INVESTIGATIONS INTO THE PATTERN OF MICROBIAL COLONIZATION OF EARLY RHIZOSPHERE WITH MODEL SYSTEMS

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ABSTRACT

The rhizosphere microflora is a result of interaction between seed surface and soil microflora. As the plant grows older the organisms from the seed slowly diminish and the soil organisms dominate. The pattern of rhizosphere colonization was analysed using model system. Pure cultures of different bacteria and moulds (Pseudomonas, Azotobacter, Arthrobactor, Bacillus, Micrococcus, Aspergillus, Mucor.) *were added to previously sterilised seeds and soil in different combinations. The relative incidence was traced in the rhizosphere. It was observed that organisms from soil can always establish better than those from seed coats. Also, that bacteria are better competitors than moulds and that among bacteria the Gram negatives were the most efficient colonizers.*

1. INTRODUCTION

The high density of microbial population in the vicinity of plant roots as compared to soil away from them (*i.e.* the rhizosphere effect) is conspicuous even within 6 hr of germination of the seed[1,2]. The primary source of these organisms may be the seed and/or the soil and the rhizosphere microflora has been generally assumed to be intermediary between seed surface and soil populations[3,4,5]. Generally speaking, the organisms on the seed surface can establish on the roots after germination but they may, however, soon disappear[6,7]. The organisms on the seed coat may also affect germination and post-emergence survival of the seedlings[8-11] but the extent to which germinating seeds may affect the microflora on their coats is not well elucidated though a few instances of antimicrobial substances exuded by germinating seeds have been reported[12,13].

Literature reports seem to suggest that the nature of the rhizosphere population in the early stages of growth is influenced by the metabolic activities of the germinating seed as well as by the interaction between the seed coat and soil microflora. The exact source of the rhizosphere microflora

is, however, indefinite and no explanation has been offered for the qualitative distribution of the microflora. Previous reports from this laboratory have brought out some interesting interrelations between soil microorganisms and the mulberry (*Morus indica*) plants. In this paper are presented the efforts made to elucidate the microbial factors affecting rhizosphere colonization with the aid of known mircoorganisms and mulberry plant as model systems.

2. Materials and Methods

Seeds. Mature mulberry fruits were collected from the Institute's garden and the seeds were extracted.

Microbiological Studies. All microbiological studies, *i. e.* sampling, plating, isolation, determination of physiological and nutritional properties, were carried out as described earlier[14].

Seed surface microflora. 5 *g* of seeds were shaken in 100 *ml* of sterile water and serial dilutions were prepared and plated on appropriate media.

Changes in the microbial population during germination. Mulberry seeds were sown in garden soil placed in petridishes. Germinating seeds were collected in sterile petridishes after 3, 7, and 10 days of sowing. Adhering soil was removed with sterile forceps and then transferred into sterile water blanks. The microbial population was studied as before.

For obtaining rhizosphere samples of the seedlings were removed aseptically and freed of loosely adhering soil by gentle tapping. The roots were then clipped off and collected into a sterile water blank and examined as usual.

Seeds were surface sterilised with 0.2% $HgCl_2$. for 3 minutes and soil by autoclaving on three days at 126° C for 30 min.

Colonization of rhizosphere by seed surface microflora. Soil was sterilised in petridishes and 30 to 40 non-sterile seeds were sown in each dish (Fifteen dishes were kept sown). Periodically all the seeds from 2 dishes at a time were removed and the microflora was studied as usual.

Colonization of rhizosphere by the soil microorganisms. In this case the seeds were surface sterilised and sown in soil kept in petridishes. Rhizosphere samples were collected and studied as usual.

Rhizosphere colonization in model systems. The proportionate contribution of soil/or seed surface microflora to the rhizosphere population and the relative ability of different microorganisms to inhabit the rhizosphere were examined in model systems. For this purpose representative isolates of different genera of bacteria and fungi, *viz.*, (1) *Pseudomonas* (2) *Azotobacter* (3) *Micrococcus* (4) *Arthrobacter* (5) *Bacillus* (6) *Aspergillus* (7) *Mucor* were chosen. All these cultures were originally isolated from mulberry rhizosphere.

Soil was sterilised in petridishes and seeds were surface sterilised with $HgCl_2$. as earlier. Soil and seeds were reinfected with a suspension one of the above organisms. In case of soil cell or spore suspension was added and mixed well. Seeds were soaked in similar suspensions for 1 hour before they were sown in soil. Uninoculated controls were kept with about 30 to 40 seeds sown per plate. In this way as many as (8×8) 64 different combinations of treatments were obtained and duplicate sets were kept. Five seeds were sampled from each set for microbiological analysis. They were suspended in sterile water and the relative incidence of the two organisms (*i.e.* the seed inoculant, and the soil inoculant) was estimated by the usual plating method.

