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Effect of Different Abiotic Factors on Chemotaxis of Bacteria Towards Fungal Propagules

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종류가 다른 무생물적 요인이 진균류에 미치는 세균의 주화성

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ABSTRACT: Chemotactic responses of five motile saprophytic and one phytopathogenic bacteria e.g. *Agrobacterium radiobacter, Bacillus subtilis, B. polymyxa, Pseudomonas aeruginosa, P. fluorescens* and *Xanthomonas malvacearum* towards exudate of *Cochliobolus sativus* conidia, *Fusarium oxysporum* f. sp. *ciceri* chlamydospores, *Macrophomina phaseolina* sclerotia and *Phytophthora drechsleri* f. sp. *cajani* oospores were determined *in vitro* at different abiotic conditions. In general, a positive correlation ($r=0.76$ to 0.89; $P=0.05$) was observed between concentration of fungal exudates and attraction of bacterial cells. Similarly, a significant ($P=0.05$; $r=+0.82$ to 0.95) positive correlation was noticed between chemotactic response and incubation period. The chemotactic response of bacteria was greatly influenced by temperature and pH of the test fungal exudate. The optimum temperature for maximum chemotaxis was 25°C for *A. radiobacter*, 30°C for *B. polymyxa, P. aerugionosa, P. fluorescens* and *X. malvacearum* and 35°C for *B. subtilis*. Fungal exudates maintained at pH 7 attracted maximum number of bacteria. The response of bacterial cells to exudates at pH 3 and 11 was not significantly ($P=0.05$) different than that to the buffer (control). Chemotaxis of bacteria was observed towards attractants (fungal propagules and their exudates) when they were kept apart and bridged with the capillaries filled with non-attractant (buffer) or attractant (exudate).

KEYWORDS: Chemotaxis, Fungal propagules, Abiotic factors.

Sensing, adaptation and regulation are important properties of biological system that are conceptually distinct and yet interrelated. The unique chemical communication system of motile microorganisms, and its role in community ecological processes such as symbiosis, predation, differentiation, sexuality and defense have a clear advantage over nonmotile microbes (Chet and Mitchell, 1976; Bergman *et al.*, 1988; Muehlstein *et al.*, 1988; Maier and Mueller, 1990). The molecular genetics and biochemistry of bacterial chemotaxis have been extensively investigated in the recent past (Mesibov and Adler, 1972; Adler, 1975; Shioi *et al.*, 1987; Vogler and Lengler, 1987; Meister *et al.*, 1987; Hess *et al.*, 1987, 1988; Berg, 1988; Lengler and Vogler, 1989; Lukat *et al.*, 1990). The role of microbial chemotaxis in rhizosphere interactions, symbiotic infection and root nodule initiation is also well understood (Ames and Bergman, 1981; Gulash *et al.*, 1984; Scher *et al.*, 1984, 1985; Bergman *et al.*, 1988; Caetano-Anolles *et al.*, 1988). Though interaction between bacteria and fungi in natural habitats has been demonstrated by several workers (Lockwood, 1968; Chet *et al.*,

1971; Siala and Gray, 1974; Diem, 1975; Wong and Griffin, 1976b; Gupta, 1992), but relatively very little is known about the chemotactic responses of bacteria towards fungal propagules and their exudates (Arora *et al.*, 1983a; Arora, 1986). The extensive colonization of fungal hyphae or sporosphere by bacteria in soil (Fradkin and Patrick, 1982) suggests that fungal propagules and hyphae could serve as a favourable niche for bacteria. Surprisingly, the role of chemotaxis in the interaction processes of fungi and bacteria is not well understood. In this study the chemotactic response of five common saprophytic and one phytopathogenic bacteria towards four fungal propagules was investigated at different abiotic conditions.

