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MICROFLORA ASSOCIATED WITH THE RHIZOSPHERE OF *CALOTROPIS GIGANTEA*

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ABSTRACT

The study of the rhizosphere of *Calotropis gigantea* revealed that during all its stages of growth the microbial population in the rhizosphere was higher than that occurring in the control soil. The bacterial, actinomycetal and mould populations showed their individual characteristic variations proving that the rhizosphere, of each plant, is a special zone of microbial activity. These population changes were accompanied by a rhizosphere effect, the degree of which varied with the development of plant.

The high count of actinomycetes in the rhizosphere, a factor which led to this systematic study, was established to be caused by a *Nocardia* sp. dependent upon glutamate for it's growth. This *Nocardia* sp. showed antibiotic activity against a culture of *Saccharomyces cerevisiae* and a yeast sp. flourishing well in the soil away from the rhizosphere influence. The reason(s) for this unusual association of a plant with a *Nocardia* sp. remains to be settled.

The HTPI method proved useful in the isolation of actinomycetes from the rhizosphere. It facilitated equally well in the isolation of the heat-resistant types (mostly sporeformers) of microorganisms occurring in rhizosphere.

All the isolated actinomycetes strains were found to be pectinolytic.

INTRODUCTION

Since Hiltner[1], in 1904, named the zone influenced by root excretions which showed increased activity of certain microorganisms as 'rhizosphere', many investigators have shown that soils in the vicinity of the roots usually support noticeably higher number of microorganisms than those away

from the plant. Early investigations have been ably reviewed by Lochhead[2], who called particular attention to the occurrence in the rhizosphere of appreciable number of actinomycetes.

During an investigation of the actinomycetal flora of soil[3], it was observed that rhizosphere of *Calotropis*, a plant of economic potential and medicinal value[4], harbours an appreciably large load of actinomycetes than does the surrounding soil. It was considered of interest to study the rhizosphere flora of this plant and the results obtained are presented in this paper.

MATERIALS, METHODS AND RESULTS

Materials. The *Calotropis* sp. used in this study was *C.gigantea* R. Br.[4,5] obtained from waste unmanured land. The soil was of red loam type. The period of study was from July, 1962 to February, 1963.

Methods. The examination of the rhizosphere was made at different stages of plant growth and development. Simultaneously, soil samples away from the roots, but within the typical zone, (control soils, *C*) were collected. For the former study, plants were carefully dug out from the soil and the superfluous soil attached to the roots was gently shaken off the root system. The entire root system was cut out and transferred into a measured amount of sterile physiological saline under aseptic conditions. The container was then shaken vigorously and the root system was further washed out into the flask before being removed and discarded. Suitable dilutions were made of this suspension and then it was filtered through a dried and pre-weighed Whatman No. 1 filter paper which, after filteration, was dried to a constant weight. Pooled control soils(*C*) from 10 borings were also treated in a like manner. In addition to this, after the plants were dug out, the surrounding soil(S) from the uprooted pit was also included in the studies with a view to ascertain the differences, if any, in the microflora of this region.

The microbial types and populations were determined by surface plating 3 suitable dilutions, in duplicate sets, on soil-extract agar(pH 7.2)[6]. One of the sets was incubated in a routine manner for estimating the total populations and the other was pre-incubated at high temperature (110°*C* for 10 mins.) according to the High Temperature Pre-Incubation(HTPI) method described earlier[3]. The latter procedure permitted isolation of actinomycetes in addition to enumeration of the heat-resistant organisms comprising principally the spore-bearers. Incubation in both the procedures was continued for 15 days at 30°*C*. Total bacterial, actinomycetal and mould counts were made by visual inspection of colonies, aided by microscopic examination wherever necessary and were expressed as number per gram

of oven dry soil and from these the microfloral population of rhizosphere to control soils ratios($R:C$) were calculated. The results are summarised in Table I. The observations on the percentage incidence of bacteria, actinomycetes and moulds in relation to the total microflora during the various developmental stages of plant are presented in Fig. 1.

Fluctuations in the microbial population

(*a*) *Bacteria.* The bacterial count increased steadily for the first 7 months, but dropped down sharply during the flowering stage and remained so in the mature plants. In the control soil the count was relatively low but constant. The percentage incidence followed the same pattern as witnessed in total counts.

(*b*) *Moulds.* The mould count was high throughout and it tended to increase gradually during the maturation and the flowering stages, whilst the control soil was showing steady population. The percentage incidence in the rhizosphere increased with the plant whereas in the control soil it remained constant.

