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Finding Practical Solution to Reduce the Effect of Fungicides Coating Crop Seeds on Bacterial Bio-Inoculants

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SEED COATING is the most frequently used method in application of bio-fertilizers in different crops. The treatment of seeds with fungicides for storage purposes or protection during germination represents a challenge for application of bio-fertilizers by seed coating. In the current work, the antibacterial properties of five commercial fungicides known to be used in seed protection were screened against ten bacterial strains used as bio-fertilizers (*Azotobacter salinestris*, *Azospirillum brasilense*, *Azospirillum lipoferum*, *Enterobacter cloacae*, *Rhizobium leguminosarum* , *Rhizobium phaseoli*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Paenibacillus polymyxa*, *Bacillus aryabhattai*) in addition, commercial sugar beet seeds dressed with unknown fungicids were also tested and the fungicide with antibacterial activity was selected and identified using gas chromatography (GC) to be used in further work. Sugar beet Seeds treated with selected fungicide were coated with different materials (Talc powder, Biochar, Charcoal, Polyvinylpyrrolidone (PVP), Beet moss) with expected adsorption properties and the effect of this materials in protection of bio-fertilizes was studied in agar plates. The most promising treatments were applied in a pot experiment using sugar beet (*Beta vulgaris*) seeds. The growth of most bacterial strains was not inhibited by already known fungicides except Rhizobium phaseoli which was inhibited by Tebuconasole 2.5% while one unknown fungicide showed a remarkable antibacterial activity against most bacterial strains under study. The GC-MS identification revealed that this fungicide is thiram. The most effective material in protection of microbial strain was charcoal.

Keywords: Bio-fertilizers; Thiram; Charcoal; GC-MS.

1. Introduction

Bio-fertilizers Inoculants aiming to partially replace mineral fertilizers and becoming gradually important in agriculture, as there is a global demand to increase sustainability **(El-Gaafarey et al., 2020**; **El-saied and Rashwan**; **Zaki et al., 2021**; **Manva et al., 2019)**.

Important enhancements in the delivery systems of the inoculants and on the finding of new microbial strains and formulations are in progress **(Rocha et al., 2019)**. On the other side, agriculture sectors will continue to release chemical pesticides with low compatibility with microbial inoculants, especially when applied as seed coats, represents a key limitation to the efficiency of bio-inoculation. (**Rani et al., 2018)**. The compatibility between chemical pesticides and bio-inoculants depend on formulation, method of application, and contact period with living microorganisms; however, in general they have a high risk on cell survival and metabolism **(Santos et al., 2021; Manva et al., 2019).**

Seed dressing with chemical fungicides has been represent a cheap solution against seed and soil-borne fungal pathogens. Many researchers have conducted

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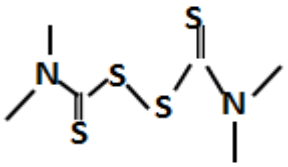
research to discover the effect of fungicide applied to seeds on number of soil microorganisms and crops yield. Here, a trial to find a solution to alleviate the effect of fungicide used for coating different seeds on bio-inoculants. **(Zaller et al., 2016)**

(Cu) based fungicides have a destructive effect on the population and activity of Nitrogen fixing bacteria (**Van Zwieten et al., 2003)**. Fungicidal residues, like, apron, captan, tend to remain in soil in contact with living organisms and disturbing the N-fixation in legume-Rhizobium (**Kyei-Boahen et al., 2001)**. Both fungicide mancozeb and chlorothalonil can decline the rate of nitrification and denitrification during 48 h incubation period (**Kinney et al., 2005)**.
Carbendazim is moderately poisonous to Pseudomonas fluorescens and *Bacillus subtilis* **(Virág et al., 2007).**
New strategies are needed to find solutions for the incompatibility between pesticides and microbial inoculants, as those that have been suggested to date are still not sufficient t in terms of demand.
Thiram [bis(dimethylthiocarbamoyl) disulfide] are a synthetic, organosulfur-based compounds used as a fungicide, to prevent fungal proliferation in seed and crops and likewise as an animal repellent to protect ornamentals crops from destruction by farm animals. Thiram also has been applied in the treatment of human scabies and act as sun screen and bactericide applied directly to the infected skin skin or mixed to soap. Thiram was also evaluated as antibacterial agents against multidrug resistant Staphylococcus aureus with MIC90 values of 4µg/ml (**long, 2017)**



