dye is, being an azo dye, degradation of such dye is very difficult, as a result, when they are disposed-off to the environment, they are included in the category of recalcitrant substances, which contributes towards the environmental pollution (Sharma *et al.*, 2011; Gill and Saini, 2017; Prabhakar *et al.*, 2013, 2014. 2020). These dyes constitute immense health risks being carcinogenic in nature (Bhatia *et al.*, 2018; Kaur, 2019). Different types of techniques have also been employed for the removal of such dyes from the wastewater (Kaur and Kamboj, 2019; Kaur and Gupta, 2017; Kumar *et al.*, 2020; Singh, 2019). These dyes due to their obvious toxic impact to flora and fauna requires a continuous incorporation of sustainable treatment technology. Dye pollution caused by usage of such chemical based dyes in various industries is thereby, a cause of concern (Kaur and Vandana, 2019). Around 1-10% of the dyes used during the production process in the textile industry are lost (Wong and Yu, 1999; Arslan *et al.*, 2000). These dyes are characterized by high stability in light, oxidizing agent and heat, recalcitrance to biodegradation and bioaccumulation and persistance in the environment, if not checked (Crini, 2006 and Annuar *et al.*, 2009). These dyes can affect the aquatic ecosystem and human health (genotoxic, mutagenic, as well as carcinogenic) via the food chain, and also reduce the oxygen concentration and light penetration when present of surface of water (Michaels and Lewis, 1985; Mathur *et al.*, 2006; Purvaneswari *et al.*, 2006; Sharma *et al.*, 2019). So, a coaction of adsorption using wheat bran (WB), a lignocellulosic waste and biological treatment with a white rot fungi, rich in ligninolytic enzyme, having the capacity to breakdown recalcitrant dye molecule could provide a suitable treatment facility which is not only eco-friendly but cost effective as well (Das *et al.*, 2019; Singh *et al.*, 2014; Nankar *et al.*, 2017; Mishra, 2019a, 2019b). Various treatment stages correspond to its individual efficiency. Therefore, in this study, reusability, toxicity and sustainability of the treated congo red is assessed by seed germination as well as pot studies. Chromosomal aberrations by treated dye is checked by *Allium cepa* study in relation to understand the cytotoxic effect caused by them (Dutta *et al.*, 2018; Chauhan *et al.*, 2017; Farooq and Sehgal, 2019a, 2019b). Thus, the purpose of the study was to check whether the bio-physically treated industrial dye and effluent has much lower or even a null toxicity level so that it can be reused and prove to be a sustainable alternative for environmental protection.

Material and Method

Materials

The reagents and chemicals used for this study were of analytical gradation and acquired from HiMedia, Bangalore, India and Genei, New Delhi, India. 98% pure congo red ($C_{32}H_{22}$ $N_6Na_2O_6S_2$) was procured from Loba Chemi, Mumbai, India. Lignocellulosic waste, wheat bran, was procured from the local market of Jalandhar Cant., Punjab, India.

Procurement of culture

The culture *Scizophyllum*-S4 was procured from the laboratory of Lovely Professional University, Phagwara, Punjab, India.

Preliminary treatment of wheat bran as an adsorbent

Wheat bran (WB), obtained from neighboring market was first cleansed with distilled water for the removal of all

dirts. The material was then overnight dried at 70°C. The dried material was sieved through mesh no. 30 (0.6 mm) and the part that was sieved was then transferred in polybags bags for future storage at room temperature in a dry and cool place (Alzeydian, 2015).

Studies on adsorption in a batch mode

Batch adsorption studies were conducted under the optimized condition of optimized pH 4, incubation time 210 min, adsorbate dose 100 mg/L, adsorbent dosage 12 gm/L, temperature 30°C.

Liquid state decolorization by Scizophyllum-S4

The decolourization of congo red is carried out under an optimized condition. In 100 ml conical flask 20ml working volume and concentration is 100 mg/l and in presence of wheat bran after autoclaved in laminar air flow culture inoculated with 2-3 pieces of button shaped bodies with help of cork-borer. Incubated at 28°C for 7 days and decolourization was monitored by taking optical density every day for a time interval of 16 days with a UV-Spectrophotometer.

