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Effect of *Rhizobium* sp., on the growth of *Vigna radiata* Stressed under Almix and detergent

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Abstract

Almix is a xenobiotic herbicide that is effective in weed control but has adverse effect on soil bacteria and plants when accumulates in the environment. It is applied along with detergent for easy dispersion in soil. The present study evaluates the application of almix and detergent on *Rhizobium* sp., and its effect on symbiosis with *Vigna radiata.* An indigenous *Rhizobium* sp., was isolated and its minimal inhibitory concentration (MIC) to herbicide almix and detergent was recorded as 1200 µg / ml of almix and 1400 µg / ml of detergent. Evaluated the effect of application of almix and detergent at varying concentrations on the growth of *V. radiata.* When concentration of almix and detergent in plants increased growth of plants reduced including both root and shoot length. Almix and detergent applications affected the production of chlorophyll a & b in experimental plants and its concentration decreased to 80% and 40% respectively. Compared to untreated plants, *Rhizobium* sp., treated plants showed overall 5% increase in chlorophyll content. The effect of application of these chemical stresses on the plant parts was more severe at 400 µg/kg of herbicide and detergent treatment. For the vigorous growth and yield of plants requires additional supply of almix and detergent resistant *Rhizobium* sp., for its stabilised plant growth and function. So the study recommends the use of almix resistant and detergent resistant indigenous *Rhizobium* sp., as biofertilizer.

Keywords: Almix, plant growth, herbicide, *Rhizobium* sp., detergent.

Introduction

Different bacterial genera are vital components of soil. They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production (Ahemad *et al.,* 2009 and Chandler *et al.,* 2008). The everlasting growth of different industries, agricultural activities and urbanization lead to increased release of pollutants in soil, air and water. Especially the usage and release of xenobiotics in the form of herbicides, pesticides, fertilizers into agriculture land affects the organisms living in soil. The most common chemicals involved in soil pollution are petroleum hydrocarbons, polynuclear aromatic hydrocarbons (such as naphthalene and benzopyrene), solvents, herbicides, pesticides, lead and other heavy metals.

Environmental pollution is the great concern and has been accepted as global problem because of its adverse effect on human health, plants, animals and exposed materials (Irshad *et al.*, 1977). On the other hand the biological approaches for improving crop production are gaining strong status among agronomists and environmentalists.

There is an ongoing rigorous research worldwide with greater impetus to explore a wide range of rhizobacteria possessing novel traits like heavy metal detoxifying potentials, pesticide degradation/tolerance, salinity tolerance, biological control of phytopathogens and insects and along with the normal plant growth promoting properties such as, phytohormone, siderophores, nitrogenase activity, phosphate solubilization etc. (Kumar *et al.*,2015).

One of such major pollutant in agriculture land is herbicides that accumulates in the soil and at elevated levels impairs the metabolic activities resulting in reduced growth of rhizobia, legumes or both. Hand weeding schedules have become impossible due to the high cost and scarcity of labour. Different types of herbicides are recommended for crops. Application of herbicides not only affects the target plants but also microbial communities in soil. Researchers reported that herbicides when applied indiscriminately had variable effects on legume production (Khan *et al.*, 2004). For instance, the photosynthesis inhibiting herbicide metribuzin affects the *Rhizobium* sp., (Heinonen-Tanski *et al.*, 1982), the plant (Rennie and Dubetz, 1984) and the legume *Rhizobium* symbiosis (Malik and Tesfai, 1985).

Diverse symbiotic (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*) and non-symbiotic (*Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum*,*Azomonas*), rhizobacteria are now being used worldwide as bio-inoculants to promote plant growth and development under various stresses like heavy metals, herbicides, insecticides, fungicides, salinity etc (Kumar, *et al.*,2015).