3. Results and Discussion

It is clear that the microorganisms proliferated actively on the seeds during germination and that they increased even more considerably on the roots. The moulds were less stimulated than the bacteria (Table 1). From the qualitative distribution of bacteria on rhizosphere of mulberry under different conditions soil and/or soil sterility (Tables 2, 3 and 4), it would seem that the Gram negative bacteria dominate on the rhizosphere soon after germination. The fast disappearance of spore formers was indeed rather striking, particularly in the sterile soil (Table 3). The growing region of the roots was consistently found to harbour microbes whose nutritional requirements was simple in comparison to those associated with the older regions of roots.

Table 1

Microbial population on seed surface during germination of mulberry

Conditon of seed	Population on seed $10^4/g$				Rhizosphere
	—	Slightly swollen	Fully swollen	Radical just emerges	Root 1-2 cm long
Days / Particulars	0 (seed)	3	7	10	3rd day after germination
Bacteria	10.2	12.8	67.8	101.8	20.6*
Actinomycetes	0.8	1.9	6.8	17.5	1.8*
Moulds	0.4	0.5	2.5	3.5	11.3

* Counts $10^6/g$

Another interesting phenomenon observed was the overall similarity in the rhizosphere population of plants grown from sterile seeds on non-sterile soils and that of plants from non-sterile seeds grown on sterile soil. But when non-sterile seeds were sown in non-sterile soils the qualitative nature of the microflora significantly differed from the other two. These results suggest that when competition from one of the habitats (*i.e.* seed coat or soil) was absent, the roots encouraged a similar flora around them. But when both were present competitions and other interactions took place resulting in a modified flora.

TABLE 2

Morphological and Nutritional Properties of bacteria on mulberry seed

Source	Mulberry Seed				Rhizosphere	
Days after sowing	0	3	7	10	3rd day after germination	Soil
No of isolates	40	45	55	48	61	50
Particulars	Positive isolates %					
Gram negative short rods	30.0	33.0	19.0	19.0	11.0	14.0
Gram negative long rods	25.0	27.0	43.0	50.0	56.0	18.0
Gram positive cocci	15.0	11.0	13.0	10.0	10.0	16.0
Gram positive nonsporing rods	10.0	6.0	5.0	6.0	6.0	22.0
Spore formers	20.0	23.0	20.0	16.0	14.0	30.0
Nutritional Groups—						
B	10.0	13.0	14.0	15.0	15.0	14.0
BA	25.0	27.0	29.0	30.0	36.0	18.0
BAG	30.0	29.0	26.0	31.0	21.0	34.0
GY	20.0	18.0	16.0	15.0	15.0	18.0
YS	15.0	13.0	13.0	11.0	13.0	16.0

TABLE 3

Population changes during germination and colonization of rhizosphere in sterilized soils

Source	Seed				Rhizosphere			
Days after sowing					3rd day after germination		7th day after germination	
	0	3	7	10	Top Half	Bottom half	Top Half	Bottom half
No. of isolates	45	50	48	50	32	39	41	43
Particulars	Positive isolates %							
Gram Negative short rods	31.0	26.0	25.0	27.0	28 0	29.0	24.0	28.0
Gram Negative long rods	24.0	34.0	40.0	43.0	34 0	32 0	34.0	39.0
Gram positive cocci	15.0	10.0	6.0	7.0	9.0	15.0	7.0	7.0
Gram positive non-sporing rods	10.0	16.0	19 0	20.0	16 0	13.0	14.0	13 0
Spore formers	20.0	14.0	11.0	4 0	12.0	13.0	12.0	13 0
Nutritional groups								
B	11.0	14.0	11.0	11.0	12.0	13.0	12.0	12.0
BA	26.0	36.0	38.0	40.0	43.0	47.0	42.0	48.0
BAG	31.0	28.0	29.0	29.0	27.0	26.0	29.0	23.0
GY	18.0	14.0	14.0	16.0	9.0	8.0	10.0	9.0
YS	13.0	8 0	8.0	5.0	6 0	8.0	8.0	7.0

TABLE 4

Colonization of rhizosphere by soil microflora

Source	Soil	Rhizosphere			
		3rd day		7th day	
		Top half	Bottom half	Top half	Bottom half
No. of isolates	50	45	42	38	36
Particulars		Positive isolates %			
Gram negative short rods	14.0	24.0	29.0	23.0	28.0
Gram negative long rods	18.0	33.0	42.0	31.0	45.0
Gram positive cocci	16.0	9.0	7.0	10.0	8.0
Gram positive non-sporing rods	22.0	17.0	14.0	18.0	11.0
Spore formers	30.0	15.0	12 0	16.0	8.0
Nutritional groups					
B	14.0	13.0	12 0	13.0	11.0
BA	18.0	44.0	48.0	39.0	45.0
BAG	34.0	26.0	26.0	23.0	22.0
GY	18.0	9.0	7.0	13.0	11.0
YS	16.0	7.0	7.0	10.0	11.0

