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A Study on Characterization, Growth Response and Nitrogen Fixing Efficacy of Fungicide Resistant Rhizobium

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ABSTRACT: Rhizobium acts as a primary symbiotic fixer of nitrogen. The present study was focused on isolation, biochemical characterization, temperature, pH, salt tolerance and Fungicide tolerance ability and nitrogen fixing efficacy of *Rhizobium* sp. from root nodules of leguminous plant Groundnut from different areas in the semi arid regions of Tamil Nadu. Out of 43 isolates, 10 strains were found to be effective in detoxifying the fungicide used in the current study, fixed nitrogen even in the presence of Carbendazim and tolerated up to 5% Nacl concentrations. Therefore such isolates may be beneficial strains to improve growth and development of leguminous plants under various environmental stress conditions.

Keywords: *Rhizobium*, Nitrogen fixation, Fungicide detoxification, Leguminous plants

I.INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is one of the main oil yielding croups. The cotyledons are rich in fatty edible oil. The oil extracted and used in cooking is known as groundnut oil. The seeds are also edible. The oil cake after the extraction of the oil is good cattle feed. The cotyledons are also rich in proteins. The plant is extensively cultivated for its underground pods. The plant (*Arachis hypogaea* L. – Papilionaceae) is a native of Brazil, but now cultivated extensively all over the world. The seeds have 40-50% oil. Oil cake is a good fertilizer.

Nitrogen is one of the most essential nutrients required for growth and development of Groundnut plants. The nitrogen can be provided in the form of chemical fertilizers and biological fixation. Among these two, biological nitrogen fixation is the less costly and ecofriendly. Leguminous plants fix atmospheric nitrogen into soil with the help of nitrogen fixing micro-organisms [1]. *Rhizobium* acts as primary symbiotic fixer of nitrogen by infecting root of leguminous plants. *Rhizobium* bacteria stimulate the growth of leguminous plants by fixing atmospheric nitrogen into soil by symbiotic interaction. Several environmental stresses may affect the nitrogen fixation in plants [2]. It includes salinity, water stress, soil pH, temperature, Pesticides and heavy metals [5]. Waraporn Payakapong [13] reported that, nearly 40% of total world land is affect due to salinity. Most of the leguminous plants are more sensitive to salinity and they require slightly acidic pH. Constant use of pesticides tend to have a negative impact on soil microflora and the "Beneficial Nitrogen Fixing Organisms" face a challenge for survival in the soil habitat as they are exposed to these pesticides. Fungicides are used to kill or inhibit the growth of fungi that cause economic damage to crop or ornamental plants. Fungicides can either be contact, translaminar or systemic. Among this Carbendazim is a broad spectrum benzimidazole carbamate fungicide with systemic activity. It inhibits fungal mitotic microtubule formation. It controls wide range of diseases on cereals, fruits, grape, sugar beet, ornamentals and vegetables. Trade names: Aimcozin, Bendazim, Triticol, Carbendazole. Mancozeb is a co-ordination product of zinc ion and manganese ethylene bisdithiocarbamate (EBDCs). It is classified as a contact fungicide with preventive activity. It inhibits enzyme activity in fungi by forming a complex with metal containing enzymes including those involved in production of ATP. Trade names: Dithane, Manzeb, Nemispot, Manzane.

In legumes, proliferous root growth and abundant lateral branching of hairy roots are considered to be useful for improving nitrogen fixation. In this process, the enzyme nitrogenase plays a key role. The ammonia may be interconnected by host cell enzymes to other usable from such as nitrate and nitrate ions, amino acids and nitrogenous bases. Leguminous plants with *Rhizobium* containing root nodules are able to fix ten times more nitrogen. It improves crop yield and enriches the surrounding soil.

In this study, *Rhizobium* isolated from different fields was characterized based on microbiological assays and tested for its tolerance towards temperature, pH and Salt. The strains were analyzed for its growth response pattern against different concentrations of Carbendazim and Mancozeb (Fungicide). This response was then analyzed on the basis of the change in specific growth rate, fungicide detoxication assay and total nitrogen fixation by Micro-kjeldahl method.

