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INTERRELATIONS OF MICRO-ORGANISMS AND MULBERRY

II. PHYLLOSPHERE MICROFLORA AND NITROGEN FIXATION IN LEAF AND ROOT SURFACES

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INTRODUCTION

The plant roots, by way of their exudations, change the environment around them. They create thereby better biological and physico-chemical conditions for the micro-organisms and themselves [11]. Whether similar condition exists in the aerial parts of the plant, notably leaf surfaces, is an interesting proposition worthy of investigation.

The leaf leachates have recently been shown to contain carbohydrates, amino acids and minerals [2 7 14 15] and, by analogy with the rhizosphere, these can be expected to stimulate preferred organisms on the leaf surface. Ruinen [12 13 14] was perhaps the first to carry out such a study and she found that on the leaves considerable proliferation of micro-organisms, especially nitrogen fixers like *Azotobacter* and *Beijerinckia*, occurs. She recognised the leaf surface as a preferred microbiological milieu and called it 'phyllosphere'. In fact she demonstrated the mutual beneficial relations of the leaf and the micro-organisms with excised leaves which gained in weight and nitrogen when kept in a mineral solution free from nitrogen. Obviously, nitrogen was furnished to the leaves by the nitrogen fixers through fixation and they in return obtained energy materials from the photosynthesising leaf.

The mulberry leaves which serve as the sole source of food for the growth of and silk (protein) synthesis by the silkworm (*Bombyx mori* L.) are rich in nitrogen content. This prompted a query whether

or not there exist on the foliage mechanisms for nitrogen nutrition of the plant apart from what is absorbed by the roots. The results of the investigations carried out on these lines are presented here. The interrelations of rhizosphere microflora and the host plant mulberry has already been a subject of a previous report from this laboratory [19].

MATERIALS AND METHODS

Enumeration of leaf-surface microflora

Mature mulberry (*Morus indica*) leaves were collected from the garden or the glasshouse maintained by the laboratory. Two varieties of mulberry, *viz* (i) the local bush variety and (ii) a Japanese graft were included in this study. In the case of the former, leaves from the glasshouse plants grown under water culture as well as those derived from garden were taken and these were directly used for estimating microbial populations. Leaves of the Japanese variety were too large for easy handling and only portions thereof, cut from the centre, were used. The leaves (after clipping off the petioles) or the cut-outs were directly dropped into 100 ml sterile water in 250-ml erlenmeyer flasks and shaken for 15 min. Serial dilutions were then prepared and plated on appropriate media. Nutrient agar, Kuster's medium [6], rose bengal – streptomycin agar [1], and yeast extract–dextrose agar (to which was added 100 μg per ml of streptomycin and penicillin) were respectively used for the counting of bacteria, streptomycetes, moulds and yeasts. Azotobacter and Beijerinckia were estimated on the nitrogen-free agar used by Becking [3] and, after purification in the usual manner, the cultures were maintained on Ashby's mannitol agar. The leaves and the cut-outs were thereafter measured for their size, dried and weighed.

Cultivation of plants under sterility conditions and inoculation methods

Mulberry seeds were surface sterilised in 0.2% $HgCl_2$ for 1 min, washed repeatedly with sterile distilled water, and sown on nutrient agar plates. Only uncontaminated seeds were used in the experiments.

Nutrient medium for plants

Two different media were used: (i) Ashby's medium and (ii) a modified Hoagland and Arnon's solution [5] used routinely in the laboratory for mulberry cultivation in the glasshouse. Both of these were used with and without the nitrogen and the carbon source as detailed in Table 1. The solutions were pipetted in 30-ml amounts into 20 × 2.5 cm tubes provided with aluminium stands for the support of seeds and autoclaved for 10 min at 121° C. In each tube were transferred 3 sterile germinating seeds and allowed to grow. When they had put forth their first pair of leaflets the following experiments were conducted with them. First set was left without any treatment as controls, in the second the root system was inoculated and in the third set the

TABLE 1

Composition (g/l) of different solutions used in the study			
Ashby's medium		Plant nutrient solution	
No nitrogen	With nitrogen	No nitrogen	With nitrogen
KH_2PO_4 . . . 0.2	KH_2PO_4 . . . 0.2	KH_2PO_4 . . 0.075	KH_2PO_4 . . 0.05
$MgSO_4.7H_2O$. 0.2	$MgSO_4.7H_2O$. 0.2	$MgSO_4.7H_2O$ 0.1	$MgSO_4.7H_2O$ 0.10
NaCl. 0.2	NaCl 0.2	KCl 0.05	KNO_3 . . . 0.015
$CaSO_4$ 0.1	$CaSO_4$ 0.1	$CaCl_2$. . . . 0.3	$NH_4H_2PO_4$. 0.05
	$Ca(NO_3)_2.4H_2O$ 1.5		$Ca(NO_3)_2$ . . 0.50