In the present study effect of an herbicide, almix on plants and rhizobacteria was evaluated. Almix is a very effective third generation herbicide widely used to control the broad leaf weeds and sedges in the paddy fields. The chemical composition of almix is 10% metsulfuron methyl, ($C_{14}H_{15}N_5O_6S$) [methyl 2-(4-methoxy-6-methyl-1, 3, 5-triazin-2-ylcarbamoyl-sulfamoyl) benzoate], 10% chlorimuron ethyl, ($C_{15}H_{15}ClN_4O_6S$) [ethyl 2-(4-chloro-6-methoxypyrimidin 2 acylcarbamoyl-sulfamoyl) benzoate] and 80% adjuvant (detergent). Permitted concentration of almix in the agriculture field is 8 gm/acre. Almix is also not prone to volatilization and does not harm adjacent crops like mustard, vegetable, fruit plants, cotton, castor, etc. unless it's directly sprayed on them. But recent reports on usage of almix inferred that long-term exposure of almix even at environment-friendly concentration may cause alterations in the digestive functions of fishes living in the rice field (PalasSamanta *et al.*, 2014).

Residual effect of almix applied in rice field may affect the microbial population in soil thereby during crop rotation affects the other plant growth too. But microorganisms develops pathway to breakdown such xenobiotics and develops resistance. Such resistant bacteria help in plant growth even at higher concentrations of xenobiotics. So the study planned to understand resistance of Rhizobium sp. to almix and detergentand its plant growth promotion in *Vigna radiata* plant.

Materials and Methods

Isolation and identification of Rhizobium species

The root nodules were collected from pea plant in a sterile container and transported immediately to the laboratory for further processing. Collected root nodules were surface sterilised using 90% ethanol and again washed with tap water for two to three times. This was crushed with sterile mortar and pestle. From the above mixture a loopful of sample was inoculated on Yeast Extract Mannitol Agar medium (YEMA) and incubated at 28^0C for 3 days. Isolated colonies were subcultured on YEMA medium, incubated at 28^0C for 3 days and plates were stored at 4^0C. The colonies formed on the solid media were identified by performing Gram's staining, motility test and various standard biochemical tests for confirmation.

Assay of herbicide resistance to Rhizobium sp. *(Khan et al., 2006)*

The sensitivity or resistance of herbicides to isolated *Rhizobium* sp., was determined by plate dilution method. The herbicide Almix (metsulfuronmethyl+chlorimuron ethyl) at various concentrations ranging from 100-6400 µg/ml were prepared and supplemented in sterilized YEM agar plates. The test organisms were grown in YEM broth for 5 days to a density of 10^8 cells/ml and were spot inoculated onto the YEM agar plates. Plates were incubated at 28°C for four days. The lowest concentration of herbicide inhibiting Rhizobial growth on YEM agar plates was defined as minimum inhibitory concentration (MIC) of the herbicide. In order to study the effect of adjuvant (detergent - used along with almix) same procedure was repeated for a detergent against *Rhizobium* sp.

Pot culture of Vigna radiata plant

Healthy seeds of *V. radiata* plant were selected, surface-sterilized in 1.3% calcium hypochlorite for 15min with constant stirring, and then rinsed with sterile distilled water. The surface sterilized *V. radiata* seeds were equispacially arranged in perforated paper cups containing 50 g of garden soil. The pots were watered regularly. Four seeds per cup were sowed and monitored physical conditions at regular intervals for optimum growth of plants.

Seed treatment

Seeds of *V. radiata* plants were surface-sterilized in 1.3% calcium hypochlorite for 15 min with constant stirring, and then rinsed with sterile distilled water. Rhizobial inoculation was performed by soaking the *V. radiata* seeds for 15 min in a freshly prepared suspension of R*hizobium* sp.,(10^8 bacterial cells/ ml). Following inoculation with *Rhizobium* sp., the seeds were transferred into pots filled with soil.

Pot culture assay on herbicide and detergent treatment (Khan et al., 2006)

The following experiments were performed in triplicates in order to analyse the variation in results. *Rhizobium* sp., was grown in YEM broth in flasks at 28°C for four days to a cell density of 4×10^8 cells per ml. Seeds of *V. radiata* were surface sterilized and air-dried before being inoculated with *Rhizobium* sp., by dipping the seeds in the liquid culture medium for 1h using 10% gum arabic as the sticker to apply approximately 10^8 cells to each seed. Using soil incorporated applications of commercial formulations to moist soil, 24 h before sowing the seeds in paper pots, the effects of the herbicides (metsulfuronmethyl+chlorimuron ethyl) were evaluated at 200 and 400 µg/kg of soil. Some pots not treated with herbicides were sown with inoculated seeds and used as control treatments for comparison. The pots were watered daily and maintained at 28°C and 60% relative humidity. For each treatment, all plants in pots were uprooted 15days after seedling. The lengths of plant parts (e.g., roots and shoots) were measured. The same procedure repeated for detergent also.