Materials and Methods

Preparation of fungal spores and exudates: Soil-borne phytopathogenic fungi *Cochliobolus sativus* (*Cs*) (Ito and Kurib) Drechs ex Dastur and *Fusarium oxysporum* Schlecht emend. Snyd. and Hans. f. sp. *ciceri* (*Foc*) (Padwick) Snyd. and Hans. were maintained on carrot agar and Komada's medium (Komada, 1975), respectively. *Macrophomina phaseolina* (*Mp*) (Tassi) Goid and *Phytophthora drechsleri* f. sp. *cajani* (*Pdc*) (Kannaiyan *et al.*) were maintained on acidified potato dextrose agar (pH 5.6). Propagules of *C. sativus* (conidia), *F. oxysporum* f. sp. *ciceri* (chlamydospores), *M. phaseolina* (sclerotia) and *P. drechsleri* f. sp. *cajani* (oospores) were obtained by a technique described elsewhere (Hsu and Lockwood, 1973; Arora *et al.*, 1983; Chauhan, 1985). Washed spores of different fungi were resuspended in 50 mM phosphate buffer (pH 7) and exudates of different concentrations were prepared by incubating the spores for a period less than that required for germination (4 h for conidia; 12 h for chlamydospores and sclerotia and 48 h for oospores). Three concentrations of fungal spores were used to prepare the exudate (1×10^4, 1×10^6 and 1×10^8 spores/m*l*). The exudate was sterilized by passing through a sterile membrane filter (0.22 μm pore size) and stored at 4℃.

Bacterial strains: The isolates of six common motile bacteria *Agrobacterium radiobacter* (*Ar*) Conn 1942 and *Pseudomonas aeruginosa* (*Pa*) Migula 1990 were maintained on nutrient yeast-extract medium. *Bacillus polymyxa* (*Bp*) Mace 1889 and *Xanthomonas malvacearum* (*Xm*) Dowson 1939 were maintained on nutrient agar medium. *Bacillus subtilis* (*Bs*) Cohn 1872 was grown on Tryptone glucose salt medium and *P. fluorescens* (*Pf*) Migula 1872 was maintained on King's medium-B. For chemotaxis experiments, bacteria were grown for 12 h in 20 m*l* of appropriate liquid medium to stationary phase. This culture (1 m*l*) was transferred to 10 m*l* of liquid medium and incubated to obtain the exponential phase of growth. Bacterial cells were centrifuged and washed with 50 mM sodium phosphate buffer. Cells were resuspended in chemotaxis medium consisting 10 mM phosphate buffer containing 10^{-4}M Na_2 EDTA (Adler, 1973).

Chemotaxis experiments: Chemotaxis was assayed by modification of a capillary method used by Adler (1973). A glass tube (0.5 cm, internal diameter (ID)×2 cm long) cemented at one end of a glass slide (7.5×2.5 cm) was used as an apparatus to measure chemotaxis. A 1 μ*l* capillary micropipete (0.2 mm ID×3 cm long), sealed at one end, was filled with fungal exudates of different concentrations and exudates adjusted at various pH (3, 5, 7, 9 and 11) or buffer solution (pH 7). The open end of the capillary micropipet was inserted in test tube containing 0.2 m*l* of bacterial suspension (1×10^7 cells/m*l*). The capillaries were incubated for 10, 30, 60 and 90 min. to observe the effect of incubation time on chemotaxis. Temperature related chemotaxis experiments were performed similarly but the apparatus was incubated in a temperature regulated BOD incubator at 15, 20, 25, 30, 35 and 40℃ for 90 min. After incubation capillary was taken out and washed exteriorly by a jet of sterilized distilled water. The capillary was crushed in 10 m*l* of 0.9% sterilized NaCl solution and 0.2 m*l* of diluted suspension (10^{-2} and 10^{-3} dilutions) was spread over Petri dishes (three plates/treatment) containing appropriate agar media. Plates were incubated at 30± 2℃ and bacterial colonies were counted after