Ferric citrate 0.5% 0.2 ml/l and micro-nutrient solution Hoagland and Arnon [5] 0.2 ml/l was used in all cases.
Mannitol and sucrose at 0.5% each were used when carbon source was added to the medium.

inoculation was done on the leaf surfaces. For inoculation, one active strain of Azotobacter isolated from mulberry leaf surface was used. In root inoculations approximately 2×10^6 cells were added to the nutrient medium and in the case of leaf inoculation a like aliquot of the cell suspension was carefully rubbed on the leaf surface. In order to know the effect of mulberry on the growth of Azotobacter one set of tubes without any seeds was also inoculated with the organism. Each set comprised of twelve tubes.

Growth measurements

After 2 months the plants were removed and measurements were made to record the shoot and root elongation. All the 3 plants in a tube were taken together for recording weight and the whole series of plants in a set were pooled for estimation of the nitrogen [16]. Representative leaf, root and nutrient solution samples were also plated for population counts.

Leaf exudations

The leaf exudations in general were collected by adopting the method of Tukey *et al* [15]. Only sterile grown untreated plants were used and the exudations were studied once at the end of the trials. Whole plants were removed from the tubes and inverted into petri dishes containing distilled water in such a manner that only the leaves were immersed. The water was changed every half hour and the leachates were collected over a 3-hour period. The total leachates were pooled, reduced to a small volume *in vacuo*, and their total amino acid contents were estimated using ninhydrin [9] and carbohydrates using anthrone [20]. The garden leaves were individually collected and the cut ends were sealed with vaseline before immersion in water. After leaching, the leaves were dried and weighed.

The results were statistically analysed following methods suggested by Moroney [8].

RESULTS

The microbial population on the leaf surfaces is given in Table 2. It is clear therefrom that the microbial load on the leaves obtained from the garden is heavier than that of the glass house. The bacteria dominated over the leaf surface. The moulds were greater in number than the streptomycetes and the yeasts. The population on the Japanese variety was in general greater but the bush leaves seemed to support more of the nitrogen fixers (Table 3). The rate of proliferation of Azotobacter on the leaf surface may be judged from Table 4.

TABLE 2

Total microbi al population of mulberry leaf surface

Source	Leaf area, cm^2	Dry weight g	Population in $10^6/cm^2$			
			Bacteria	Strepto-mycetes	Yeasts	Moulds
Bush, garden	85.0	0.49	35.2	1.3	0.72	1.82
Bush, water culture	90.0	0.45	19.8	0.8	0.30	1.10
Japanese variety	370.0	3.40	45.7	2.2	0.84	1.90

TABLE 3

Incidence of nitrogen-fixing bacteria on the mulberry leaf surface

Source	Population, $10^6/cm^2$	
	Azotobacter	Beijerinckia
Bush, garden	2.9	4.8
Bush, water culture .	1.8	2.9
Japanese variety . .	2.1	4.3

TABLE 4

Azotobacter population in the leaf surface of inoculated plants

Medium	Azotobacter population, $10^6/cm^2$ of leaf from			
	Without nitrogen		With nitrogen	
	Without C	With C	Without C	With C
Ashby's	3.0	4.2	3.6	5.2
Plant nutrient solution	3.2	4.6	3.1	5.9

Even though only the first pair of leaves were inoculated, it was evidenced that all the leaves got well loaded with the organism revealing the ease with which the foliage gets contaminated. Only in a very few cases the root system of the leaf inoculated plants got

TABLE 5

Azotobacter population on the root surface and in nutrient solution								
Source	Ashby's medium				Plant nutrient solution			
	Without N		With N		Without N		With N	
	Without C	With C	Without C	With C	Without C	With C	Without C	With C
Root surface *	120.0	240.0	160.0	310.0	130.0	238.0	143.0	297.0
Nutrient solution **	68.0	210.0	118.0	265.0	79.0	205.0	105.0	268.0
Unplanted nutrient solution **	nil	200.0	nil	210.0	nil	188.0	nil	210.0

