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Binding of soil microorganisms and red blood cells by the gelatinous matrix and eggs of *Meloidogyne javanica* and *Rotylenchulus reniformis*

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Summary – Egg masses of both *Meloidogyne javanica* and *Rotylenchulus reniformis*, and the gelatinous matrix (GM) suspension of *M. javanica* bound various bacteria, as well as human red blood cells (RBC). *Enterobacter aerogenes* and *Escherichia coli* did not have the ability to be agglutinated by the egg masses or the GM. Four rhizobacteria isolates from tomato roots which did not multiply on the GM, had the ability to agglutinate by the GM. However, four other rhizobacteria isolates which did not agglutinate, were able to utilize the GM for growth. The agglutination intensity of RBC by the GM was not inhibited by several carbohydrates, depleted with preheated GM, but increased after trypsinization. The possibility of antibiotic(s) involvement in the GM-bacteria interaction was ruled out. The authors suggest that the agglutination phenomenon may partly explain the GM ability to protect the eggs from surrounding microorganisms in soil.

Résumé – ***Agglutination de bactéries du sol et d'hématies humaines par la matière gélatineuse et les œufs de* Meloidogyne javanica *et de* Rotylenchulus reniformis** – Les masses d'œufs de *Meloidogyne javanica* et de *Rotylenchulus reniformis*, de même que des suspensions de matière gélatineuse (MG) provenant de *M. javanica*, agglutinent différentes bactéries ainsi que les hématies humaines. *Enterobacter aerogenes* et *Escherichia coli* ne sont agglutinés ni par les masses d'œufs ni par la MG. Quatre rhizobactéries isolées de racines de tomate, et ne se multipliant pas sur la MG, sont agglutinées par cette dernière. Cependant, quatre autres isolats de rhizobactéries, non agglutinés, sont capables d'utiliser la MG pour leur croissance. L'agglutination des hématies humaines par la MG n'est pas inhibée par différents hydrates de carbones; elle est diminuée si la MG est préchauffée et augmentée après trypsination. La possibilité de l'intervention de substance(s) antibiotique(s) dans l'interaction GM-bactéries est écartée. Les auteurs suggèrent que le phénomène d'agglutination peut expliquer la capacité de la MG à protéger les œufs des microorganismes présents dans le sol.

Key-words : Nematodes, *Meloidogyne, Rotylenchulus*, soil microorganisms.

Females of several sedentary nematodes of plants envelop their eggs with a gelatinous matrix (GM). In the root-knot nematode, *Meloidogyne,* the GM is synthesized in a voluminous quantity by the female anal glands and secreted through its anus just before and during egg laying; eggs are deposited into the GM to form the egg-mass (Maggenti & Allen, 1960). Information about the chemical composition of the matrix is very limited. In the root-knot nematode, *Meloidogyne,* it was found to be a tanned protein which contains carbohydrate, but no lipids, and appeared to be morphologically homogeneous (Bird, 1958; Bird & Rogers, 1965).

Several studies have been performed on the production process of the matrix (Maggenti & Allen, 1960; Maggenti, 1962). Orion *et al.* (1987) showed that the GM dissolved cells in the gall cortical parenchyma to form a canal through which the egg-mass protruded outside the gall surface. Later, Orion and Franck (1989) described the cell lysis process by electron microscopy observations.

The biological function of the GM is still speculative. Recently, Orion and Kritzman (1991) found that *M. javanica* GM was able to agglutinate two microorganisms, thus interpreting that this property of the GM might enable it to protect the nematode eggs from the microbial fauna in the soil.

In the current paper we explore the agglutination ability of *Meloidogyne javanica* and *Rotylenchulus reniformis* intact egg masses, GM suspensions, and separated eggs – using identified microorganisms, rhizobacteria, and human red blood cells (RBC).

Materials and methods

BACTERIA

The following bacteria were utilized in this study : *Aeromonas caviae, Bacillus cereus, B. subtilis, Enterobacter aerogenes, Escherichia coli, Micrococcus* sp., *Pseudomonas putida, Serratia marcescens* and several unrecognized tomato rhizobacteria. The rhizobacteria were isolated from tomato roots as follows : one gram of tomato roots, grown for a month in a natural non-infected sandy soil, was carefully washed with water and shaken on a rotatory shaker (250 rpm), for 30 min, in 50 ml saline with 50 glass beads (5 mm diameter). The mixture was then transferred through a 15 µm-pore sieve, centrifuged

(10 000 *g;* 10 min) and washed twice with 0.1 M phosphate buffer saline (PBS), pH7.2 which included Ca++ and Mg++.