Estimation of chlorophyll content (Sadhasivam and Manickam, 2005)

Approximately 20 mg (0.6 cm^2) of fresh leaf material collected from each uprooted plant and was weighed in eppendorf. Samples were well ground to extract plant sap using mortar and pestle. In next step 1 ml of 100% of acetone mixed with each sample. The homogenate was filtered through two layer cheese cloths and was centrifuged at 2500 rpm for ten minutes. The supernatant was separated and the absorbance was read at 645 and 662 nm. The amount of chlorophyll a and b pigments were calculated using the formula,

$$\text{Chlorophyll a (g/l)} = 11.75\ A_{662} - 2.350\ A_{645}$$
$$\text{Chlorophyll b (g/l)} = 18.61\ A_{645} - 3.960\ A_{662}$$

Results and Discussion

From the past few decades *Rhizobium* sp., was considered as the agriculturally friendly bacteria and considered as biofertilizer for plant growth. These soil bacteria stimulate plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them, improving soil structure and bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds (like herbicides) (Ahemad *et al.*,2012, Hayat *et al.*,2010, and Braud *et al.*,2009).

Due to drastic release of agricultural and household pollutants such as herbicides and detergents into the land affects the growth of bacteria living in the soil too. Thereby establishment of symbiotic association between plant and bacteria also affected. But some of the soil bacteria develops resistance to these pollutants and enhance plant growth. The present study investigates stress resistance in *Rhizobium* sp and assistance to growth of a leguminous plant, *Vigna radiata* under herbicide almix and detergent applied soil.

Identification of Rhizobium sp.

Bacteria isolated from root nodule produced circular, mucoid, white, translucent, large, elevated colonies. Under microscope, Gram negative, long individual, motile rods were observed. In biochemical tests, the isolate gave positive result to indole, methyl red, citrate, catalase, oxidase, urease, sugar fermentation and nitrate reduction tests. Thereby the isolate was confirmed as *Rhizobium* sp.

Herbicide resistance assay

The Rhizobacteria differed considerably in their sensitivity towards different concentration of herbicides on YEM agar plates and showed a greater variation in MIC. Almix herbicide was most damaging at and above 1200 µg/ml (fig- 1). The *Rhizobium* sp., was able to grow at different concentrations of detergents. Density of growth was decreased with increasing concentration of detergent. *Rhizobium* sp., was strictly inhibited to grow above 1800 µg/ml concentrations of detergent in plate assay (fig-2). So, the MIC value of *Rhizobium* sp., is 1200 µg/ml and 1400 µg/ml for almix and detergent respectively. The herbicide was the most damaging while detergent the least. The results reported here are based on studies conducted on YEM agar plates.