Phytotoxicity Studies on *Vigna radiata* (moong dal)

Healthy moong (*Vigna radiata* or *Phaseolus aureus*) seeds were collected from local market. The seeds were sterilized on the surface with sodium hypochlorite (1%) solution for 5-10 minutes. They were then rinsed carefully with water (distilled) for three times for the removal of excess amount of chemical, before being used for the experimentation (Salam *et al.,* 2011).

Petri dish Experiment for seed study

In this experiment, the effect of treated synthetic textile dye effluent as well as various dyes on germination of *V. radiata* was evaluated. The surface sterilized seeds were evenly placed in sterile 10 cm petri dishes containing untreated congo red, and wheat bran treated dye (1st cycle and 3rd cycle) and organism treated dye layered with double layer of Whatman filter paper 1 and water from the tap was taken as control and kept for incubation at 28°C in the dark to allow germination. The relative seed germination %, relative toxicity %, relative root growth %, percentage phytotoxicity, germination index and tolerance index was then calculated using the plant growth data after 48 hrs and 168 hrs. Seeds were accounted as germinated when both the epicotyl and hypocotyl were visible together (Jadhav *et al.,* 2016; David and Rajan, 2015).

Relative toxicity (%R.T.) of the dye (congo red) on the germination of the seed and growth of the seedling of the plant was done to evaluate the degree of inhibition over control can be calculated using the following formula:

$$\% \text{ of Relative Toxicity} = \{(a-b)/a\} \times 100$$

a = length of seedling in control at certain period of time

b = length of seedling in the presence of effluent/dye at the same period

The Relative seed germination percentage calculated using the formula:

Relative seed germination (%) =

$$\frac{\text{Number of seeds germinated in presence of effluent/dye}}{\text{Number of seeds germinated in control}}$$

The Relative root growth percentage calculated using the formula:

Relative root growth (%) =

$$\frac{\text{Mean root length in synthetic dye effluent}}{\text{Mean root length in control}}$$

The percentage of Phytotoxicity calculated using the formula:

Percentage phytotoxicity =

$$\frac{\text{length of radical of control} - \text{length of radical of test}}{\text{length of radical of control}} \times 100$$

The Germination index (GI) calculated using the formula:

Germination index (GI) =

$$\frac{(\% \text{ Relative seed germination}) \text{ X } (\% \text{ Relative root growth})}{100}$$

The tolerance index of seedlings calculated by the formula:

Tolerance index =

$$\frac{\text{Mean length of the longest root in test}}{\text{Mean length of the longest root in control}}$$

Pot Study

The phyto-toxicity assay was carried out in triplets using medium sized plastic container. Soil in each container was incubated with 10 seeds of moong (*Vigna radiata* or *Phaseolus aureus*). The assay was checked using untreated, unmodified wheat bran treated (1st cycle, 2nd cycle and 3rd cycle), modified (acid and alkali) wheat bran treated and organism treated effluent/dye samples. The assay was executed at room temperature (30 ± 2 °C) and 5 ml of sample was watered separately per day. At the same time plain water was used to carry out the control set. After 10 days of incubation, length of plumule (shoot), radicle (root), Chlorophyll content (mg/g) and germination (%) was recorded (Oleszczuk, 2008; Rajeswari *et al.,* 2014).

$$\text{Germination \%} = \frac{\text{Number of seeds germinated in presence of effluent/dye}}{\text{Number of seeds germinated in control}}$$

$$\text{Chlorophyll a} = \frac{(12.3 \text{x} D_{663} - 0.86 \text{x} D_{645}) \text{ x V}}{\text{D x 1000 x W}}$$

$$\text{Chlorophyll b} = \frac{(19.3 \text{x} D_{645} - 3.60 \text{x} D_{645}) \text{ x V}}{\text{D x 1000 x W}}$$

Whereby, D_{645} is the Optical density at 645 nm, D_{663} is the Optical density at 663 nm, V is the final volume in ml, W is the fresh weight of leaf and D is the path length (cm).