Thiram

Fig. 1. Chemical structure of thiram

Currently, there is a need for innovative and demand-driven research in bio fertilization science, for facilitating of high-quality eco-friendly research by creating a conducive and trustworthy work atmosphere, thereby rewarding productivity and merits and one major challenge is to make the chemicals and biological compatible

2. Experimental

Agar diffusion method for screening commercial fungicides for its antibacterial activity

The agar plate's surfaces were inoculated by spreading (100 µl) of the 24h old broth culture over the entire agar surface. Then, wells with a diameter of 8 mm were punched aseptically with a sterile tip, and a volume (100 µl) of tenfold diluted fungicide solution is introduced into the well. Then, agar plates are incubated at 10 C° for two hours to permit fungicide diffusion then transferred to 30 C° for 24h **(Magaldi et al., 2004)**
Random commercial sugar beet and wheat seed samples treated with fungicides were tested against bacterial strains under study by agar diffusion method.

In vitro assay the potential of different materials in protection of bacterial inoculum.

Sugar beet seeds coated with the most effective fungicide (thiram) were immersed in sterilized 0.1 gum Arabic solution then rolled in adsorbent materials (Charcoal, beat moss, Talc powder, PVP) then the seeds were gently infused in the pre inoculated nutrient agar plates and incubated at 30 C° for 24h, the diameter of clear zone around the seeds were measured. The most effective material in protection of bacterial strains was applied in pot experiment.

Gas chromatography-Mass spectrometry for Identification of the most effective fungicide

Commercial Sugar beet seeds treated with unknown fungicide and showing antibacterial activity against tested strains were sieved vigorously to separate a part from a dressed fungicide then a stock solution of (400mg l^{-1}) was prepared in 25 ml methanol. The chromatographic analysis was performed using Agilent 7890B gas chromatograph equipped with mass detector (MSD) Agilent 5977A.
A fused silica capillary column with dimensions (30m x 0.25mm HP-5-0.25 microm -60 to 325 C°) was used. The injector port temperature was 280 C°, the flow rate of helium carrier 1.0 ml min^{-1}, solvent delay 4min and the injected sample volume was 1µl. the temperature of column was maintained at 50 C° for 0.5 min then programed at 10 C° min. to 190 C° the 10 C° min ramp to 210 C° for one min followed by 10 C° min ramp to 300 C° and kept for 2 minutes. The analysis time was 29.5 minutes. The resulted separated peaks were identified using wiley mass spectral data.

Pot experiment

Plastic pots of 0.5 kg capacity were filled with washed sterilized sand (sand was used instead of clay soil to prevent interference with the adsorption capacity of clay soil particles), sugar beet seeds were surface sterilized using 3% sodium hypochlorite for about 2 min followed by rinsing in sterile distilled water containing a few drops of hydrogen peroxide then distilled water for three successive times. **(Ghazi et al., 2021)** then the seeds were treated with witted fungicide powder and dried immediately with air gun.

Preparation of bacterial inoculums on charcoal carrier and seed inoculation

Bacterial cultures in late log phase were inoculated in pre-sterilized charcoal to obtain 35% moisture content. Then sugar beet seeds were rolled in the carrier containing the bacterial strains under study, 0.1% gum Arabic solution was used as a sticky agent. The control treatments received the same density of bacterial inoculum in the sticky agent (gum Arabic), after planting; full strength hoagland solution was used to irrigate plants alternately with distilled water. The experiment continued for 21 days the plant growth parameters (plant height, fresh and dry weight) were measured. The bacterial count in the rhizosphere was estimated using plate count technique.