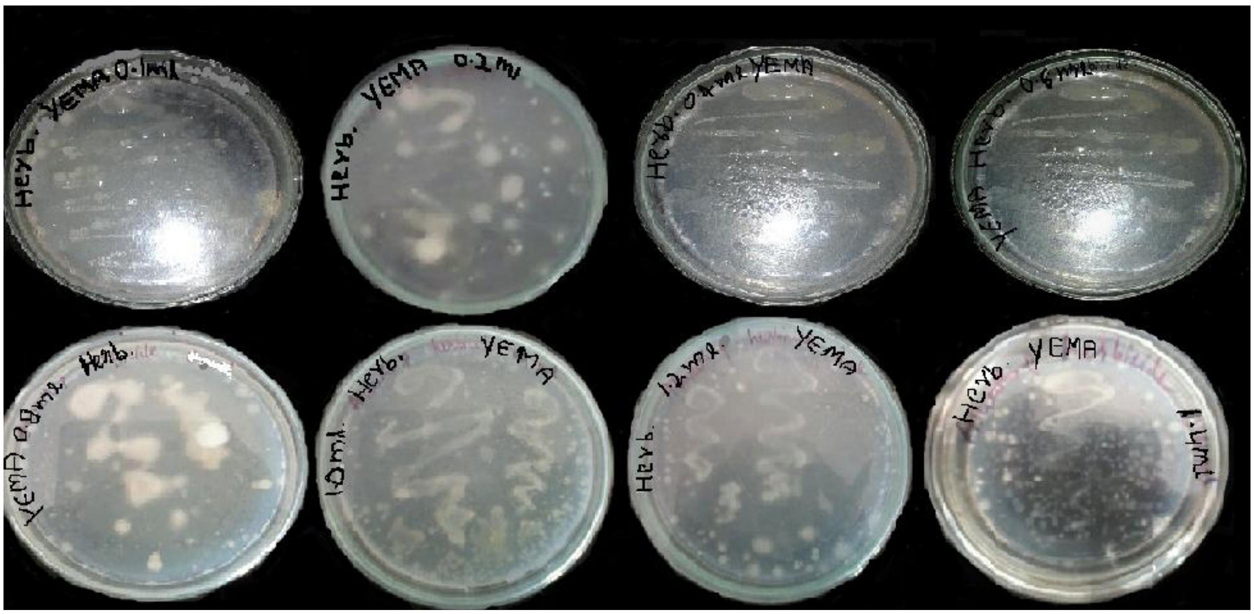

Fig. 1. Herbicide resistance of *Rhizobium* sp., against almix

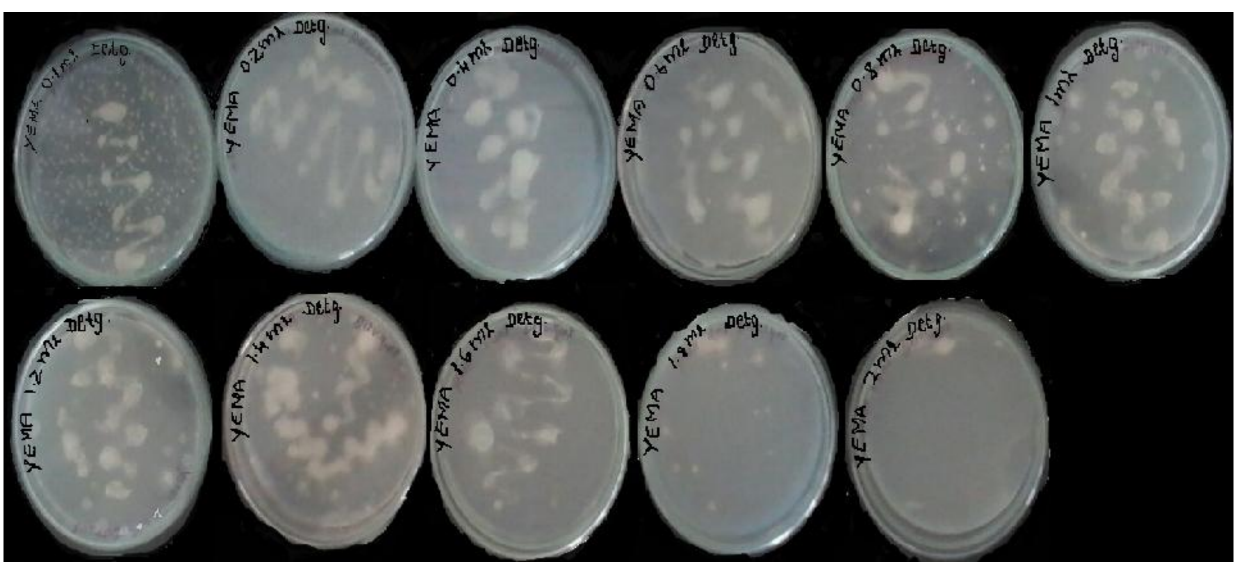

Fig. 2.Detergent resistance of *Rhizobium* sp.

Analysis of pot culture assay on almix treatment

Rhizobium sp., inoculated plants showed 58% and 14% of root and shoot length over uninoculated plants. In plants applied with almix and *Rhizobium* sp., showed 9% and 6% increase in root and shoot growth than untreated plants. At lower concentration (200 µg of almix/kg of soil), *Rhizobium* sp., treatment enhanced the plant growth to achieve normal growth characters. Above this concentration *Rhizobium* sp., treatment was unable to improve plant growth. Minimum plant growth was observed in the plants treated with 400 µg/ kg of almix (Table 1 & fig.3). A common variance of $\pm$ 0.002 recorded among the all calculated values given in table 1. The results shows that *Rhizobium* sp., had developed resistance to lower concentration of almix and could enhance plant growth. Butat higher concentrations (due to bioaccumulation) of almix, drastically affected the growth of both microbial and plant population.