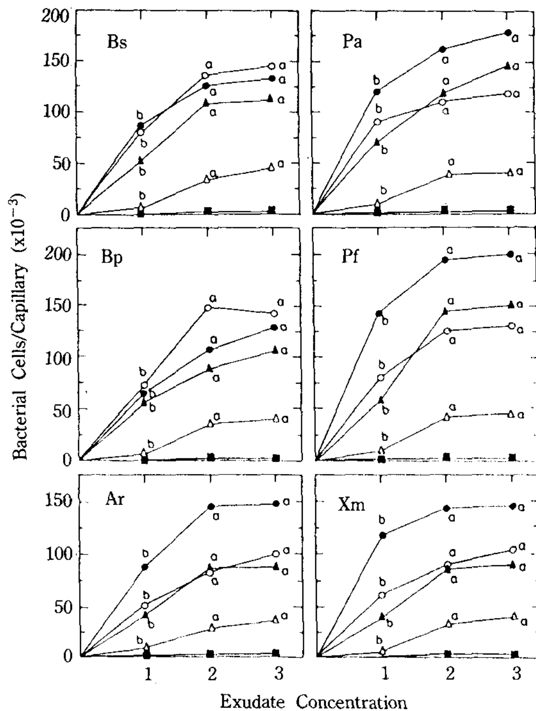

Fig. 1. Acumulation of bacteria in capillaries containing exudates from *Cochliobolus sativus* conidia (●), *Fusarium oxysporum* f. sp. *ciceri* chalmydospores (○), *Macrophomina phaseolina* sclerotia ▲), *Phytophthora drechsleri* oospores (△), and buffer solution (■); 1, 2 and 3 = 1×10^4, 1×10^6 and 1×10^8 fungal propagules/m*l* used to prepare the exudate, respectively; *Ar*=*Agrobacterium radiobacter*; *Bp*=*Bacillus polymyxa*; *Bs*=*Bacillus subtilis*; *Pa*=*Pseudomonas aeruginosa*; *Xm*=*Xanthomonas malvacearum*. Similar symbols folowed by different letters are significantly (P= 0.01 and 0.05) different, using Duncan's new multiple range test. Separate analysis was made for different fungal propagules.

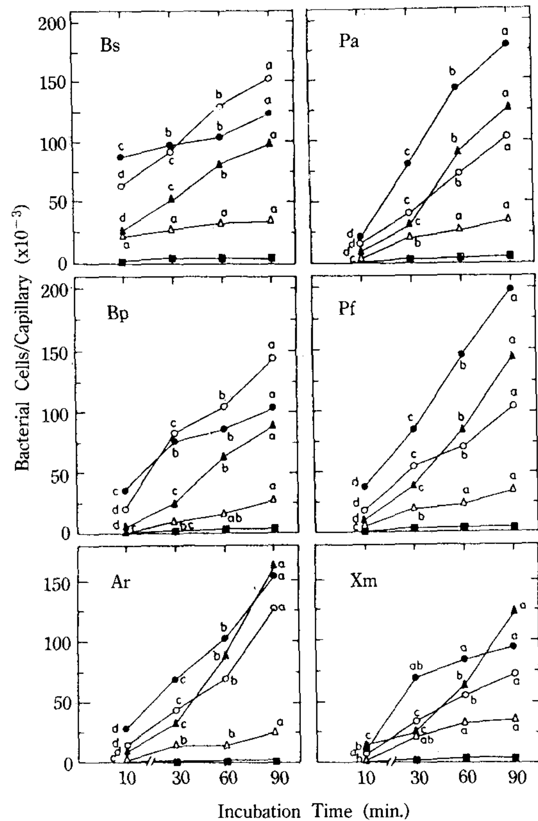

Fig. 2. Effect of incubation time on accumulation of bacterial cells in the capillaries containing exudate from *Cs* conidia (●), *Foc* chlamydospores (○), *Mp* sclerotia (▲), *Pdc* oospores (△), and buffer (■); exudate was prepared from 1×10^6 propagules/m*l*. Other footnotes and abbreviations are same as mentioned in Fig. 1.

