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SHORT COMMUNICATION

Enumeration of N_2-fixing spirilla

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A semi-solid malate medium has been recommended to enrich and detect N_2-fixing spirilla in soil and the rhizosphere (Day and Dobereiner, 1976). In a survey on the occurrence of these bacteria in Egypt, *Azotobacter* developed vigorously in such enrichments. Therefore, we tested the effects of decreasing the concentration of malate (usually 0.5%) on the proliferation of *Azotobacter*. We did this because it is known that in dilute conditions spirilla are able to compete successfully with other bacteria for nutrients (Krieg, 1976). Methods to count N_2-fixing spirilla have been insufficiently investigated; nitrogenase activity in enrichment cultures is used to estimate abundance of this organism in soil or roots (Dobereiner *et al.*, 1976). We have used the most probable number (m.p.n.) technique to count N_2-fixing spirilla in soils, rhizospheres, phylloplanes and on seeds.

Nine samples; three representative of Egyptian soil, one Belgian soil, rhizospheres and phyllospheres of maize and sugar cane, and seed of maize were tested (Table 2). Phyllosphere samples were obtained by first insertion and separation of the vegetative part of full grown plants into autoclaved large paper bags. The remainder of the plant stem together with the root system was removed and transferred to another bag.

The semi-solid medium of Day and Dobereiner (1976) was used. K-Malate as C-source was added to obtain final concentrations of 0.05, 0.1, 0.25 and 0.5% (w/w), and 8 ml medium was distributed per 25 ml tube (15 mm dia) which were then plugged with cotton and autoclaved.

Table 1. Effect of malate concentration on m.p.n. estimates of spirilla in Fayum clay soil

Concentration of malate in culture media (%)	m.p.n. $10^5 . g^{-1}$ oven dry soil
0.50	15.8
0.25	184.0
0.10	70.0
0.05	70.0
0.025	2.2

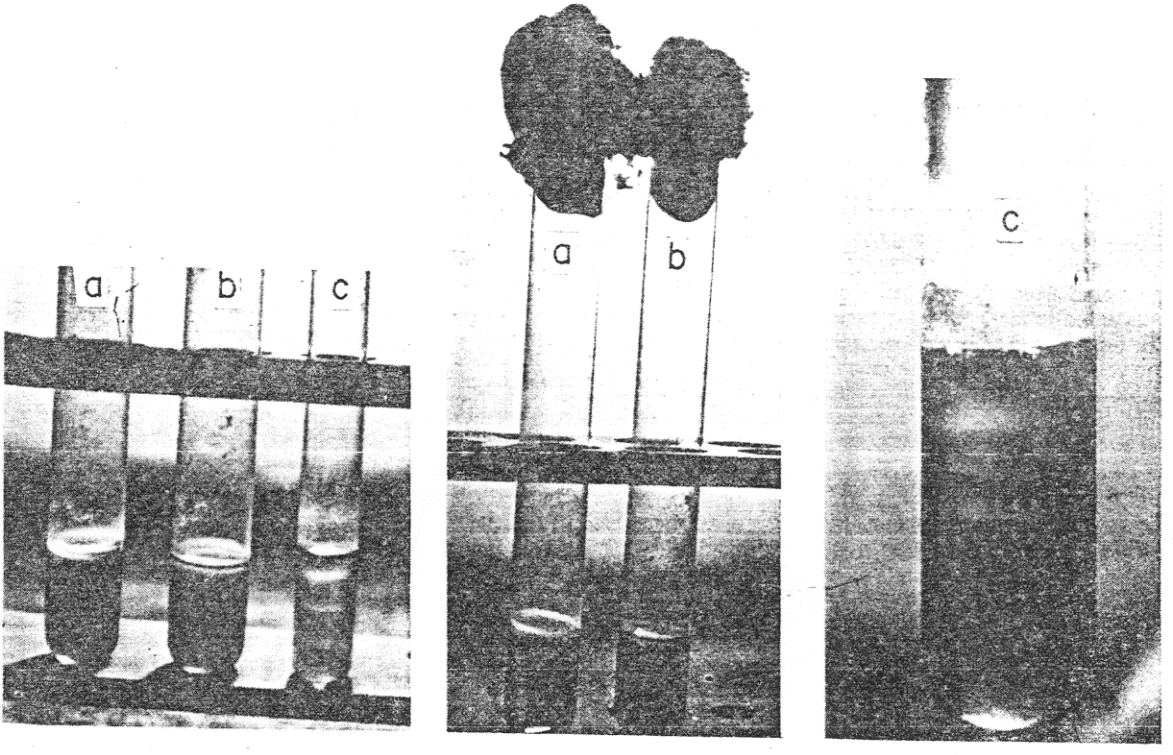

Fig. 1. MPN tubes representing different types of pellicle: (a) A profuse pellicle of *Azotobacter* masks that of spirillum; (b) A characteristic white, thin pellicle of spirillum 2 mm. below surface; (c) In addition to the sub-surface pellicle of spirillum three more have formed at depth by bacteria other than spirillum.