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TABLE 5

Relative ability of seed and soil microflora to colonize rhizosphere during early stages of growth

Soil Inoculation / Seed Inoculation		Nil		*Pseudomonas*		*Azotobacter*		*Micrococcus*		*Arthrobacter*		*Bacillus*		*Aspergillus*		*Mucor*	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
	1		...	...	Nil	...	Nil	...	Nil	...	Nil	...	Nil	...	Nil	...	Nil
	2		...	...	1600	..	580	...	280	...	780	...	620	...	230	...	180.0
Nil	3	Completely	.	...	4.8×10^{3}	...	3.6×10^{3}	...	2.9×10^{3}	...	3.5×10^{3}	...	2.1×10^{3}	...	1.2×10^{3}	...	1400.0
	4	Sterile	..	...	8.2×10^{4}	...	4.2×10^{4}	...	4.7×10^{4}	...	4.3×10^{4}	...	4.3×10^{4}	...	4.9×10^{3}	...	3.9×10^{3}
	5		...	...	10.7×10^{4}	...	6.9×10^{4}	...	5.8×10^{4}	...	6.1×10^{4}	..	5.1×10^{4}	...	6.1×10^{3}	...	4.8×10^{3}
	1	Very High	...			100.0	...	100.0	...	100.0	...	100.0	...	100.0	..	100.0	...
	2	Very High	...			95.0	5.0	92.0	5.0	92.0	8.0	95.0	5.0	95.0	5.0	94.0	6.0
Pseudomonas	3	1.6×10^{3}	...	*Pseudomonas*		50.0	50.0	50.0	50.0	45.0	55.0	55.0	45.0	80.0	20.0	76.0	24.0
	4	2.2×10^{3}	...			25.0	75.0	30.0	70.0	30.0	70.0	32.0	68.0	50.0	50.0	50.0	50.0
	5	3.1×10^{3}	...			30.0	70.0	35.0	66.0	28.0	72.0	30.0	70.0	40.0	60.0	45.0	55.0
	1	Very High	...	100.0	...			100.0	...	100.0	...	100.0	...	100.0	...	100.0	...
	2	Very High	...	90.0	10.0			90.0	10.0	95.0	5.0	90.0	10.0	90.0	10.0	90.0	10.0
Azotobacter	3	2.8×10^{3}	...	25.0	75.0	*Azotobacter*		45.0	55.0	40.0	60.0	50.0	50.0	75.0	25.0	70.0	30.0
	4	2.2×10^{3}	...	20.0	80.0			30.0	70.0	30.0	70.0	30.0	70.0	45.0	155.0	50.0	50.0
	5	8.9×10^{3}	...	10.0	90.0			25.0	75.0	30.0	70.0	20.0	80.0	40.0	160.0	50.0	50.0
	1	Very High	...	100.0	...	100.0	...			100.0	...	100.0	...	100.0	..	100.0	...
	2	Very High	..	90.0	10.0	80.0	20.0			75.0	25.0	85.0	15.0	85.0	15.0	90.0	10.0
Micrococcus	3	1.4×10^{3}	...	20.0	80.0	40.0	60.0	*Micrococcus*		35.0	65.0	45.0	55.0	75.0	25.0	80.0	20.0
	4	1.9×10^{3}	...	20.0	80.0	25.0	75.0			30.0	70.0	28.0	72.0	40.0	60.0	45.0	55.0
	5	2.4×10^{3}	...	15.0	85.0	20.0	80.0			18.0	82.0	14.0	86.0	50.0	50.0	35.0	65.0
	1	Very High	...	100.0	...	100.0	..	100.0	...			100.0	...	100.0	...	100.0	...
	2	Very High	...	88.0	12.0	95.0	5.0	90.0	10.0			92.0	8.0	95.0	5.0	90.0	10.0
Arthrobacter	3	1.6×10^{3}	...	30.0	70.0	50.0	50.0	40.0	60.0	*Arthrobacter*		60.0	40.0	75.0	25.0	75.0	25.0
	4	1.8×10^{3}	...	25.0	75.0	35.0	65.0	30.0	70.0			45.0	55.0	60.0	40.0	68.0	132.0
	5	3.1×10^{3}	..	15.0	85.0	30.0	70.0	30.0	70.0			24.0	76.0	45.0	55.0	50.0	50.0
	1	Very High	...	100.0	..	100.0	..	100.0	...	100.0	...			100.0	...	100.0	...
	2	Very High	...	50.0	50.0	60.0	40.0	65.0	35.0	62.0	38.0			95.0	5.0	95.0	5.0
Bacillus	3	1.3×10^{3}	...	20.0	80.0	40.0	60.0	43.0	57.0	45.0	55.0	*Bacillus*		80.0	20.0	73.0	27.0
	4	1.8×10^{3}	...	20.0	80.0	30.0	70.0	20.0	80.0	30.0	70.0			55.0	45.0	65.0	35.0
	5	2.9×10^{3}	..	10.0	90.0	15.0	85.0	15.0	85.0	17.0	83.0			40.0	60.0	55.0	45.0
	1	Very High	..	100.0	...	100.0	...	100.0	..	100.0	..	100.0	...			100.0	...
	2	Very High	...	60.0	40.0	65.0	35.0	62.0	38.0	60.0	40.0	65.0	35.0			85.0	15.0
Aspergillus	3	2,200	...	15.0	85.0	30.0	70.0	40.0	60.0	45.0	55.0	40.0	60.0	*Aspergillus*		45.0	55.0
	4	3,600	...	10.0	90.0	18.0	82.0	35.0	65.0	20.0	80.0	30.0	70.0			35.0	65.0
	5	2,400	.	5.0	95.0	10.0	90.0	15.0	85.0	9.0	91.0	20.0	80.0			28.0	72.0
	1	Very High	...	100.0	.	100.0	..	100.0	...	100.0	...	100.0	...	100.0	...		
	2	Very High	...	70.0	30.0	80.0	20.0	75.0	25.0	60.0	40.0	60.0	40.0	90.0	10.0		
Mucor	3	1,900	...	35.0	65.0	40.0	60.0	40.0	60.0	20.0	80.0	20.0	80.0	40.0	60.0	*Mucor*	
	4	2,200	...	20.0	80.0	30.0	70.0	30.0	70.0	14.0	90.0	10.0	90.0	35.0	65.0		
	5	1,800	...	5.0	95.0	15.0	85.0	18.0	82.0	8.0	92.0	8.0	92.0	15.0	85.0		