3-4 days.

To determine whether or not bacteria could move towards fungal propagules and their exudates when these attractants were kept at a distance and bridged with a capillary containing buffer or exudate "combination" experiment was conducted.

The combination studies performed similarly except the chemotaxis apparatus consisted of two glass chambers cemented at the both ends of the slide. One chamber was filled with 0.2 m*l* of bacterial suspension (1×10^7 cells/m*l*) and other chamber was filled with 0.2 m*l* of spore suspension (conidia and chlamydospores 1×10^5/m*l*, sclerotia 5×10^3/m*l* and oospore 1×10^3/m*l*). Glass capillary micropipet open at both ends, was filled with buffer or exudates by capillary action, and inserted into the glass chamber containing bacterial suspension. The another end of capillary was inserted into second glass chamber contained 0.2 m*l* of spore suspension.

Treatments were replicated three times per experiment, and experiments were repeated twice to establish the reproducibility of results. The differences between means were distinguished using Duncan's multiple-range test.

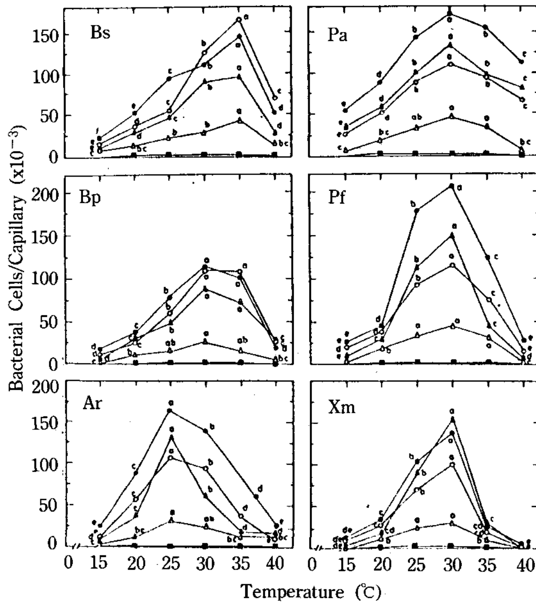

Fig. 3. Effect of different temperatures on a accumulation of bacterial cells in capillaries containing exudate from *Cs* conidia (●), *Foc* chlamydospores (○), *Mp* sclerotia (▲), *Pdc* oospores (△), and buffer (■). Accumulation after 90 min; exudate was prepared from 1×10^6 propagules/m*l*. Other footnotes and abbreviations are same as mentioned in Fig. 1.

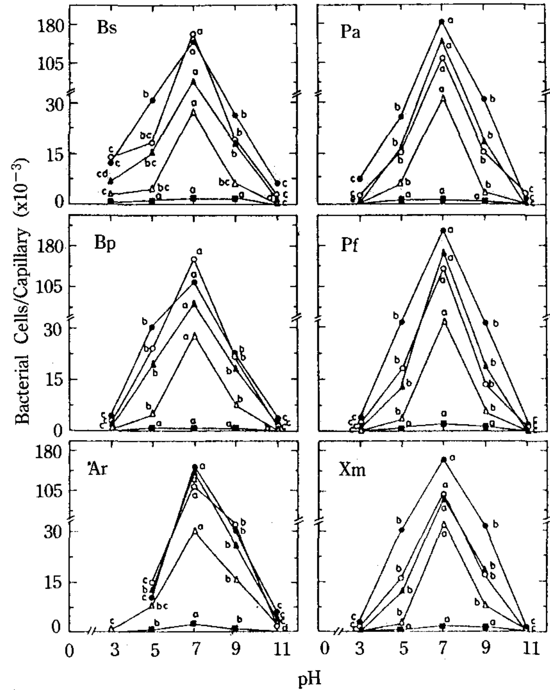

Fig. 4. Effect of different pH on accumulation of bacterial cells in capillaries containing exudate from *Cs* conidia (●), *Foc* chlamydospores (○), *Mp* sclerotia (▲), *Pdc* oospores (△), and buffer (■).
Accumulation after 90 min; exudate was prepared from 1×10^6 propagules/m*l*. Other footnotes and abbreviations are same as mentioned in Fig. 1.