Table 2. Numbers* of N_2-fixing spirilla and *Azotobacter* estimated by m.p.n. technique in different samples

Sample	pH	m.p.n. of spirilla Malate concentration (%) 0.25	0.10	0.05	m.p.n. of *Azotobacter***
Soil					
Fayum (clay)	7.4	216,000	84,000	84,000	30,000
Giza (clay)	7.2	ND	570	160	1760
Sinai (sand)	6.7	390	1430	540	1980
Behira (calcareous)	8.0	26	29	29	18
Flanders (Belgium-clay)	7.2	1	18	1	0.2
Rhizosphere					
Sugar cane (Giza)	6.7	4000	2000	3000	ND
Maize (Giza)	6.9	400	400	2400	ND
Phylloplane					
Sugar cane	5.9	4	1	2	ND
Maize	6.3	1	1	0.2	ND
Maize seed	6.5	0.1	0.2	0.1	0

* Confirmed by microscopic examination; $\times 10^2\ g^{-1}$ dry matter.
** Ashby liquid medium (Hegazi and Niemela, 1976).
ND, Not determined.
0, Not detected.

Enrichments were prepared for qualitative assay by introducing 0.5 g soil, small segments of roots with the adherent rhizosphere soil or segments of leaves to the 0.25% malate semi-solid tubes. They were incubated at 30–32°C and examined during the course of 2–7 days by eye for the characteristic white sub-surface pellicle of spirilla and microscopically for spirilla.

For m.p.n. estimations, 10-fold dilutions were prepared in Winogradsky solution (Holm and Jensen, 1972). Five semi-solid malate tubes were inoculated with aliquots of 1 ml from each appropriate dilution and incubated for 4 days at 30–33°C. Positive tubes were those showing the characteristic white sub-surface pellicle of N_2-fixing spirilla (Fig. 1, b). Such tubes always gave positive results in the C_2H_2-reduction assay (Postgate, 1972). Fresh preparations were prepared from all m.p.n. tubes and examined microscopically for the presence of the very-active motile curved-rods or spiral-shaped cells of spirillum. Estimates were derived from the statistical tables of Cochran (1950).

N_2-fixing spirilla were detected in enrichment cultures prepared from all samples. Counting them on dilution plates was difficult as these bacteria are easily overgrown by other bacteria, particularly when an available N-source, such as yeast extract or ammonium salt, is added. In addition, as these organisms fix N_2 under microaerophilic conditions (Okon *et al.*, 1976) their colonies develop slowly, reaching 2 mm dia at most, when grown aerobically on N_2-deficient malate agar. Therefore the m.p.n. technique is thought to be suitable to count spirilla. In malate semi-solid medium the spirilla migrate toward the region of O_2 concentration which ensures maximal growth where eventually a white, thin sub-surface pellicle is formed. An experiment indicated 0.25% malate but not 0.5 or 0.1%, was favourable for the enrichment of spirilla (Table 1). In further experiments comparing malate concentrations of 0.25, 0.1 and 0.05% (Table 2), N_2-fixing spirilla developed well with all concentrations including the lowest one. Most tubes showed the characteristic pellicle of spirillum (Fig. 1, b) were positive by microscopic examination. There was a very significant correlation coefficient (0.988) between m.p.n. estimates based on pellicle and microscopic observations. Nevertheless, either microscopic examination or C_2H_2 reduction assay for m.p.n. tubes is recommended as a confirmatory test, particularly in tubes where the pellicle is not well-developed. This was found to be the case in samples where the microbe exists in low numbers, such as the phyllosphere and seed.

Although *t*-tests showed no significant differences attributed to malate concentration, we favour using low concentration (0.05–0.1%). They significantly eliminated development of *Azotobacter* which are abundant in soils of the Nile Delta. With high concentrations of malate *Azotobacter* develop a profuse surface pellicle which masks the white, thin sub-surface pellicle of spirillum (Fig. 1, a). Our results presented indicate the abundance of N_2-fixing spirilla in Egyptian soils, particularly those of the Nile Valley.

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