Soil Study with Himedia K054 soil test kit

Soil obtained after pot study (period of 10 days) was further analysed using Himedia K054 soil test kit whereby the pH, organic carbon, available phosphate, potassium and ammoniacal nitrogen and nitrate present as a source of nitrogen was evaluated which is denoted essential for agriculture and horticulture soil efficiency. The color of the final solution was compared with the color of the respective chart and the nearest color match indicated the result.

***Allium cepa* study**

Purple color medium sized onion (*Allium cepa*-2n = 16) varieties were selected to carry out the study. The external loose scales and old roots present at the base were shaved shaved off using sharp knife to immerse the exposed root primordia to the test sample. The bulbs (3 bulbs for each set) was germinated in the 100 ml beaker containing distilled

water at 25 ± 1 C till new roots reached about 1 cm in length. The root tips were then immersed in the beaker containing test sample and distilled water sample was taken as control. After 48 hrs of incubation, the bulbs were taken out, root tips collected and heated in 1N HCl for 15 min and again with acetocarmine stain for 15 min at 65 °C in water bath. Around 1 mm of the root tip was carefully cut using a forecep and was then placed in clean glass slide and a drop of the stain was poured before placing the cover slips and nail polish sealing. Cells were then examined under microscope at 1000X magnification for aberrations in chromosome and photographs were taken. The total number of cells observed were calculated for mitotic index and the frequency of chromosomal aberrations (Dutta and Ahmad, 2016; Akinboro and Bakare, 2007).

Mitotic Index (MI)

$$= \frac{\text{Number of cells in dividing phase}}{\text{Total number of cells counted}} \times 100$$

Total Chromosomal aberration (TCA)

$$= \frac{\text{Number of cells aberrant}}{\text{Total number of cells counted}} \times 100$$

Result and Discussion

Under the optimized condition wheat bran has shown 97.02±0.8% removal of dye at a concentration of 100mg/L. Under liquid state fermentation of the dye adsorbed bran the percentage reduction was around 93.39±0.4% and 88.59±0.5% after 2nd and 3rd cycle of treatment after adsorption. Under all the conditions that were taken into consideration for treatment of seeds like wheat bran treated dye after 1st and third cycle for the assessment of reusability and Scizophyllum-S4 treated dye, it has shown 100% relative seed germination excepting on treatment with untreated congo red which has shown only 90% relative seed germination. 3.38% germination index, with relative toxicity around 39.53% and 25.76% after 48 and 168 hrs with 62.90% phytotoxicity with only 6.4% tolerance index. Which is around 53% more relative root growth, 56.94% increase in germination index, with zero percent relative toxicity after 48 and 168 hrs of exposure with 53.23 % less phytotoxicity with around 90.6% increase in tolerance index on treatment with Scizophyllum-S4 of the dye adsorbed bran (Table1; Figure 1).

The result obtained after the treatment of the permeate, obtained after the treatment on the bran and organism treatment, on the seeds for the growth of the plant (*Vigna radiata*) has again shown around 88.37% increase in radical and 41.4% plumule growth when compared with the control (the normally growing plant) (Table 2; Figure 2).

In phytotoxicity assessment the effect of dye (treated and untreated) was evaluated even on the basis of chlorophyll a and chlorophyll b content. Chlorophyll a on treatment with organism has shown an increase by 3% but a decrease of around 4.1% in chlorophyll b has been shown under the same condition. On the contrary, permeate obtained after the 1st cycle of treatment of the dye on wheat bran has shown an increase in chlorophyll a and b by 6.6 and 34.84%, respectively.

Soil analysis has shown that on 10 days of exposure of the permeate obtained after the adsorption study and organism treatment of the dye adsorbed bran has shown the pH to be acidic (7.5) whereas under normal condition it is around 9 (Table 4). Organic carbon on the other hand has shown not much variation as per Table 5 after 10 days of exposure, apart from that on 3rd cycle exposure, which has shown a decrease in the organic carbon content. Organic phosphate, on the other hand has shown a decrease after the usage of permeate obtained after the third cycle of adsorption but one obtained after the organism treatment has shown a comparable result with that of the control (Table 6). Table shows the effect on potassium content in the soil was good with a very high content around 392 kg/Ha as comparable with that of the control (Table 7) and Table 8 depicts the effect on ammoniacal nitrogen in the soil which is comparable with that of the control, depicting not much difference been created because of the dye content in the soil.