Statistical analysis

Using one way complete randomized analysis of variances (ANOVA), data were analyzed by CoStat computer software type 6.303 for Windows Variances at $p < 0.05$ were measured to be significant.

3. Results

Effect of the most frequently used commercial fungicide on the in vitro growth of PGPB

Most of tested bacterial strains did not show growth inhibition toward the applied fungicides. The strain of *Rhizobium phaseoli* was the most sensitive strain among the tested bacteria where it was inhibited by Tebuconasole 2.5%. The situation was also the same in case of commercial sugar beet and wheat seed samples treated with unknown fungicide except one sample showed inhibition in growth with most tested bacterial strains. The strains of *E. cloacae*, *R.leguminosarum* and *A. lipoferum* showed obvious good growth under all tested fungicides.

GC-MS chromatographic analysis (Fig. 1) of the unknown fungicide

the unknown fungicide separated from treated sugar beet seeds with antibacterial activity to the most bacterial strains under study was identified using GC-MS as thiram [Bis(dimethyle thiocarbamyl) sulfide] (Fig 2) , then it was kindly obtained from the Central Agricultural Pesticides Laboratory and used for complete the study.

Potential of different materials in protection of bacterial isolates.

Among the tested materials, charcoal recorded the highest protection for the most strain against the used fungicide. As shown in Table (2), the degree of protection by charcoal varied between the different strains under study. Whoever it completely protect some strains as in case of *R.leguminosarum*, and *P. polymyxa* , it partially protect other strains as B. amyloliquefaciens, *Bacillus aryabhattai* while it had no obvious effect in case of A. brasilense and R. phaseoli which was the most sensitive strain. The growth of other strains (*E. cloacae* and *A. lipoferum*) were not affected by the presence of fungicide at all, moreover the growth of strain (*A. lipoferum*) seems to be enhanced in the fungicide diffusion area fig (3).

***In vivo* evaluation of the Impact of charcoal as adsorbent for fungicide (thiram)**

The results obtained from the pot experiment showed a great agreement with the results of agar diffusion, where the treatments using charcoal as a carrier of bacterial inoculants showed an increase in the number of bacteria in the root zone in addition to the reflection of this increase on the vegetative characteristics of the beet plant at the end of the experiment such as root length, plant height and fresh weight of the plant. This increase in vegetative parameters is likely due to the role of microbial inoculants as stimulators of plant growth. There were no significant differences in shoot and root length in case of *A. lipoferum* between treatments because of this strain is not sensitive to thiram at all. The results also showed that the best growth parameters were recorded for plants which inoculated with bacterial strains in absence of fungicide and charcoal followed by those inoculated with bacterial strains mixed with charcoal in presence of fungicide. (Table 3)

The total bacterial count was greatly affected by the presence of thiram without protecting agent (charcoal), while in the presence of charcoal, a slight reduction in microbial count was noticed. As expected, the strain of *A. lipoferum* was the most tolerant strain in the presence of thiram without charcoal (Table 4).

Table 1. Screening of the antibacterial properties of the most frequently used fungicides used in seed dressing.

	Tebuconasole 6%	Tebuconasole 2.5%	Difenoconasole 9.2% + Metalaxyle M 2.3%	Triticonasole	Diniconazole
A. lipoferum	NS	NS	NS	NS	NS
E. cloacae	NS	NS	NS	NS	NS
A. brasilense	NS	NS	NS	NS	NS
P. polymyxa	NS	NS	NS	NS	NS
A. salinestris	NS	NS	NS	NS	NS
R.leguminosarum	NS	NS	NS	NS	NS
R. phaseoli	NS	S	NS	NS	NS
B. subtilis	NS	NS	NS	NS	NS
B. amyloliquefa-ciens	NS	NS	NS	NS	NS
P. polymyxa	NS	NS	NS	NS	NS

Table 2. Effect of seed dressing with different adsorbent materials on the protection of microbial inoculants against fungicide (Thiram).