II. MATERIALS AND METHODS

II .1.SAMPLE SITES:

The samples were collected from 15 different groundnut fields of Vellore, Thiruvallur and Kancheepuram districts of Tamil Nadu. The samples were randomly collected, mixed thoroughly packed in polythene bags and labelled the location, dated and brought to the laboratory and stored for further studies (Figure 1).

FIGURE 1 SAMPLE SITES

II. 2. ISOLATION OF *RHIZOBIUM* FROM LEGUMINOUS ROOTS:

Roots of leguminous plants were washed with running tap water to remove adhering soil particles. Healthy, unbroken and firm root nodules were selected and washed with running tap water. The nodules were immersed in 0.1%$Hgcl_2$ for five minutes to surface sterilize. The nodules were washed repeatedly in sterile water for 3-4 times to get rid of the sterilizing agent. The nodules were placed in 70% Ethyl Alcohol for 3 minutes. The nodules were washed repeatedly in sterile water. The nodules were crushed in 1ml of water with a sterile glass rod. An aliquot of the sample is then diluted and streaked on the surface of YEMA plates containing Congo red. The plates were incubated at 28^0 C for 3-5 days until colonies appear. The isolates were identified and preserved for further investigations [6].

II. 3. MICROBIOLOGICAL ASSAYS:

The morphological traits evaluated comprised colony morphology, mucous production and change in pH of medium during growth and growth rate. Mucous morphology analysis was based on type, elasticity and appearance, while colony morphology parameters were diameter, form, transparency and color [6]. Gram staining reaction was performed to evaluate type of strain.

II.3.1 METHYLENE BLUE AND GENTIAN VIOLET TREATMENT

In this assay, methylene blue dye was added to a concentration of 0.1% to the growth medium and inoculated with *Rhizobium*. Incubation was given at 29.4°C for 2-7 days and observations were made. Similar experiment was done with gentian violet at the concentration of 0.1% [9].

II.3.2 GLUCOSE PEPTONE AGAR (GPA) AND LACTOSE ASSAY

GPA assay was performed to determine the capability of the micro- organism to utilize glucose as the sole carbon source for its growth. GPA medium (40 g/L glucose, 5 g/L peptone, 15 g/L agar, pH 7.0) was inoculated with *Rhizobium* cultures, incubated and growth was observed. Similarly lactose assay was performed to determine the capability of the micro-organism to utilize lactose present in medium (10 g/L lactose, 5 g/L peptone, 3 g/L beef extract, 15 g/L agar, Ph 7.0) as the sole carbon source for its growth.

II.3.3. STARCH HYDROLYSIS

The test was performed so as to determine capability of micro- organism to use starch as carbon source [8]. Starch agar media (5 g/L peptone, 2 g/L potato starch, 3 g/L beef extract, 15 g/L agar, pH 7.0) were inoculated with Rhizobium, incubated and analyzed. In the presence of starch,

the production of extra- cellular enzymes occurs indicating the potential of the organism to use starch as carbon source. Iodine test was used to determine capability of microorganisms to use starch. Drops of iodine solution (0.1 N) were spread on 24 h old cultures grown on Petri-plates. Formation of blue color indicated non-utilization of starch and vice versa.

II.3.4. FLUORESCENCE ASSAY

The test was performed to determine the ability of the isolates to fluoresce [14]. King's Medium (2 g/L peptone, 1.5 g/L MgSO4, 1.5 g/L K2HPO4, 10 mL/L glycerol, 15 g/L agar, pH 7) was prepared and inoculated aseptically with Rhizobium cultures. Culture was incubated at 29.4°C and after 48 h observations were made under UV- light source.

II.3.5. GELATINASE TEST

The test was performed to determine capability of microorganisms to produce gelatinase enzyme and use gelatin as media source. Degradation of gelatin indicates the presence of gelatinase enzyme [6]. The actively grown cultures were inoculated in nutrient gelatin medium (5 g/L peptone, 3 g/L beef extract, 12 g/L gelatin) and grown for 48 h. On subjecting the growing culture to low temperature treatment at 4°C for 30 min, the cultures which produce gelatinase remains liquefied while others due to presence of gelatin becomes solid.