Note:—a & b in vertical columns represent ... a = seed inoculant; b = soil inoculant

1 to 5 in horizontal columns denote stage of sampling

1 – on sowing; 2 – 10th day after sowing; 3 – 3rd days after germination: 4 – 7th days after germination – Top half; 5 – 7th days after germination – Bottom half

Figures in Top horizontal row and left vertical columns are actual population counts, all other figures are percentages.

In order to clarify the nature of interactions and to gain an insight into the pattern of microbial colonization of the rhizosphere an experiment was conducted with model systems. The basic principle of the study was to sterilise both soil and the seeds initially and reinfect them with pure cultures of easily recognizable organisms and follow the course of their establishment and survival in the rhizosphere. The results are presented in Table 5.

Microorganisms derived from soil proved to be better colonizers of roots than those of the seed surface. This is very clearly brought-out in Table 5. The same test organism when it originated from soil was found in much larger numbers on the roots than when it was derived from inoculated seeds (compare top row with first column in Table 5). This may be due to the fact that whereas in soil majority of the organisms may occur in an active state those on the seed surface, apart from their lower population, are likely to be in a lower state of activity and for that reason can only slowly migrate along the roots in order to colonize it.

Among the bacteria the Gram negative species, notably *Pseudomonas* species, were very versatile. The population on the roots, whether from soil or seeds, was higher than that of other organisms. *Arthrobacter*, which appears to be very common soil inhabitant, as also better colonizer than *Bacillus* or *Micrococcus* or the moulds but relatively less capable than the Gram negatives. *Azotobacter* species faired well in their competition with other organisms except for *Pseudomonas* and *Arthrobacter* species These observations were in agreement with the direct microscopic examinations made simultaneously. These experiments bring out in a neat way the competitive ability of these forms and explain the predominantincidence of Gram negatives in the rhizosphere, though it is not yet known if it endows *Pseudomonas* the ability to dominate the rhizosphere.

Chan *et al* [15,16] in order to investigate the same phenomenon, observed that in mixed cultures *Pseudomonas* (a typical rhizosphere organism) inhibited the growth of *Arthrobacter* (a typical soil organism). This inhibition was intensified when root extract was added to the medium and diminished when soil extract was added instead. The authors offered this as a possible explanation of what is taking place in nature. Our studies conducted with actual seeds and soil bring out the competitive ability of different soil microorganisms, though what intrinsic properties enable them as such remains to be explored. From the present results it would seem that antagonistic properties and faster growth rate of some species may in fact be factors involved in the phenomenon.

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