Treatments	Control	Charcoal	PVP	Peat moss	talc	bio char
A. lipoferum	0	0	0	0	0	0
E. cloacae	0	0	0	0	0	0
A. brasilense	2.3	1.4	1.7	2.1	2.2	2.2
P. polymyxa	3.2	0.7	2.9	2.8	2.4	2.4
A. salinestris	1.8	0.9	1.6	1.6	1.7	1.7
R.leguminosarum	1.9	0.8	1.4	1.2	1.5	1.4
R. phaseoli	3.1	1.2	2.3	2.5	2.7	2.8
B. subtilis	2.7	1.6	2.3	2.4	2.6	2.6
B. amyloliquefaciens	٣.٣	2.4	2.3	3.1	2.9	3
B. aryabhattai	2.9	1.8	2	2.1	2.3	2.2

NS= Not sensitive S= Sensitive

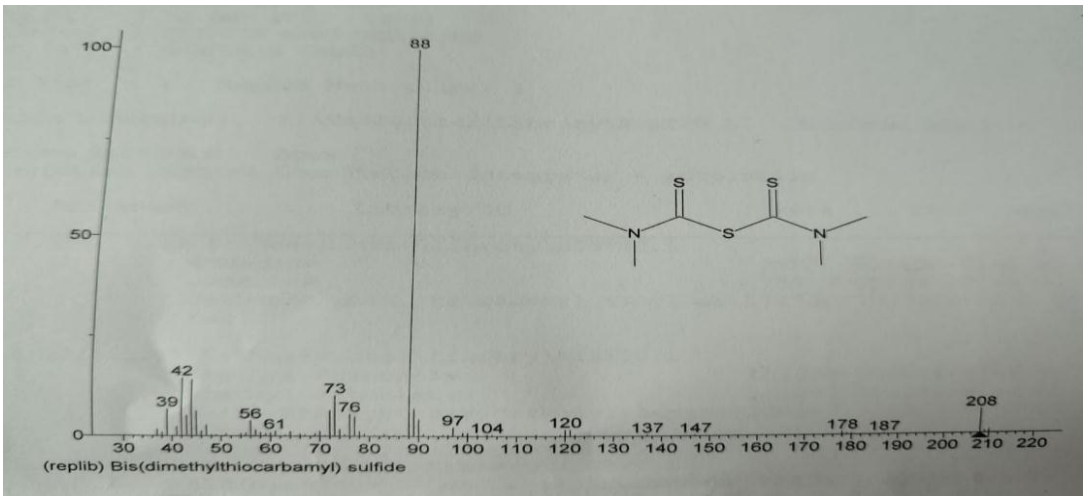


Fig. 2. Chromatographic analysis showing that the unknown fungicide possess antibacterial activity is thiram.