Allium cepa studies have shown that the effect of dye on chromosomes rise to the generation of sticky chromosome (Figure 4, E) with fragments of chromosome (Figure 4, F), vagrant chromosome in (Figure 4, G) and improper anaphasic bridge (Figure 4, H), depicting the adverse effect of the dye on the chromosomes of *Allium cepa*. Figure 5,6,7 has shown the effect of dye obtained after the first cycle, 3rd cycle as well as the permeate obtained after Scizophyllum-S4 treatment with disturbed spindle fibres of the *Allium cepa*. Though in a study, a negative influence of the sludge was observed on plant development when the dose applied was around 24%. A significant limitation in plant development and growth was observed when the sludge dose was increased from 6-24%, confirming the negative influence of sludge on the plant tested (Oleszczuk, 2007).

The study thereby showing that congo red after treatment may be used for plant growth but it can show effect on the chromosome of the plant as has been depicted after the organism treatment even, thus ornamental plants are proper to be applied with the permeate obtained rather than the cereal or any important vegetations.

Table 1: Relative seed germination %, relative root growth %, GI, relative toxicity after 48 and 168 hrs., % phytotoxicity, and tolerance index of plant seeds treated with CR (100 mg/L) (laboratory scale treated).

Congo Red (100 mg/L)	Relative seed germination %	Relative root growth %	Germination Index (GI)	Relative toxicity % after 48 hrs	Relative toxicity % after 168 hrs	Percentage phytotoxicity	Tolerance Index
Control	100	100	100	0	0	0	100
Untreated	90	37.09±0.5	33.38±0.3	39.53±0.5	25.76±0.6	62.90±0.2	6.4±0.4
WB (First Cycle)	100	80.64±0.5	80.64±0.4	13.95±0.5	20.85±0.4	19.35±0.5	96±0.2
WB (Third Cycle)	100	98.38±0.3	98.38±0.3	2.32±0.6	0	1.61±0.6	84±0.3
Organism Treated	100	90.32±0.3	90.32±0.5	0	0	9.67±0.4	97±0.4

Table 2: Radical length, plumule length and germination % of plant treated with CR (100 mg/L) (laboratory scale treated)

Plant treatment	Radical (cm)	Plumule (cm)	Germination %
Control	4.3 ± 0.5	12.8 ± 2.21	100
Untreated	1.5 ± 0.3	8.9 ± 1.6	100
Unmodified WB (first cycle) treated	2.0 ± 0.8	9.8 ± 2.7	100
Unmodified WB (second cycle) treated	2.1 ± 1.1	13.1 ± 1.7	100
Unmodified WB (third cycle) treated	2.2 ± 0.3	16.5 ± 0.8	100
Acid modified WB treated	1.2 ± 0.3	11.1 ± 1.1	100
Alkali modified WB treated	1.6 ± 0.6	9.4 ± 0.4	100
Organism treated	5.3 ± 0.4	14.2 ± 1.2	100

Table 3: Chlorophyll content of plant treated with CR (100mg/L) (laboratory scale treated).

Plant treatment	Chlorophyll a	Chlorophyll b
Control	0.945 ± 0.084	0.264 ± 0.016
Untreated	0.288 ± 0.013	0.096 ± 0.008
Unmodified WB (first cycle) treated	1.011 ± 0.099	0.356 ± 0.021
Unmodified WB (third cycle) treated	0.887 ±0.035	0.118 ± 0.011
Organism treated	0.974 ±0.057	0.253 ± 0.012