Results

Attraction of bacteria towards fungal exudates, as measured by the number of cells accumulated in the capillary, was significantly ($P=0.01$) greater than that towards Na-phosphate buffer, the blank experiments (Figs. 1 to 4). In all the treatments (concentration of exudate, assay time, temperature and pH) attraction of bacteria towards fungal exudates was in the order of conidia>sclerotia>chlamydospores>oospores. Whereas, accumulation of *Bs* and *Bp* in the capillaries containing exudate was in the order of chlamydospores>conidia>sclerotia>oospores.

Effect of Concentration of Exudate. Attraction of bacterial cells was found to be directly related ($P=0.05$; $r=+0.76$ to 0.89) to the concentration of fungal exudates (Fig. 1). In general, accumulation of bacterial cells was almost 2 to 8 fold greater in the capillaries containing exudates prepared from 10^8 propagules/m*l* than capillaries containing exudates prepared from 10^4 propagules/m*l*. However, no significant ($P=0.05$) difference between the number of bacterial cells accumulated in the capillaries containing exudates from 10^8 and 10^6 propagules/m*l* was observed (Fig. 1).

Effect of Incubation Time: Fig. 2 illustrates the rate of accumulation of each bacteria in the capillaries containing exudate or buffer differed greatly. The chemotactic response of the bacterial cells, after each incubation period, i.e. 10, 30, 60 and 90 min, towards the exudates was significantly ($P=0.01$) greater than towards buffer solution (Fig. 2). A significant ($P=0.05$; $r=+0.92$ to 0.98) positive correlation was observed between chemotactic response and chemotactic duration time. For instance, k28, 68, 102, and 154×10^3 *Ar* cells per

capillary were attracted towards the exudate of *Cs* conidia after 10, 30, 60 and 90 min, respectively. However, in few cases, chemotactic response was not increased significantly ($P=0.05$) with increasing incubation time (Fig. 2).

In general, all the test bacteria showed significant ($P=0.05$) greater response after 90 min than after other incubation periods. For instance, accumulation of bacterial cells in the exudates increased from 1 to 7 fold with increasing incubation time from 10 to 90 min. The response of *Ar, Bp, Pa, Pf* and *Xm* towards the exudate of *Mp* sclerotia increased two-fold with increasing incubation time i.e. from 30 to 60 min. Thus, shorter incubation period e.g., 10 and 30 min, generally resulted accumulation of fewer bacterial cells in the exudate-filled capillaries. The accumulation of bacteria in capillaries filled with fungal exudate was not due to multiplication.

Effect of Temperature: Fig. 3 shows the effect of different temperatures on chemotactic response of the test bacteria towards the exudate of fungal propagules. In general, temperature greatly influenced the chemotactic response of all the bacteria. For instance, 24, 87, 164, 134, 60 and 25×10^3 *Ar* cells per capillary were attracted towards the exudate of *Cs* conidia at 15, 20, 25, 30, 35 and 40℃, respectively. The optimum temperature for active chemotaxis was 25℃ for *Ar* cells, 30℃ for *Bp, Pa, Pf* and *Xm* cells and 35℃ for *Bs* cells. The response of bacterial cells at their respective optimum temperatures was 1 to 78 fold greater than at other temperatures. The response of *Ar, Bs, Pa, Pf* and *Xm* cells towards the exudate of *Cs* conidia, *Foc* chlamydospores and *Mp* sclerotia was greatly influenced by any change in optimum temperature, while response of *Bp* cells at 35℃ was not significantly ($P=0.05$) different than the response at optimum temperature i.e. 30℃. *Bacillus polymyxa, Bs* and *Pa* cells showed minimum response at 15℃. Whereas *Xm* was found to be minimum at 40℃ (Fig. 3).