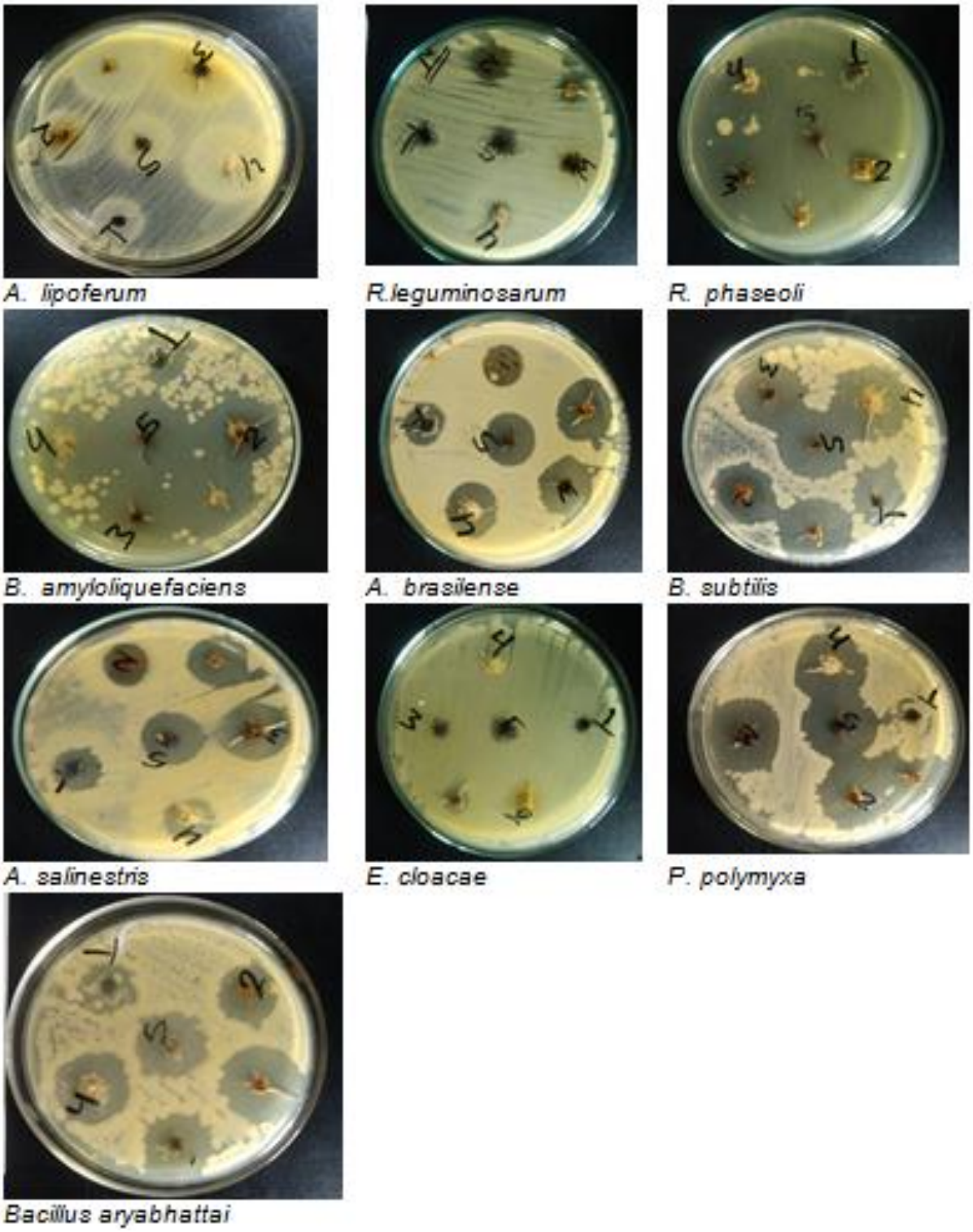


Fig. 3. agar diffusion technique for assaying the effect of fungicide dressed sugar beet seeds on different bacterial strains

Table 3. ***In vivo*** **assay of the charcoal potential to alleviate fungicide toxicity on bacterial bio-inoculums applied on sugar beet plant.**

Treatments	Root length 9cm)									
	A. lipoferum	*E. cloacae.*	*A. brasilense.*	*P. polymyxa.*	*A. salinestris.*	*R.leguminosarum.*	*R. phaseoli.*	*B. subtilis.*	*B. amyloliquefaciens*	*B. aryabhattai*
A	19.47	17.50	18.33	15.77	16.80	16.23	16.03	14.30	14.13	13.80
B	19.30	17.93	15.83	13.47	12.40	12.50	12.10	10.97	12.27	11.83
C	18.97	15.50	14.53	12.57	14.53	13.80	12.57	12.30	12.83	11.93
L.S.D. 0.01	n.s	2.08*	2.11**	1.72**	3.7*	3.72**	3.08**	1.5**	n.s	1.68**
	Shoot length (cm)									
A	13.03	11.97	12.47	12.50	13.13	11.40	12.10	10.20	12.30	12.57
B	13.83	11.47	11.40	10.80	10.63	9.97	10.27	9.67	10.80	11.17
C	13.70	11.57	12.17	11.03	11.47	10.20	10.97	8.83	11.23	10.83
L.S.D. 0.01	n.s	n.s	n.s	1.65*	1.1**	1.4*	1.5*	n.s	1.6*	1.8*
	Fresh weight (g/plant)									
A	1.80	1.50	1.31	1.20	1.10	0.93	0.87	0.77	0.67	0.65
B	1.73	1.41	1.22	1.04	0.90	0.81	0.74	0.63	0.60	0.61
C	1.39	1.17	1.07	1.03	0.77	0.67	0.59	0.55	0.53	0.52
L.S.D. 0.01	0.21**	0.18**	0.2*	n.s	0.23**	0.2**	0.28*	0.12**	0.1**	0.07**