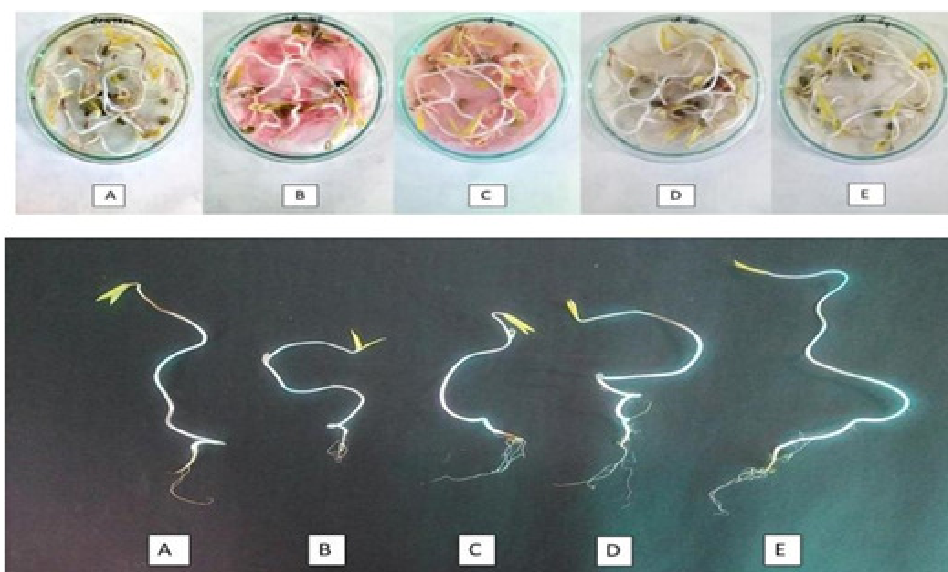

Figure 1: Seed germination in presence of CR (100 mg/L); A-Control, B-Untreated, C- Unmodified WB treated (1st cycle), D- Unmodified WB treated (3rd cycle), E-Schizophyllum-S4 treated

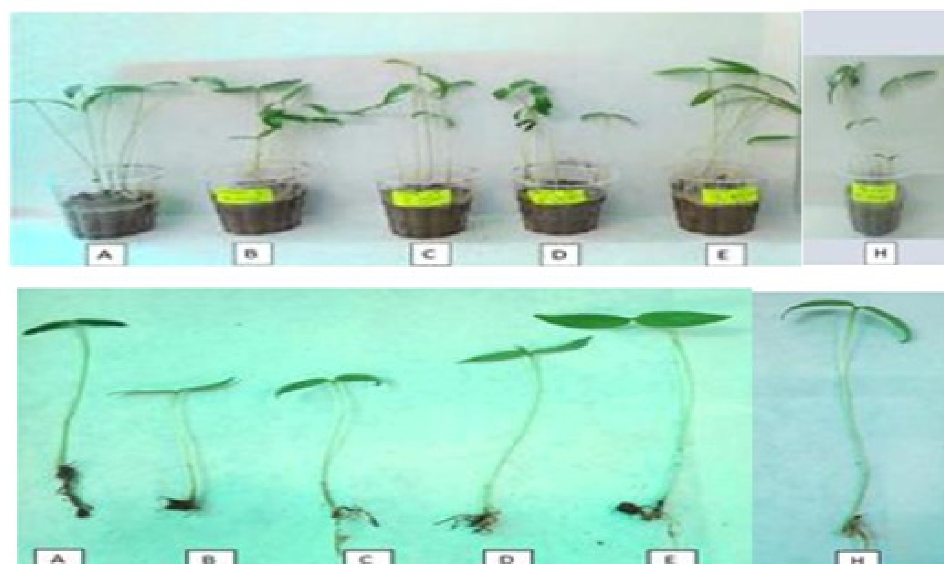

Figure 2: Effect of CR (100 mg/L) (laboratory scale treated) on plant; A-Control, B-Untreated, C-Unmodified WB treated (1st cycle), D-Unmodified WB treated (2nd cycle), E- Unmodified WB treated (3rd cycle), F- Acid modified WB treated, G-Alkali modified WB treated, H-Organism treated.

Figure 3: Effect of CR (100 mg/L) (laboratory scale treated) on chlorophyll content; A-Control, B-Untreated, C-Unmodified WB treated (1st cycle), D-Unmodified WB treated (2nd cycle), E- Unmodified WB treated (3rd cycle), H-Organism treated.