II.3.6. TRIPLE SUGAR IRON AGAR TEST

The test was performed to determine the capability of isolates to use various carbohydrate sources e.g. sucrose, glucose, lactose, etc as media for growth. Triple Sugar Iron Agar media consisted of beef extract 3 g/L, yeast extract 3 g/L, peptone 15 g/L, NaCl 5 g/L, lactose 10 g/L, sucrose 10 g/L, dextrose 1 g/L, ferrous sulfate 0.2 g/L, sodium thiosulfate 0.3 g/L, phenol red 0.24 g/L, agar 15 g/L, pH 7.0 [15]. After inoculation and incubation, color on the butt and the slant was observed. On this basis capability of organisms to use carbohydrates, three possible ob- servations could occur.

Alkaline (red) slant and acid (yellow) butt:

Only glucose fermentation has taken place. In the slant, acid produced is oxidized and alkali is formed. In the butt, acid reaction is maintained because of less oxygen and the slow growth of organisms.

Acid (yellow) slant and acid (yellow) butt:

Lactose and sucrose fermentation has occurred. Because of presence of these substances in higher concentrations in the media, acid reaction is maintained in both slant and butt.

Alkaline (red) slant and alkaline (red) butt:

No carbohydrate fermentation occurred. Peptones are catabolised to form alkaline pH.

II.3.7. MANNITOL - MOTILITY TEST:

Mannitol motility test medium was prepared. Appropriate cultures were inoculated and left for overnight incubation

II.3.8 . BROMOTHYMOL BLUE TEST:

Bromothymol blue was added to the growth medium. The culture was streaked onto the agar plates and checked for growth. Plates were incubated at room temperature for 24 hours.

II.1.3.9. CATALASE, OXIDASE AND UREASE TEST:

Catalase activity was observed by stirring the culture in a drop of hydrogen peroxide (10% by w/v), while oxidase activity was tested. Production of ammonia from urea was examined by Christensen urea agar with phenol red as an indicator [16].

1I.4. TEST FOR NACL, PH AND TEMPERATURE TOLERANCE

Rhizobium culture was grown in triplicates on YEM medium having different concentrations of NaCl ranging from 1 to 6% (w/v). Growth was determined by measuring the optical density at 600 nm after 48 h of inoculation [17].

In order to analyze the effect of pH variations on the growth of the organism, media were prepared with pH 4.0, 7.0 and 9.0. After inoculation, the plates were kept at 29.4 and 37°C separately to analyze the effect of temperature along with pH [17].

I1.5. IMPACT ANALYSIS OF FUNGICIDE ON THE RHIZOBIAL CULTURES

50ml of Yeast Extract Mannitol broth was prepared, sterilized by autoclaving and cooled to room temperature. For the analysis, each of the isolate was inoculated into the various flasks containing different concentrations of the fungicide (100 – 1000µg/ml). The flasks were kept for incubation at room temperature on a rotary shaker overnight. Yeast Extract Mannitol Agar with Congo red plates was prepared. A loop full of the broth from the different flasks was streaked onto the agar plates correspondingly labeled to check for growth. The plates were incubated at room temperature overnight and checked for growth.

II.5.1. FUNGICIDE DETOXICATION ASSAY

To study the ability to detoxicate the fungicide by the resistant isolates of rhizobia selected from the 1st analysis (resistance study), the broth cultures of the selected isolates were maintained and used. 50ml of Yeast Extract Mannitol Broth was prepared in 125ml Erlenmeyer flasks, sterilized by autoclaving and cooled at ambient temperature. Strains resistant up to 1000µg/ml of the fungicide

were selected for the study and inoculated into the broth, incubated at room temperature of 30°Celsius for 24 hours. The growth was measured turbidimetrically for every half an hour. The specific growth rate constant and generation time was calculated using the formula:

$$K = \frac{\text{max log value} - \text{min log value}}{t}$$

$$\text{Generation time} = \frac{0.3010}{K}$$

II.6. ESTIMATION OF TOTAL NITROGEN FIXED BY MICRO-KJELDAHL METHOD

The total nitrogen fixed by the selected fungicide resistant rhizobial cultures were estimated by Micro-Kjeldahl's method. The resistant strains of rhizobia were inoculated into YEMB with 1000µg/ml of fungicide and into YEMB with fungicide. All the above cultures were incubated for 48 hours in a rotary shaker at room temperature.