* Population 10^6/g roots

** Population 10^6/ml

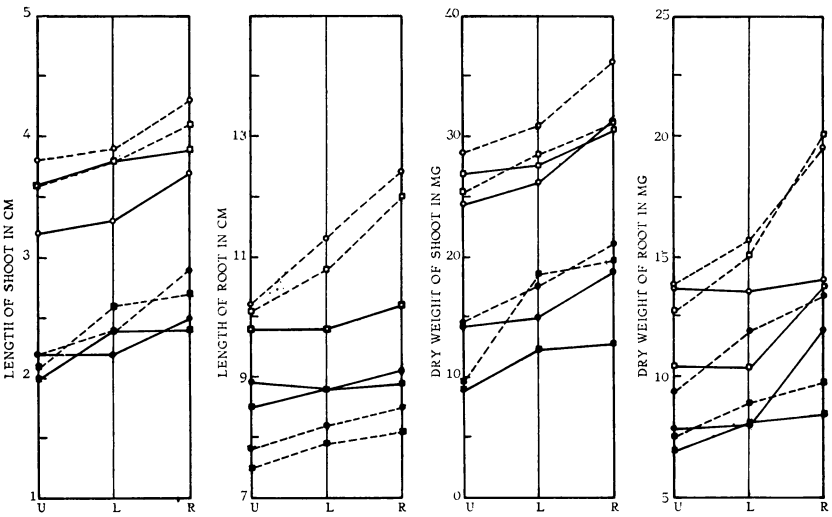

Fig. 1. Effect of Azotobacter inoculation on growth of mulberry.

Legends:

U = Uninoculated
L = Leaf inoculated
R = Root inoculated

○———○ Ashby's medium, with nitrogen, no carbon
○- - - -○ Ashby's medium, with nitrogen, with carbon
●———● Ashby's medium, no nitrogen, no carbon
●- - - -● Ashby's medium, no nitrogen, with carbon
□———□ Plant nutrient solution, with nitrogen, no carbon
□- - - -□ Plant nutrient solution, with nitrogen, with carbon
■———■ Plant nutrient solution, no nitrogen, no carbon
■- - - -■ Plant nutrient solution, no nitrogen, with carbon

contaminated and in no case the leaves of the uninoculated plant had any bacteria. The ready proliferation of Azotobacter in the root surface of mulberry is shown in Table 5. Azotobacter, it would appear, thrived on the root surface of the plant. The stimulatory effect of the plant on the organism is seen clearly from the higher density of their population in the nutrient solution in which the plants were growing than that witnessed in tubes containing the same, but without the mulberry plants. Similarly, unplanted tubes containing media with no carbon source could not support Azotobacter in contradistinction to those in which mulberry was allowed to grow proving thereby that the plant, by way of its root exudations, furnished adequate energy material to the rhizosphere organisms.

The effect of Azotobacter inoculation on the growth and dry wt of mulberry is depicted in Fig. 1 and its influence on the nitrogen content is presented in Table 6. The overall results of the statistical analysis are presented in Table 7. Inoculation, it is clear, distinctly brought about increases in the weight of plants. The weight gain was greatly enhanced when a readily assimilable carbon source was present in the medium. Root inoculation was always found to be superior to leaf inoculation, though the beneficial effect of the latter was evident and considerable.

The carbohydrate and amino acid content of the leaf leachates

TABLE 6

Effect of Azotobacter inoculation on the nitrogen content of mulberry plants grown in various media (per cent dry wt).

Source		Ashby's medium				Plant nutrient solution			
		Without nitrogen		With nitrogen		Without nitrogen		With nitrogen	
		Without C	With C	Without C	With C	Without C	With C	Without C	With C
Shoot	R	1.41	1.85	2.80	3.10	1.90	2.15	2.85	3.00
	L	1.32	1.71	2.75	2.95	1.75	1.95	2.70	2.85
	U	1.20	1.40	2.60	2.80	1.60	1.70	2.60	2.70
Root	R	0.92	0.95	1.90	1.95	0.90	1.10	1.60	1.80
	L	0.85	0.84	1.70	1.80	0.85	0.90	1.50	1.50
	U	0.81	0.79	1.70	1.70	0.84	0.90	1.45	1.50

R = Root system inoculated
L = leaf surface
U = Uninoculated control

varied with the variety used, as may be noticed from the results presented in Table 8. The nutrient solution appeared to affect somewhat the leaf exudation of plants. The nature of the leachates from