MEDIA AND CULTIVATION CONDITIONS

For the experiments described herein, the different bacteria strains were grown in 250 ml Erlenmeyer flasks, each containing 50 ml of Difco Bacto Nutrient Broth. The flasks were shaken on a rotatory shaker (150 rpm) at 25 °C, and after 24 h the bacteria were separated from the medium by centrifugation (10 000 *g;* 10 min) and washed twice with PBS. The cells were resuspended in sterile PBS. Bacterial concentrations, *ca* 10^8 colony-forming units (cfu)·ml^{-1}, were determined spectrophotometrically at 560 nm.

EGGS AND GELATINOUS MATRIX

Meloidogyne javanica and *Rotylenchulus reniformis* were monoxenically cultured on tomato (*Lycopersicon esculentum* Mill) cv. Hosen Eilon and on sunflower (*Helianthus annuus* L.) excised roots, respectively. Egg-masses were dissected from 4-week-old cultures. Gelatinous matrix suspension was prepared as follows: 150 egg masses, drawn with a sterile Pasteur pipet from 28-day-old cultures, were suspended in 1 ml PBS and vigorously shaken for 1 min. The suspension was then centrifuged at 1000 *g* for 1 min, and the supernatant fraction was separated from the eggs by decantation. Eggs were resuspended in PBS.

INTERACTION OF EGGS, EGG-MASSES AND GM OF *M. JAVANICA* WITH RED BLOOD CELLS (RBC)

Binding.

Human red blood cells (RBC) (A, B, and O), were obtained from Zerifin Medical Center Blood Bank (Zerifin, Israel). RBC were washed four times with PBS to remove serum proteins and used without fixation. Binding to eggs and egg-masses were performed at 37 °C, in a 24 multi-well plate (Becton Dickinson Labware, CA, USA). Final volume was 400 µl, containing 200 µl of 0.3 % suspension of RBC and 200 µl of egg suspension (100 eggs per well) or three to four egg-masses in PBS. Binding was observed under a dissecting microscope after incubation for 60 min.

Agglutination

Agglutination titers with fresh RBC were recorded only with *M. javanica* GM. Assays were carried out at 37 °C, in a 96 multi-well u-shape plate (Becton Dickinson Labware, CA, USA), in a final volume of 0.1 ml containing 50 µl of 0.5 % suspension of RBC and 50 µl of GM suspension diluted serially in two-fold increments up to 1024 times. Agglutination was observed after 60 min.

To verify the specificity and to assess the nature of the observed agglutination and binding phenomena, eggs and GM suspensions in PBS were incubated for 120 min, at 37 °C, with fucose, galactose, N-acetylgalactosamine, glucose, N-acetylglucosamine and alpha-methyl-mannoside at final concentrations of 0.2 M. As a control, PBS was substituted for the GM suspension.

Eggs were pre-treated with : a) 0.5 % sodium hypochlorite solution for 1 min and washed three times with PBS, and b) thoroughly washed with PBS.

Trypsinization

Eggs or GM were incubated in a water-bath shaker for 150 min, at 37 °C, with 0.5 and 0.05 mg/ml trypsin (Sigma), in PBS, respectively.

Each treatment was replicated in four wells, and the experiments were repeated three times.

INTERACTION OF EGG-MASSES AND GM WITH DIFFERENT BACTERIA

Binding assays

Egg-mass (one per volume), suspended in 25 µl PBS, was mixed on a microscope slide with the same volume (25 µl) of bacterial suspension in PBS. The slide was maintained at 25 °C, in a covered Petri plate to prevent evaporation. The same tests were performed with 25 µl of GM suspension. Observations were made under a microscope, after incubation for 30 min.