II.6.1. SAMPLE PREPARATION FOR ESTIMATION OF NITROGEN FIXED

A known weight of the sample was taken and mixed with 7.5g of sodium sulphate and 0.5g of copper sulphate. Digestion procedure was carried out with 15ml of concentrated sulphuric acid. Digested samples were distilled with 32% sodium hydroxide (90ml) until all ammonia passed over into boric acid (50ml).The mixture was then titrated with the standard 0.1N Hydrochloric acid along with the blank.

Calculation:

$$N_2 = \frac{(\text{sample} - \text{blank}) \times 1.4007 \times \text{N of Hcl}}{\text{weight of the sample}}$$

III. RESULTS AND DISCUSSION

The isolates obtained from the root nodules of Groundnut plants were designated according to the sites involved(Table 1).

TABLE 1: ISOLATES OBTAINED FROM SAMPLE SITES

S.NO	DISTRICTS	AREA OF COLLECTION	ISOLATES
1	THIRUVALLOOR	Pallipet(W)	TRh1, TRh2,TRh3, TRh4
2	THIRUVALLOOR	Thiruvallor(c)	TRh12,TRh13,
3	THIRUVALLOOR	Kizhicheri(S)	TRh5,TRh6,TRh7,TRh8,
4	THIRUVALLOOR	Gummidipoondy(N)	TRh14, TRh15
5	THIRUVALLOOR	Red hills(E)	TRh9,TRh10,TRh11
6	KANCHIPURAM	Uttiramerur(W)	KRh10,KRh11,KRh12
7	KANCHIPURAM	Chengalpet(c)	KRh1,KRh2,KRh3,KRh4,KRh5
8	KANCHIPURAM	Tiruporur(E)	KRh13,KRh14
9	KANCHIPURAM	Cheyur(S)	KRh15,KRh16
10	KANCHIPURAM	Kunnathur(N)	KRh6,KRh7,KRh8,KRh9
11	VELLORE	Gudiyattam(N)	VRh3,VRh4,VRh5
12	VELLORE	Nemili(w)	VRh7,VRh8,VRh9
13	VELLORE	Katpadi(C)	VRh1,VRh2
14	VELLORE	Thirupattur(S)	VRh6,VRh10,VRh11
15	VELLORE	Jolarpet(E)	VRh12

Colonies of *Rhizobium* sp. Were obtained on YEMA medium after incubation at 30ºC for two days. General microscopic view of the isolates showed them to be rod cells and Gram negative in nature and highly motile. On Yeast Extract Mannitol Agar, 2mm, circular, colourless, raised, smooth, translucent, highly mucoid colonies with musky odor were observed. Rhizoidal cells were able to grow on the GPA media showing the utilization of glucose as the carbon source by the *Rhizobium* . It is a confirmatory test for *Rhizobium* and these are able to utilize glucose as carbon source [20]. It was observed that rhizobial cells do not produce gelatinase enzymes as medium containing gelatin solidified when kept at 4ºC for 30 as well as 60 min. Negative gelatinase activity is also a feature of *Rhizobium* [18]. Positive results were obtained from the starch hydrolysis assay. On subjecting inoculated plates to iodine test, clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas, blue colour appears on no growth areas. This indicates that the isolates have the potential to hydrolyze starch present in the medium. [8] *Rhizobium* strains can utilize starch obtained from different sources. Yellow slants and red butt was obtained showing the utilization of glucose and sucrose in the triple sugar ion agar medium [19]. Methylene blue, Gentian blue and Bromothymol blue tests showed negative results. Earlier studies also indicated that rhizobial cells were unable to grow in the presence of such dyes [22]. The results of microbiological assays were tabulated as in **TABLE NO: 2.**