(*c*) *Actinomycetes.* The population increased at a steady rate for about 3 months and at the flowering stage registered a sudden increase. The fully mature plant carried the highest count in it's rhizosphere. The control soils, on the other hand, either revealed a fall in numbers or maintained the usual level. Here, the percentage incidence dropped after the first month and thereafter slowly increased until the maturation of the plant. Their incidence was almost constant in the control soils.

The surrounding soil (S) showed comparatively less number of micro-organisms than those encountered in the rhizosphere, the density of population showing the trend,

moulds > bacteria > actinomycetes

An interesting observation made was that the high incidence of actinomycetes was attributable to the dominations of a particular species and on a thorough examination of 9 specimens it became abundantly clear that a *Nocardia* species was responsible for the occurrence. A brief description of the organism studied[7,8] is given in Table II. A study of it's nutritional requirements using Stokes and Gunness basal medium[9] revealed that although the organism did not demand any vitamins for it's growth, it had a specific requirement for glutamic acid.

R:C ratio. In case of actinomycetes and moulds this ratio increased with the age of the plant, but in case of bacteria it decreased at the maturation and flowering stages of the plant.

TABLE

Developmental Variation in Microbial

Age of plant in months	Height of plant (cm)	Root length (cm)	pH of soils		Bacterial population per gram of		R:C Ratio (Bacteria)
			Rhizo-sphere soil (R)	Control soil (C)	R	C	
$\frac{1}{2}$	4.5	3.5	5.8	5.7	2.5×10^8	2.5×10^8	1:1
1	6.5	6.0	5.8	6.0	6.8×10^8	2.4×10^8	2.7:1
$3\frac{1}{2}$	11.0	8.0	6.0	6.0	1.6×10^9	2.6×10^8	6.1:1
5	15.5	10.5	5.9	5.8	5.3×10^9	2.1×10^8	25.2:1
$7\frac{1}{4}$	17	16.5	6.1	5.8	8.7×10^9	1.9×10^8	45.7:1
F*	20	18.0	5.8	5.7	4.5×10^9	2.3×10^8	19.5:1
M*	180	48.0	6.0	5.8	6.4×10^8	2.2×10^8	2.8:1
S*	.....	.....	.....	6.2	4.1×10^6	.....	.....
							HTPI –
$\frac{1}{2}$	,,	,,	,,	,,	1.5×10^8	1.3×10^8	11:1
1	,,	,,	,,	,,	1.4 ,,	2.6 ,,	53.1
$3\frac{1}{2}$	,,	,,	,,	,,	0.56 ,,	2.1 ,,	26:1
5	,,	,,	,,	,,	1.8 ,,	1.2 ,,	15:1
$7\frac{1}{4}$	,,	,,	,,	,,	11.5 ,,	2.3 ,,	50:1
F*	,,	,,	,,	,,	12.5 ,,	1.5 ,,	83:1
M*	,,	,,	,,	,,	16.0 ,,	3.8 ,,	42:1
S*	,,	,,	,,	,,	0.26×10^6	.....	.....