Effect of GM on bacteria growth and viability

The experiments described herein were accomplished with *M. javanica*. Two growth media were used : Nutrient Agar (Difco) (Na) and Mueller Hinton (MH) (Diagnostics Pasteur), which was used to determine bacterial antibiotic sensitivity. Various experimental combinations were conducted to determine the qualitative effect of GM suspension on bacteria growth and viability : M*a*) different bacterial suspensions (25 µl) were spread onto agar plates and two or three egg masses were then positioned in the center 15 mm apart each other; M*b*) three filter paper discs (5 mm diameter) were absorbed with 25 µl GM suspension, allowed to dry, and then placed in each agar plate with MH, which had already contained the bacteria; M*c*) GM suspension (10 µl) was pipetted on the agar (NA) which had already contained the bacteria; M*d*) GM suspension (10 µl) was pipetted on the agar (NA) and allowed to dry before the different bacterial suspensions were spread onto the agar; M*e*) equal volumes of GM and bacteria suspension were mixed and 10 µl were pipetted on the agar (NA) plates; M*f*) GM and bacteria suspensions (50 µl, each) were mixed, and incubated at 25 °C for 60 min; 10 µl of the mixture was then pipetted on agar (NA) plates.

Within all treatments, plates were incubated at 25 °C for 24 h before the observations were recorded.

In order to evaluate quantitatively the effect of GM suspension on the viability of *B. subtilis, Micrococcus* sp., *P. putida,* and the tomato rhizobacteria, 90 µl of the GM suspension and 10 µl of the bacteria suspension were mixed in two ml u-shape tubes and incubated at 28 °C, for 48 h. Vigorously mixed suspensions were di-

luted ten-fold and 10 µl were pipetted on agar (NA) plates and cfu were counted.

Results

INTERACTION OF EGGS, EGG-MASSES AND GM SUSPENSION WITH RED BLOOD CELLS (RBC)

Eggs and egg-masses of *M. javanica* (Fig. 1 A, B) and egg-masses of *R. reniformis* bound RBC. The binding was not blood-group-specific. Binding of RBC decreased when *M. javanica* eggs were pre-exposed to sodium hypochlorite or pre-washed extensively with PBS (Table 1). The intensity of RBC binding to *M. javanica* eggs decreased after pre-incubation with fucose, glucose, N-acetyl-glucosamine, alpha-methyl-mannoside and trypsin (0.5 mg/ml) (Table 1). Galactose and N-acetyl-galactosamine increased the binding intensity (Table 1).

GM suspension of *M. javanica* agglutinated RBC. The agglutination was not blood-group-specific. Agglutination occurred up to the × 128 dilution. None of the sugars mentioned above was found to inhibit the agglutination. Trypsinization, however, caused a partial increment of the agglutination intensity (two times increment), while boiling the GM (15 min in boiling water) completely inhibited the agglutination.

INTERACTION OF EGGS, EGG-MASSES AND GM WITH DIFFERENT BACTERIA

Binding ability of several bacteria to *M. javanica* (Fig. 1 C) and *R. reniformis* egg masses is summarized in Table 2. *A. caviae, B. cereus, B. subtilis, Micrococcus* sp., *P. putida* and *S. marcescens* bound to *M. javanica* egg-mass, while *E. aeorogenes* and *E. coli* did not.

The agglutination capability of *M. javanica* GM suspension was identical to the bacterial binding pattern recorded with the egg mass.

Bacterial growth was not affected by whole egg masses, or GM suspensions when tested by the paper disc (*Mb)* or method *Md* (see above). However, when GM suspension drops were applied onto the agar plates (*Mc*), or by other methods described in the " Materials and methods " section (*Me, Mf*), the bacteria which had the ability to bind to egg masses (Table 2), showed a different growth pattern within the GM drop zone – as compared to the non-agglutinating bacteria (Fig. 1 D). Pre-heated GM, however, did not exhibit this effect on bacterial growth.

The viability of *B. subtilis* and *P. putida* slightly decreased in the presence of the GM suspension : cfu decreased by one order of magnitude, after incubation for 48 h, compared with the bacteria viability in the presence of PBS (Table 3). *Micrococcus* sp. growth was

Table 1. Agglutination and binding of human red blood cells (RBC) to *Meloidogyne javanica* gelatinous matrix (GM) and eggs.

Treatment	Agglutination to the GM	Binding to eggs
Non-treated (control)	+++	+++
Eggs pre-exposed to NaOCl		--
Eggs thoroughly washed with PBS		--
GM suspension heated up to 100 °C	--	
Fucose (0.2 M, 2 h)	+++	++
Galactose (0.2 M, 2 h)	+++	++++
Glucose (0.2 M, 2 h)	+++	+
N-acetyl-galactosamine	+++	++++
N-acetyl-glucosamine	+++	++
-methyl-mannoside (0.2 M, 2 h)	+++	++
Trypsin (0.05 mg/ml, 2.5 h)	++++	+++
Trypsin (0.5 mg/ml, 2.5 h)		++

Tests were performed at 37 °C. Binding was recorded 1 h after incubation with RBC – (+ = detectable agglutination/binding. ++, +++, and ++++ = increasing intensities of agglutination/binding. -- = No agglutination/binding to GM or eggs, respectively).