TABLE NO.2
MORPHOLOGICAL CHARACTERISTICS OF ALL THE RECOVERED ISOLATES OF *RHIZOBIUM*

S.No	CHARACTERISTICS	RESULTS
1.	Colour of colony	Colorless mucoid colonies
2.	Elevation	Convex
3.	Opacity	Opaque
4.	Spore formation	Non spore forming
5.	Oxygen demand	Aerobic
6.	Size of colony	2-3 mm
7.	Gram staining	Gram negative
8.	Motility	Highly motile
9.	Capsule	+
10.	Generation Time	344 – 460 minutes
11.	Gelatin Hydrolysis	-
12.	Starch hydrolysis	+
13.	1% Methylene blue treatment	-
14.	1% Gentian violet treatment	-
15.	King's medium	-
16.	Mannitol test	+
17.	Bromothymol blue test	-
18.	Catalase	+
19.	Oxidase	+
20.	Urease	-
21.	GPA Medium	+
22.	TSI	Positive with gas production

It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. Salt stress may decrease the efficiency of the Rhizobium- legume symbiosis by reducing plant growth and photosynthesis survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function[23].
Some rhizobial isolates have been shown to grow under high salt conditions 4-5% [5]. Results indicated that cells were able to grow up to 5 % NaCl containing medium but unable to grow on higher concentrations, showing that the isolates were sensitive to higher salt(**TABLE NO. 3**). pH is an important parameter for the growth of the organism. Slight variations in pH of medium might have enormous effect on the on the growth of organism. Superior growth of Rhizobium has been reported at neutral pH. Results showed that cells were able to grow only at pH 7.0 at 30ºC temperature. Growth of Rhizobium was observed at 37 ºC after 2 days of incubation. No growth was observed in medium with pH 4.0 and 9.0. Similar observations were made in previous investigations [21] [7].

TABLE NO.3 TESTS FOR SALT, TEMPERATURE AND PH TOLERANCE

PARAMETERS / STRAINS	SALT %						pH						TEMPERATURE		
	1%	2%	3%	4%	5%	6%	4	5	6	7	8	9	29 ºC	30 ºC	37 ºC
Vellore strains(VRh1 - VRh12)	++	++	++	+	+	-	-	++	++	++	++	-	++	++	++
Thiruvalloor strains(TRh1-TRh15)	++	++	++	++	+	-	-	++	++	++	++	-	++	++	++
Kancheepuram Strains(KRh1-KRh16)	++	++	++	+	+	-	-	++	++	++	++	-	++	++	++

++ GOOD GROWTH + MODERATE GROWTH - NO GROWTH

The rhizobial cultures isolated from Groundnut plants, from different areas were analyzed for resistance to the different concentrations of the fungicide. Out of the total 43 isolates obtained from the 3 districts, 10 strains showed resistance to 1000 µg/ml fungicide **(**CARBENDAZIM 12 % + MANCOZEB 63 %) concentration, Procured from Hari Seeds, Red hills. Those 10 strains were further processed for Fungicide detoxification studies. Specific growth rate constant and Generation time was calculated for the 10 strains in the presence and absence of fungicide. There were also sensitive strains which showed no resistance. This impossibility to survive could be due to incompatibility and failure to get acclimatized to the stress of addition of the fungicide. The metabolism of the organism would have been halted fully and the bacteria might have entered into a stage from where it could not come back into life. Odeyemi Olu [3] worked on the impact analysis of thiram on rhizobia which stated resistance only upto 200µg/ml of thiram. The resistance hence is due to the capacity of the organism to overcome the stress of fungicide addition, utilize/detoxicate it to grow and multiply. Pandey P.K [20] said through their experiment trails that the number of nodules increased with lower concentration of pesticide but increased concentration reduces the growth and therefore the morphology also changes. But the resistance of over 1000µg/ml can be considered as a promising scope for detoxication of fungicide polluted habitats. Olu Odeyemi[3] and Martin Alexander in 1977 surveyed and studied the detoxication efficacy of the resistant strains at maximum concentration of fungicide used for experiment. The resistant strains upto 1000µg/ml of fungicide showed detoxication efficacy which is usually suggested to occur in the late log phase or early log phase so that once the bacteria gets over the metabolic stress they multiply. But due to the marked decrease in the number of cells showed initially was due to the metabolic stress and the extended lag phase is the time required by the organism to overcome the stress, breakdown the fungicide and utilize it for itself. The results are tabulated in **TABLE NO.4** and **TABLE NO. 5 and FIGURE NO. 2.**