A- Control (without pesticide without charcoal), **B**- pesticide+ charcoal and **C**- pesticide

Table 4. ***In vivo*** **assay of the effect of seed dressing with charcoal on the survival of bacterial inoculums in the sugar l rhizosphere.**

	A. lipoferum	*E. cloacae.*	*A. brasilense.*	*P. polymyxa.*	*A. salinestris.*	*R.leguminosarum.*	*R. phaseoli.*	*B. subtilis.*	*B. amyloliquefaciens*	*. B. aryabhattai*
A	7.39	7.39	7.34	7.39	7.32	7.36	7.29	7.34	7.29	7.34
B	7.38	7.29	7.07	7.21	7.21	6.95	6.81	6.93	6.31	6.35
C	6.08	5.99	5.78	5.59	5.69	5.77	5.71	5.91	5.68	5.76
L.S.D. 0.01	0.09**	0.09**	0.09**	0.07**	0.08**	0.15**	0.08**	0.1**	0.13**	0.08**

A- Control (without pesticide without charcoal), **B**- pesticide+ charcoal and **C**- pesticide

4. Discussion

Fungicidal applied as seed dressing help to improve the early plant emergence are often detrimental to microbial inoculums applied. Some reports statement little injury, which may reflect the variation in between different microbial strains in their sensitivity to fungicides **(Mishra et al., 2014)**.

The lack of effect of the tested fungicides on the growth of microbial strains under study may be due to the mechanism of action of the antifungals, which target biological structures in fungi that are not present in bacteria. Sometimes the fungicide may affect

some non-target microbial groups, such as what happened in the case of the fungicide thiram, where some reports proved its ability to affect bacterial activity and growth **(long, 2017)**.

It is noticeable that the sensitivity of some strains of Rhizobia towards some chemical compounds extends to other compounds. In the case of Rhizobium phaseoli, some researchers reported their sensitivity to chemical compounds present in the faba bean seeds defusate (**Azza, 2017**). In the current study, Rhizobium phaseoli strain was the most sensitive to the fungicide thiram, as the fungicide was able to completely prevent its growth in a petri dish even when the seeds were treated with adsorbent materials, which means that the minimum inhibitory concentration (MIC) is very low where the remaining amount of the pesticide after adsorption was able to completely prevent the growth
of this strain.

Pyrolysed carbonaceous materials like biochar and charcoal has been used for a long time to absorb pesticides from the environment. (**Taha et al., 2014)** used biochar and charcoal to adsorb 15 different pesticides from polluted water The results showed that the pesticides were adsorbed successfully by both biochar and charcoal but the adsorption efficiency was higher in case of biochar. (**Azza, 2017)** used biochar as carrier materials to ameliorate the antimicrobial effect of germinating Kidney bean seeds on rhizobia.

Gonzhlez-Pradas, (1987) evaluated the potential of the activated carbon in removing the fungicide thiram from aqueous solution and he found that activated carbon is effective in removing thiram from aqueous solution because of their higher adsorption ability, large surface area and removal efficacy values.

5. Conclusion

Most bacterial inoculums are not affected by antifungals compounds because they do not possess the target sites of antifungal, but there are some fungicides that have a dual effect on both fungi and bacteria, such as thiram. The toxic effect of these compounds can be reduced by using activated charcoal to adsorb and limit them away from bacteria.

6. Conflicts of interest

The authors declare that they have no competing interests.

7. Formatting of funding sources

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