The study results from media inoculation and soil assay varies drastically. This is due to media in which the organisms were inoculated. Researchers have shown that the effect of herbicides on the growth of Rhizobia can be quite different depending upon the method used. Martensson *et al.* (1992) obtained quite different results in liquid broth and in agar based methods and was of the view that the presence of agar may influence the mode of action of the investigated compounds, probably due to the complex formation with the agar. However, the report suggests that the persistence of herbicides when applied in soil is influenced by its adsorption by the soil and many other factors including volatalization, photodecomposition, leaching, and degradation by soil microorganisms. In 2002, Singh and Wright reported that evaluation of the herbicide effects on legume cultivation is complicated because herbicide may not only affect the *Rhizobium* sp., in the free living state in soil and within root tissues but also affect plant growth.

Table 1. Growth rate of *V. radiata* in soil

S.No.	Almix concentration (µg/kg of soil)	Seedling growth (in cm)			
		Rhizobium sp., treated		*Rhizobium* sp., untreated	
		Root length	Shoot length	Root length	Shoot length
1	0	8.1	19.5	5.1	17
2	200	4.8	17.4	4.3	16.5
3	300	4.4	14.5	4.0	13.9
4	400	3.8	13.1	3.4	12.2

Fig. 3. Pot culture assay on almix treated soil

Analysis of pot culture assay on detergent treatment

Maximum seedling growth was recorded in the *Rhizobium* sp., inoculated plants than the uninoculated plants and in the plants treated with different concentration of detergent. Under the given concentrations of detergents, variation in growth was scanty in both *Rhizobium* sp., treated and untreated plants (table 2 & fig - 4). An average variation of 3% and 6.3% increase of root and shoot length was recorded among *Rhizobium* sp., treatment. High concentration of detergent was toxic to seed germination and seedling growth. A common variance of ± 0.002 recorded among the all calculated values given in table 2. These results shows that *Rhizobium* sp., is stressed under detergent treatment because of that there was not much variation in growth of *Rhizobium* sp., treated and untreated plants. The reason according to Nagada *et al*. reported in 2006 that the soap factory effluent was toxic to seed germination and seedling growth when it was diluted it enhanced the seed germination and seedling growth. Inhibition of germination may be due to osmotic pressure of the effluent at higher concentration of total salts making inhibition.

Table 2. Growth rate of *V. radiata* on detergent treatment

S.No.	Detergent concentration (µg/kg of soil)	Seedling growth (in cm)			
		***Rhizobium* sp., treated**		***Rhizobium* sp., untreated**	
		Root length	**Shoot length**	**Root length**	**Shoot length**
1	0	8.1	19.5	5.1	17
2	200	5	18	5	16.8
3	300	4.8	16.8	4.6	16
4	400	4.4	16.4	4.1	15.2