Table 2. Bacteria binding to *Meloidogyne javanica* and *Rotylenchulus reniformis* egg masses.

Bacteria	*M. javanica*	*R. reniformis*
Aeromonas caviae	+	+
Bacillus cereus	+	+
Bacillus subtilis	+	n.t.
Enterobacter aerogenes	–	n.t.
Escherichia coli	–	–
Micrococcus sp.	+	+
Pseudomonas putida	+	n.t.

+ = positive binding; – = negative binding; n.t. = not tested.

Table 3. Viability of different bacteria populations after exposure to *Meloidogyne javanica* gelatinous matrix (GM) suspension.

Bacteria	Counts at time 0 (log)	Counts after 48 h (log)	
		GM	PBS**
Bacillus subtilis	7.34	5.72	6.38
Micrococcus sp.	6.93	2.8	4.95
Pseudomonas putida	7.38	5.2	6.76
Tomato rhizobacteria	6.55	7.69	6.55

* Counts were recorded in colony-forming units (cfu) per ml –
** PBS (0.1M phosphate buffer saline, pH 7.2) was used as a control.

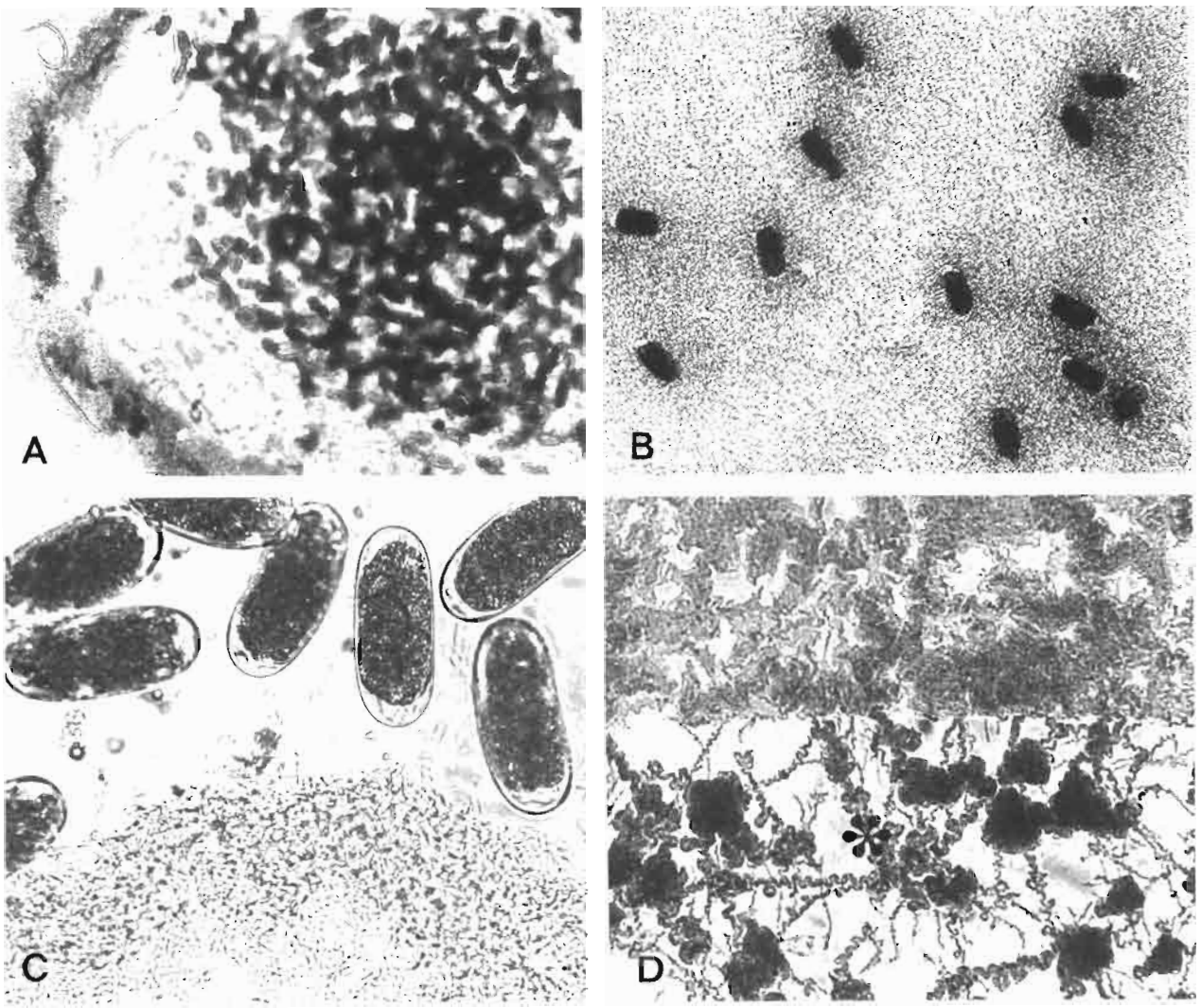

Fig. 1. Red blood cells binding, A : To the gelatinous matrix of *Meloidogyne javanica* egg-mass; B : To separated eggs; C : *Bacillus cereus* binding to the gelatinous matrix of *M. javanica* egg-mass; D : Colonies of *B.cereus* exposed to *M. javanica* gelatinous matrix suspension (area signed by star) compared with non-exposed colonies.

tremendously inhibited, while the tomato rhizobacteria growth increased (Table 3).

Eight morphologically different colonies isolated from the rhizobacteria population, were further tested for their agglutination ability by the GM. Four isolates which were not able to multiply on the GM suspension were able to agglutinate, while the bacteria which did not have the agglutination ability, were able to utilize the GM for growth.

Discussion

Egg masses of both *M. javanica* and *R. reniformis* had the ability to bind various bacteria and RBC to their surfaces (Table 2, Fig. 1). These results confirm preliminary observations reported by Orion and Kritzman (1991).

The resemblance between the binding reactions to *M. javanica* and *R. reniformis* egg-masses may hint a presence of a similar agglutination factor in the GM of both nematode species, in spite of their distant phylogenic relations.

Erythrocytes have been used as a tool to trace carbohydrate recognition domains (CRD; Jungrey & Weatherall, 1986) since the preponderance of their membrane carbohydrate consists of oligosaccharides linked to three types of glycophorine molecules, also known as sialoglycoproteins (Mirelman, 1986). RBC agglutination by the