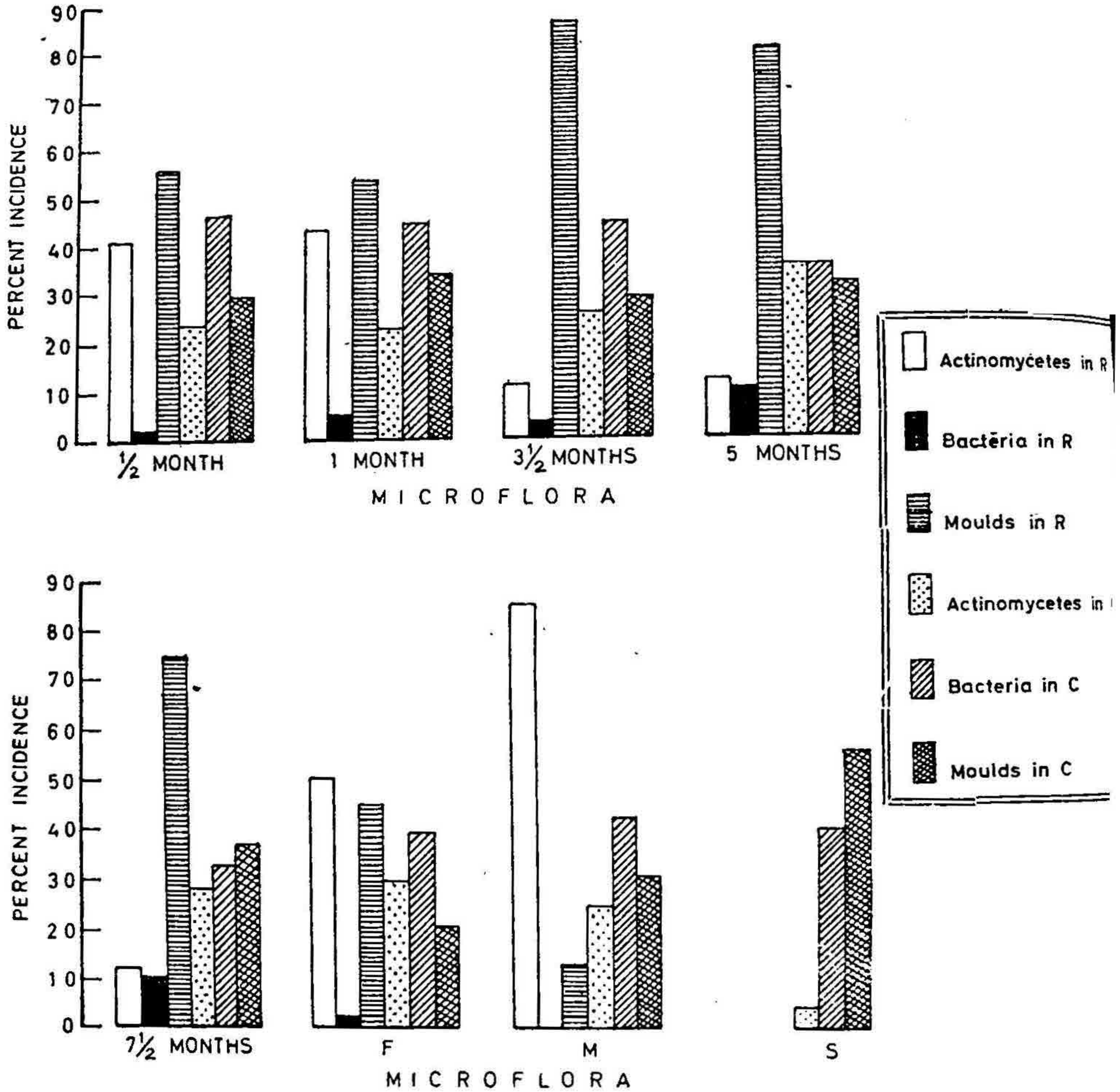
(Same as above)

I

Population in Rhizosphere and Control Soils

*F = Flowering plant
*M = Mature plant.
*S = Surrounding soil after the plants were dug out.

Actinomycetal Population per gram of		R:C Ratio (Actino-mycetes)	Mould population per gram of		R:C Ratio (Moulds)
R	C		R	C	
5.0×10^9	1.3×10^8	38.8:1	6.7×10^9	1.6×10^8	41.8:1
6.8×10^9	1.2×10^8	56.6:1	8.5×10^9	1.8×10^8	47.2:1
4.5×10^9	1.5×10^8	30 :1	3.4×10^{10}	1.7×10^8	200 :1
6.3×10^9	2.1×10^8	30 :1	4.2×10^{10}	1.9×10^8	221 :1
8.0×10^9	1.6×10^8	50 :1	5.6×10^{10}	2.1×10^8	266 :1
8.9×10^{10}	1.7×10^8	52.3:1	7.8×10^{10}	1.8×10^8	411 :1
6.9×10^{11}	1.3×10^8	53.3:1	1.2×10^{11}	1.6×10^8	750 :1
5×10^6	.....	.....	5.6×10^6	.....	.....
Method :					
2.2×10^9	0.62×10^8	35:1	2.1×10^9	1.2×10^8	17:1
1.7 ,,	1. 0 ,,	17:1	1. 0 ,,	1. 5 ,,	66:1
1.1 ,,	0. 8 ,,	13:1	0.56 ,,	0.82 ,,	68:1
3.2 ,,	1. 0 ,,	32:1	5. 3 ,,	0.76 ,,	72:1
3.8 ,,	0.52 ,,	73:1	19 ,,	0.54 ,,	35:1
35 ,,	0.87 ,,	402:1	37 ,,	0.93 ,,	397:1
76 ,,	1. 0 ,,	760:1	137 ,,	1. 2 ,,	1141:1
1.4×10^6	.....	.....	2.3×10^6	.....	.....



F—Flowering plant. M—Mature plant. S—Soil after the plants were dug out. R—Rhizosphere. C—Control.