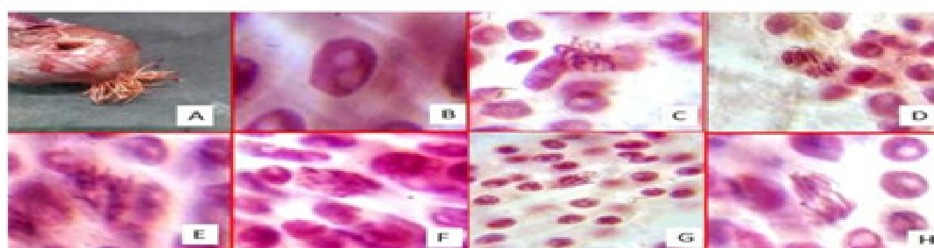

Figure 4: Effect of untreated CR on cells; A-Onion, B-Prophase, C-Metaphase, D-Anaphase, E- Sticky chromosome, F-Chromosome fragments, G- Vagrant chromosome, H- Anaphase bridges.

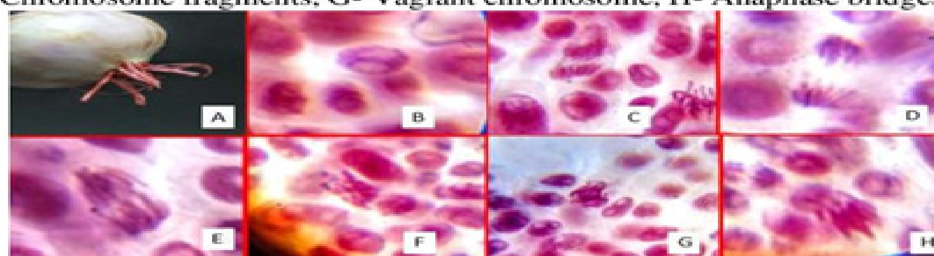

Figure 5: Effect of WB treated CR (first cycle) on cells; A-Onion, B-Prophase, C-Metaphase and anaphase, D-Anaphase, E- Anaphase bridges, F-Sticky chromosome, G- Vagrant chromosome, H- Disturbed spindle

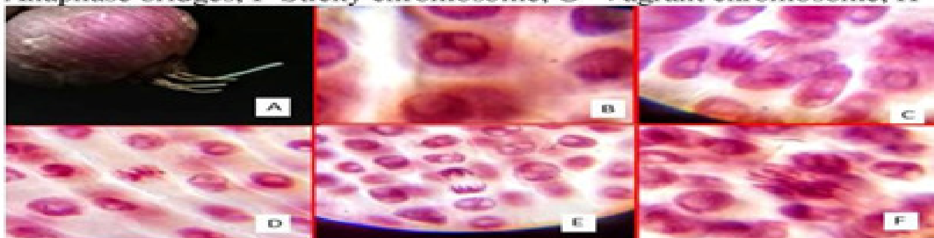

Figure 6: Effect of WB treated CR (third cycle) on cells; A-Onion, B-Prophase, C- Anaphase, D-Metaphase, E-Telophase, F-Disturbed spindle or fragments.

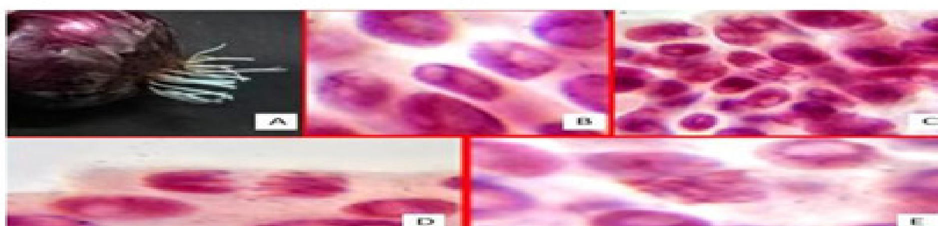

Figure 7: Effect of organism treated CR on cells; A-Onion, B-Prophase, C- Anaphase, D- Telophase, E-Disturbed spindle or fragments

Acknowledgement

The authors concede their sincere gratitude to the School of Bioengineering and Biosciences, Department of Microbiology of Lovely Professional University, Phagwara, India, for offering infrastructural facilities and active ethical support for the achievement of this work. The authors are also pleased to all faculty members and nonteaching staff for their assistance for the execution of the present research.

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