TABLE 7

Results of statistical analysis						
Nature of effect	Source		Plant parameter studied			
			Shoot		Root	
			Length	Weight	Length	Weight
Main factors	Nitrogen	(N)	HS	HS	HS	HS
	Carbon	(C)	S	HS	S	S
	Medium	(M)	NS	HS	NS	HS
	Inoculation	(I)	HS	HS	HS	HS
I order interaction	C × I		S	S	HS	S
	N × I		S	S	S	S
	N × C		S	HS	HS	HS
Pairs of factors II order interaction, triplets	N × C × I		NS	NS	NS	S

Other interactions of the I, II, and III order were not significant in all cases.
HS = Significant at 1%
S = Significant at 5%
NS = Not significant

TABLE 8

Amino acids and carbohydrates in the leachates of mulberry leaf		
Source	μg/g dry wt of leaf	
	Amino acids	Carbohydrates
Bush, garden	48	584
Bush, water culture	33	382
Japanese variety	58	682

TABLE 9

Carbohydrates and amino acids (μg/g dry weight of leaf) in the leaf leachates of mulberry plants grown in different solutions								
	Ashby's				Plant nutrient			
	Without Nitrogen		With nitrogen		Without nitrogen		With nitrogen	
	Without C	With C	Without C	With C	Without C	With C	Without C	With C
Carbohydrates	120	140	280	295	140	150	290	310
Amino acids	14	18	28	28	18	18	31	34

plants grown in different solutions is given in Table 9. The leaf leachates, like the root exudates, contained a number of aminoacids and a few sugars; a detailed report on these results, however, has been reserved for another occasion.

DISCUSSION

The results clearly show to what extent the plants encourage and provide proper environment for the nitrogen fixing organisms to grow around them and to contribute to their nitrogen economy. In difference with Rovira [10] who reported that Azotobacter to be a poor colonizer of plant roots under pure culture conditions, we find that the mulberry roots were readily colonized by the organisms under similar conditions. But our results agree with his other findings to the effect that the inoculation of crops with Clostridium or Azotobacter has a beneficial effect. In fact, the positive response to Azotobacter observed in fertile soil made him suggest their benevolence to extend beyond the mere fact of their ability to supply nitrogen. Likewise, present findings, *viz* the favourable influence on plants by the inoculated organisms even in the presence of nitrogenous plant nutrients is at once interesting and significant and suggests the occurrence in the culture solutions of microbial metabolites. Possibly the beneficial effect manifests in the form of enhancement of uptake of nutrients by the plants. At any rate the ability of Azotobacter to elaborate plant hormones and other substances affecting growth has recently been reported [4 17 18]. The presence of sugars in the medium would undoubtedly tend to enhance the effect of Azotobacter in addition to plant, which itself would contribute by way of its root exudations.

Leaf inoculation clearly endows benefits on the plant growth though it is not commensurable with those obtaining under the conditions of root inoculations. Our studies with whole plants have brought out more clearly than the earlier ones with excised leaves [14] that the phyllosphere may prove to be yet another interesting zone of plant-microbe interaction.

The presence of amino acids and carbohydrates in the leaf leachates indeed shows them to be rich sources of nutrition to the microbes. It is possible, that larger the leaf surface, the better the plant serves the purpose of supporting higher microbial population; this

seems to be borne out by the comparatively higher population on the Japanese than the indigenous mulberry leaves.

Though it may seem that the conditions of the experiment were somewhat artificial and the beneficial effects of inoculations under field conditions are difficult of demonstration, there is adequate evidence in support of the view that the plants and microbes benefit each other from their foliar interaction and thus help in the nitrogen economy of the soil.

SUMMARY

The mulberry leaves were shown to harbour substantial populations of bacteria, streptomycetes, yeasts, and moulds. Azotobacter and Beijerinckia were observed to contribute to nearly 5 to 10 per cent of the bacterial population. When grown in water culture under sterile conditions, Azotobacter inoculation on the leaf or root surface was found to increase plant growth, dry wt, and nitrogen content of the mulberry. The beneficial effect of Azotobacter was largely influenced by the presence of a carbon source in the plant nutrient solution. The root inoculation in comparison to leaf application was found to confer greater benefits to the growing plant. The presence of carbohydrates and amino acids in the leaf leachates of mulberry was shown. The mutual beneficial nature of the association of the plant and Azotobacter has been brought to light.

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