GM was not inhibited by several carbohydrates, which, within our experimental conditions, excluding the possibility of CRD involvement in the agglutination phenomenon.

M. javanica separated eggs also bound RBC (Fig.1 B); however, the binding could be easily eliminated by washing thoroughly the eggs either with PBS or a hypochlorite solution. Agglutination of RBC by GM increased after trypsinization but was not affected by carbohydrates; RBC binding to eggs, however, was a trypsin- and carbohydrate-dependent process (Table 1), much like the RBC binding to several plant-parasitic nematodes described elsewhere (Spiegel *et al.*, 1991). This suggests that the tested egg surface contained a binding factor different from that in the GM.

Bacterial growth inhibition by antibiosis is generally expressed by the appearance of a halo around the source of the antibiotic material. Gelatinous component derived from the egg-mass or the GM suspension which had been placed on the agar plate – already containing the different bacteria – did not react in this manner. Since a halo was not observed in our experiments beyond the egg-mass or the GM drop zone, the authors concluded that the results did not support the possibility of the involvement of antibiotic activity in the GM-bacteria interaction. The agglutination phenomena expressed by the ability of the GM suspension to agglutinate bacteria (and RBC), and thus to reduce their growth or viability, may explain part of the GM capability to protect the eggs from surrounding microorganisms in soil. The highly viscous GM, which forms a hard layer around the egg-mass surface, could support a further physical (or mechanical) barrier.

Out of the different rhizobacteria isolated from tomato roots, several populations were able to utilize the GM suspension as a sole nutrient source, and multiply. Agglutination tests with populations which either multiply on the GM suspension or did not, revealed a good correlation between the ability of growth on the GM and agglutination. This observation further supports the role of the agglutination phenomenon in the GM protection process.

Additional chemical characterization of the GM suspension is underway to further understanding of the agglutination phenomenon described in this paper.

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