FIG. 1

Distribution of Microflora in the Rhizosphere of Calotropis plant and Control soil, 1962-63

TABLE II

Some characteristics of the isolated dominant actinomycete

1. Morphological characteristics	Gram positive (Stained unevenly, so Gram's character not certain). A true mycelium is produced. Spores formed, but not in sporangia. Vegetative mycelium remains divided.
	Family. *Actinomycetaceae.*
	Aerobic, bacteria-like colonies with dough-like consistency.
	Genus. *Nocardia.*
(*a*) Vegetative mycelium	Limited mycelium. Branches break into segmentation spores. Non acid-fast.
(*b*) Aerial mycelium	White. Rapidly dividing into rods and cocci. Rapidly growing.
2. Cultural characteristics	
Growth on :	
(*a*) Inorganic salts – starch agar plates.	Nature of sporophere : Sporophores absent.
	Colour of spores 'en masse' : Pinkish white, appears pink from the back.
(*b*) Iorn-peptone agar slants.	Blackening of slants : (–)ve. So no H_2S produced.

Table II—(*contd.*)

3. Antibiotic activity by cross-streak plate method on Asparagine – glucose agar against :	Zone of inhibition in mm	Results read after	Effectivity
(i) *Staphylococcus aureus*	.....	12 hrs.	Not effective against bacteria tested.
(ii) *Escherichia coli*	.....		
(iii) *Bacilius subtilis*	.....		
(iv) *Candida albicans*	.....	24 hrs.	Not effective.
(v) *Saccharomyces cerevisiae*	1.0		Effective.
(vi) An Yeast sp.(unindentified)	1.3		Effective.
(vii) *Pencillium notatum*	.....	3 days	Not effective against moulds tested.
(viii) *Aspergillus niger*	.....	,,	
(ix) *Mucor* sp.	.....	,,	

4. Carbon studies (Pridham and Gottlieb's basal medium) with :	Nature of growth	Pigment, if any
(i) Dextrose	Good	Pinkish from the back of the slant.
(ii) Glycerol	,,	
(iii) Arabinose	,,	No diffusible pigment.

Pectinolytic activity of the isolated actinomycetes. Using HTPI method, 15 apparently dissimilar types of actinomycetes in addition to the dominant type described earlier, were isolated with ease. All of them were found to contain pectin polygalacturonase and pectin methylesterase activity.[10]

DISCUSSION

The observation that *Nocardia* species dominates over all others in the rhizosphere of the *Calotropis* plant becomes a matter of great interest when it is considered that the species of *Nocardia* are negligible in soils as compared to those of *Streptomyces.* Furthermore, *Nocardia* sp. as such have not been closely associated with any rhizosphere systems so far studied. It is not, however, unlikely that its domination might have been influenced by its ability to excrete an antibiotic antagonistic to an yeast sp. normally occurring in the

soil surrounding the roots and its capacity to overwhelm the growth of others by drawing in large measure the glutamate excreted by root system on which its growth is dependent. Whether or not the medicinal property of the plant can be ascribed to the *Nocardia* cannot be decided from the meagre results collected so far from this study. Likewise, the significance of pectinolytic property of the organisms or in the environments cannot be explained as yet. It may however be emphasized that, in general agreement with our earlier observations[3, 10, 11, 12, 13], all the actinomycetes isolated were found to possess pectinolytic activity.

The application of HTPI method in the study of rhizosphere flora has offered an unique advantage in the sense that not only the isolation itself of the actinomycetes has been rendered easy, but an idea of the fluctuations in the heat-resistant flora has been gained therefrom. It is of interest to note that the heat-resistant bacterial species find the environs not favourable during the first three months of the plant growth, whereas thereafterwards, *i.e.*, during the maturation and flowering stages of the plant, they flourish almost to the exclusion of the non-resistant species.

Several investigators have reported an increase in the microbial population of rhizosphere with the gradual growth of the plants studied.[14] The results presented here also support the earlier findings in that population changes in the rhizosphere were clearly evidenced. The initial increase in bacteria followed by a post-flowering decline has its parallel in the case of cotton[15,16] even as the increase in the actinomycetal and mould populations throughout the developmental stages observed here has its parallel in the rhizosphere of peanut.[17]

A characteristic rhizosphere effect was witnessed in this plant in that the effect was seen in the fluctuations of bacteria whereas the actinomycetes and moulds registered a conspicuous increase in their populations. Similar increase in populations due to the rhizosphere effect has been reported in several other plants by Katznelson, *et al.*, though much remains to be understood on the subject itself. It is in the rhizosphere, where not only the main interaction occurs between soil microorganisms and the growing plant, but where the action of various soil-borne pathogens or toxic factors occurs to complicate and accentuate associative or antagonistic influences.

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