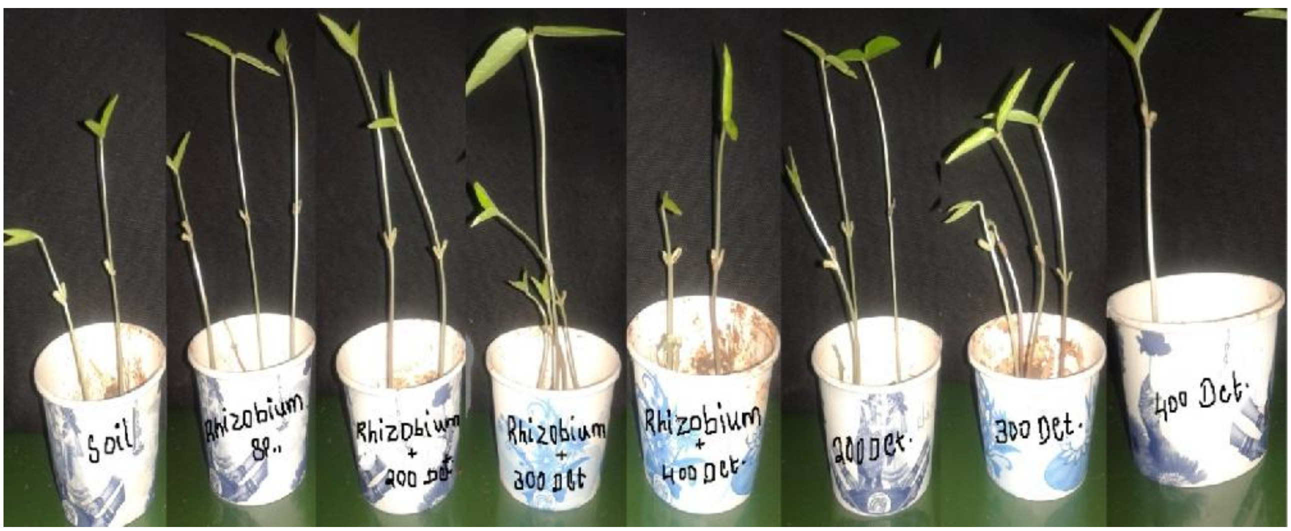

Fig. 4.Pot culture assay on detergent treated soil

Estimation of chlorophyll content

Table-3 presents the data regarding chlorophyll contents of *V. radiata* plants treated with different concentrations of almix and detergent doses. A common variance of ± 0.005 recorded among the all calculated values given in the table 3. The data revealed that maximum chlorophyll content was found in *Rhizobium* sp., inoculated plants. Under stressed conditions, chlorophyll content was estimated to be high at lower concentration of almix and detergent treatment. The plants treated with *Rhizobium*sp., enhanced the accumulation of chlorophyll pigment in the plant. Leaves of plant growing at higher concentrations of almix and detergent (400µg/kg) appeared pale green due to the very low concentrations of chlorophyll. *Rhizobium* sp., treatment in normal plants increased the chlorophyll a & b content to 82% in each. But almix and detergent applications affected the accumulation of chlorophyll a & b decreased to 80% and 40% respectively.

The significance fall in the chlorophyll content under the higher percentage of the detergent concentration might have been due to inhibitory effects of toxicants on chlorophyll synthesis in exposed plants (Singh *et al.,* 2004). But compared to *Rhizobium* sp., untreated plants, treated plants showed overall 5% increase in overall chlorophyll content. The data from this study thus supported the concept that the detrimental effect of herbicide and detergent is primarily bacterium mediated that resulted in the indirect effects on nodulation and yield in leguminous plants (Alonge, 2000).

Table 3. Effect of almix and detergent on photosynthetic pigment in *V. radiata*

Treatment Substance	concentration (µg/kg of soil)	Seedling growth (in cm)			
		***Rhizobium* sp., treated**		***Rhizobium* sp., untreated**	
		Chl. a	**Chl. b**	**Chl. a**	**Chl. b**
Normal plant	**0**	8.4	13.2	4.6	7.2
Almix	200	2.8	4.4	2.6	4.2
	300	1.9	3.9	1.7	2.8
	400	1.2	2.4	0.4	0.5
Detergent	200	4.6	7.6	3.8	6.5
	300	4.1	6.3	3.2	5.1
	400	2.5	3.9	2.4	3.7

Key: Chl. Chlorophyll (g/l)

Conclusion

The use of different chemicals in agriculture field affects both cultivated plants and rhizhosphere microorganism. The present study investigate the effect of almix on development of stress resistant symbiotic bacteria and its effect on *V. radiata*. The MIC value of the isolate ranged between 1200 µg/ml of herbicide and 1400 µg/ml of detergent. When *V. radiata* was grown in soil amended between 200 - 400 µg /kg of almix and detergent variable effect on plant growth were recorded. Higher concentration of both herbicide and detergent reduced the root and shoot length of the plants. The effect of stress on the plant parts was more severe at 400 µg/kg of soil of herbicide and detergent treatment. This work exemplifies the inhibitory effect of herbicide and detergent on nodule bacterium and inoculated in *V. radiata*, resulting in a substantial decline in the seed germination, seedling growth and chlorophyll content of *V. radiata*. It can be concluded that the higher concentration of detergent and almix was toxic to the plant growth. The study recommends the use of highly damaging herbicide almix resistant and detergent resistant indigenous *Rhizobium* sp., for commercialization as biofertilizer.

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