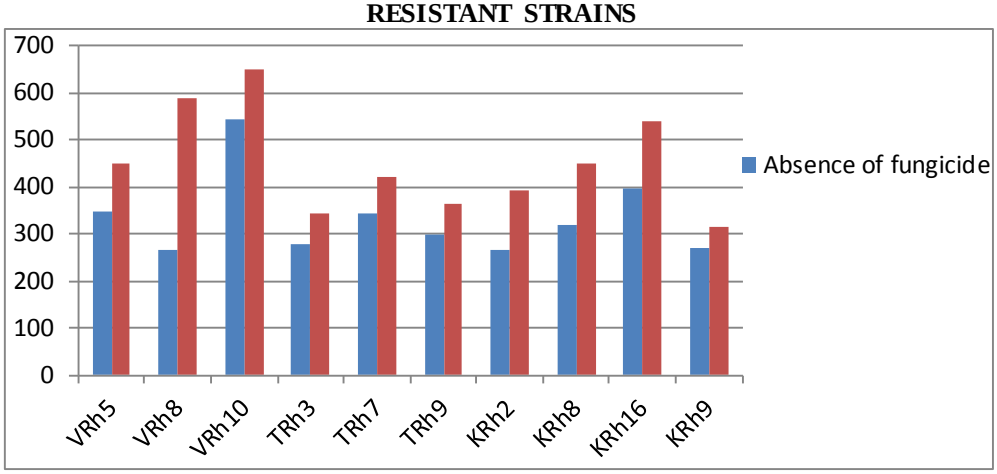
TABLE NO.4 IMPACT ANALYSIS OF FUNGICIDE ON THE RHIZOBIAL CULTURES

STRAINS	Concentration of fungicide in µg/ml									
	100	200	300	400	500	600	700	800	900	1000
VRh5	+	+	+	+	+	+	+	+	+	+
VRh8	+	+	+	+	+	+	+	+	+	+
Vrh10	+	+	+	+	+	+	+	+	+	+
TRh3	+	+	+	+	+	+	+	+	+	+
TRh7	+	+	+	+	+	+	+	+	+	+
TRh9	+	+	+	+	+	+	+	+	+	+
KRh2	+	+	+	+	+	+	+	+	+	+
KRh8	+	+	+	+	+	+	+	+	+	+
KRh16	+	+	+	+	+	+	+	+	+	+
KRh9	+	+	+	+	+	+	+	+	+	+

TABLE NO.5 IMPACT ANALYSIS OF FUNGICIDE ON GENERATION TIME OF THE RHIZOBIAL CULTURES

STRAINS	GENERATION TIME IN MINUTES	
	Absence of fungicide	Presence of fungicide
VRh5	348.9	450
VRh8	266.6	587.2
Vrh10	543.1	650.8
TRh3	280	345
TRh7	345	420
TRh9	298	365
KRh2	265	392
KRh8	320	450
KRh16	398	540
KRh9	270	315

FIGURE 2 IMPACT ANALYSIS OF FUNGICIDE ON GENERATION TIME (MINUTES) OF RESISTANT STRAINS



RESISTANT STRAINS

Total nitrogen assay by Micro-kjeldahl method [11] evaluated the levels of nitrogen fixed easily. The strains which showed resistance upto 1000 µg/ml fungicide were used for estimation studies on total nitrogen fixed by Micro – Kjeldahl method. It was found that there was a marked increase in nitrogen fixation by the organism cultured in the presence of fungicide. In this context, most of the isolated strains markedly enhanced the nitrogen fixation efficacy in the presence of 1000µg/ml of fungicide than in the absence of fungicide **(TABLE NO.6)**. Thus we have to agree to the work done by Linda. B. Lennox and Martin Alexander [12] which stated that there occurs an enhancement in nitrogen fixation due to the resistance towards the fungicide. It is said that presence of small amounts of nitrogen initiates the nitrogen fixing efficacy. The enhancement of the nitrogen fixing efficacy observed could be attributed to the presence of small amount of combined nitrogen in fungicide as well as the modification of genes due to the presence of fungicide or its degradation products.

Few strains showed only a marginal nitrogen fixation in the presence of fungicide. Absence of nitrogen fixation or low nitrogen fixation in the presence of fungicide could be related to the toxicity due to the fungicide itself or the degradation products**(FIGURE NO.6 & 7)**. Earlier studies reported the in vitro effect of seed dressing fungicide thiram and its degradation products which does suggest the organism's sensitivity to either the fungicide on the whole or its degradation products. Inability to fix nitrogen could also be related to the suppression of the genes related to nitrogen fixation by the degradation products of fungicide [4].

The combined use of rhizobia with fungicides also produced greater number of nodules than used alone [10]. There are also reports where seed treatment with fungicides can cause an increase, a reduction or no effect on nodulation [24, 25].

This investigation brings to notice the continuous exposure of fields to the pesticides and thus enlightening the scientific community to frame remedies to overcome such pesticidal pollutions as well as promote the use of biofertilizers specially rhizobia. Even more significantly rhizobial strains resistant to fungicide which also showed enhanced nitrogen fixing capacity at the right concentration of the fungicide for developing seed dresses and can be made available to farmers.

The results obtained are a part of successful efforts to contribute to screen bacteria from root nodules which can be future candidates for increasing productivity of agricultural crops. The results on screening of rhizobial strains resistant to various stress factors and also to determine its tolerance levels to fungicide and nitrogen fixation efficacy may help to grow crops in highly polluted soils.

TABLE NO. 6 ESTIMATION OF TOTAL NITROGEN FIXED BY MICRO – KJELDAHL METHOD

STRAIN NO:	AMOUNT OF NITROGEN FIXED IN %	
	ABSENCE OF FUNGICIDE	PRESENCE OF FUNGICIDE
VRh5	0.28	0.23
VRh8	0.06	0.12
VRh10	0.07	0.08
TRh3	0.8	0.8
TRh7	1.1	0.77
TRh9	0.9	0.67
KRh2	0.75	0.68
KRh8	0.45	0.39
KRh16	0.05	0.07
KRh9	0.82	0.65

FIGURE NO.6 NITROGEN FIXATION EFFICACY BY RESISTANT STRAINS

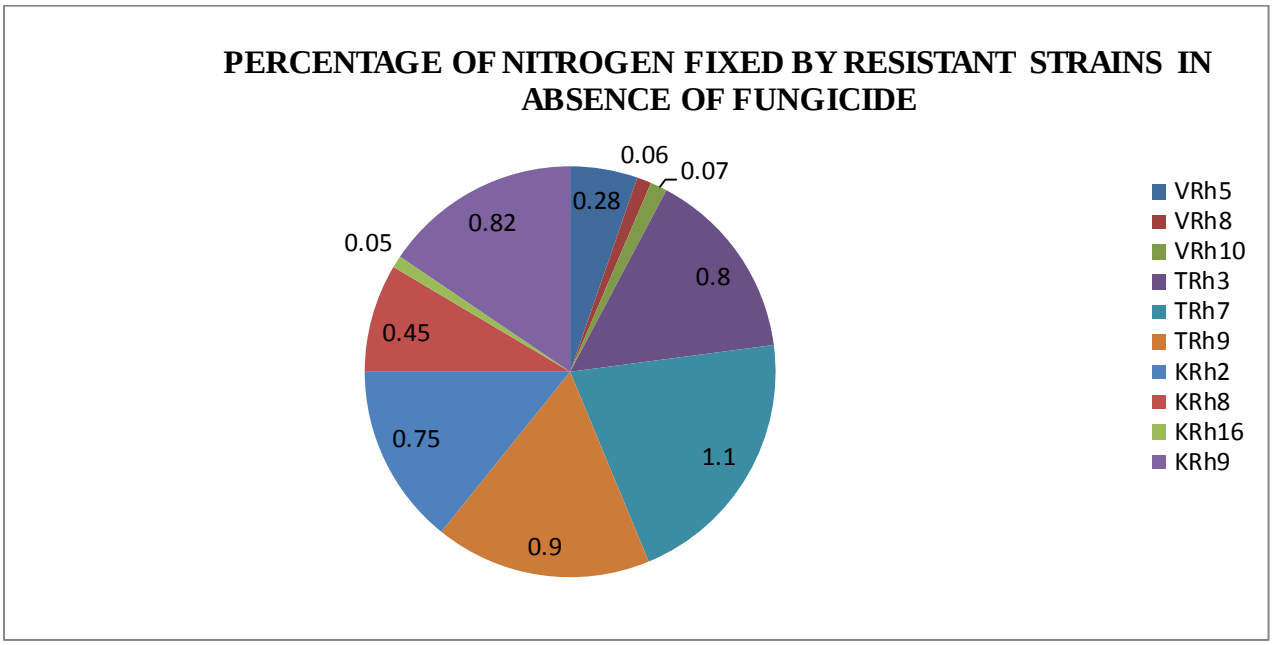
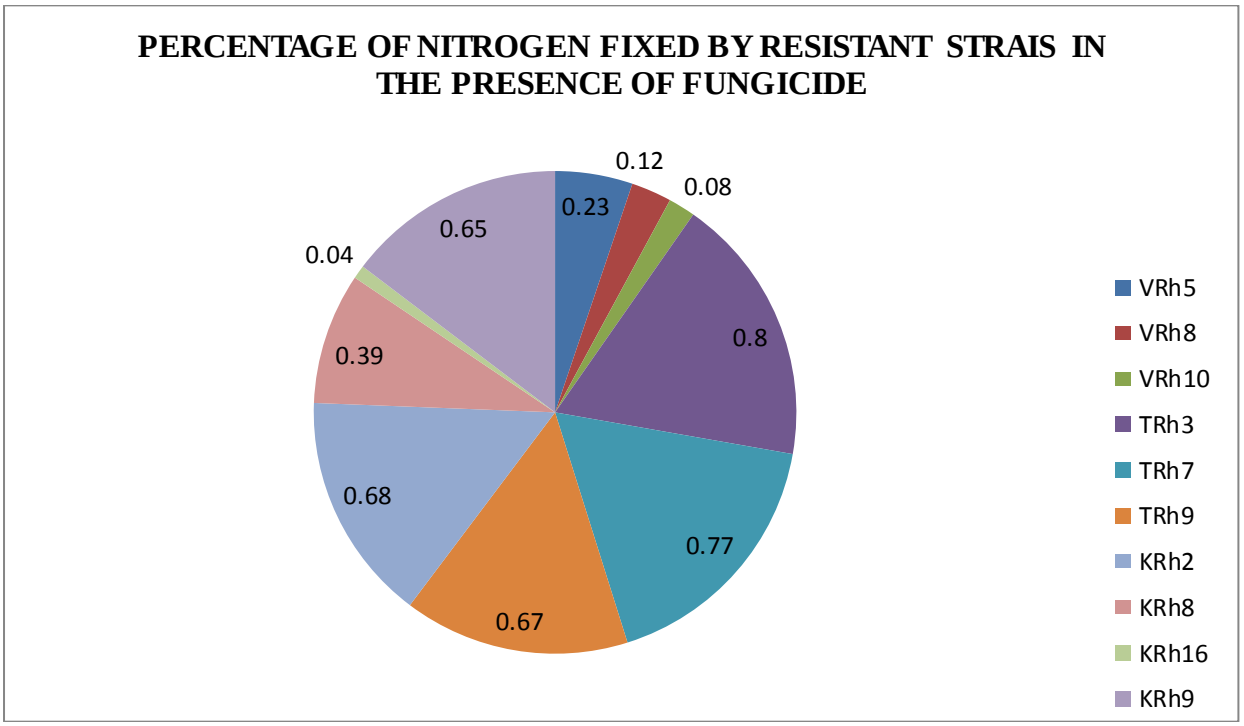


FIGURE NO.7 NITROGEN FIXATION EFFICACY BY RESISTANT STRAINS IN PRESENCE OF FUNGICIDE



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