Effect of pH on chemotactic response: Maximum chemotactic response of the test bacteria was noted when the fungal exudate was maintained at pH 7 (Fig. 4). Response of the bacterial cells towards the exudate maintained at the pH 3, 5, 9 and 11 was significantly ($P=0.05$) less than to the exudate maintained at pH 7. For instance, 4, 25, 181, 30 and 2×10^3 *Pa* cells per capillary were attracted towards the exudate of *Cs* conidia at pH 3, 5, 7, 9 and 11, respectively (Fig. 4). Accumulation of bacterial cells was 2 to 15 fold greater in the exudate maintained at pH 5 than at pH 3. Similarly, 3.5 to 15 fold greater accumulation was observed in the exudates maintained at pH 7 than in the exudate maintained at ph 5. Further increase in the pH, i.e. from 7 to 9, reduced the accumulation of cells up to 2 to 8 fold.

"Combination" Experiment: The chemotactic response of motile bacteria towards attractants (propagules or exudates) when they were kept apart and bridged with the capillaries filled with nonattractant (buffer) or attractant (exudate) (Table 1). All the four bacteria accumulated in significant ($P=0.05$) greater number in capillaries containing fungal exudate than in those containing buffer. For example, capillaries filled with the exudate of *Cs* conidia and inserted in chamber containing buffer contained 84×10^3 *Ar* cells/m*l*, whereas, capillaries filled with buffer and inserted in chamber containing buffer contained only 2.5×10^3 *Ar* cells/m*l*. Capillaries filled with the exudate of *Cs* conidia and inserted into a suspension of *Cs* conidia accumulated 95×10^3 *ar* cells/m*l*, and capillaries filled with buffer and inserted in chamber containing conidial suspension contained 42×10^3 *Ar* cells/m*l*. Similarly, chambers containing conidia and exudate had greater number of bacterial cells (130, 96, and 80×10^3 *Ar* cells/m*l*) than did chambers containing buffer (35 and 1×10^3 *Ar* cells/m*l*). The accumulation of bacterial cells in chambers in different combinations was in the order: exudate-propagules>buffer-propagules>buffer-exudate>exudate-buffer. Similarly, accumulation of bacterial cells in capillaries in different combinations was in the orders: exudate-propagules>exudate-buffer>buffer-propagules>buffer-exudate (Table 1).

Accumulation of bacterial cells in different combinations was greater towards propagules than their exudates. For example, 96×10^3 *Ar* cells/m*l*

Table 1. Accumulation of bacteria in chambers containing fungal propagules, their exudates or buffer and in capillaries containing fungal exudates or buffer

Fungi	Attractant		Bacterial cells/ml (x10^{-3})							
	In capillary[x]	In chamber[x]	*Ar*		*Bs*		*Pf*		*Xm*	
			I	II	I	II	I	II	I	II
Cochliobolus	Exudate[y]	Conidia	[z]95a	130a	77b	150a	125a	178a	86a	154a
Sativus	Buffer	Conidia	42c	96b	58c	129b	66c	155b	52c	126b
	Exudate	Buffer	84b	35d	114a	35d	100b	23d	69b	35d
	Buffer	Exudate	34c	80c	50c	101c	59c	114c	30d	94c
	Buffer	Buffer	3d	1e	2d	1e	3d	2e	2e	2e
Fusarium oxysporum f.	Exudate	Chlamydo-spores	88a	94a	58b	162a	110a	129a	70a	110a
sp. *ciceri*	Buffer	Chlamydo-spores	46c	89a	40c	107b	48c	87b	28c	77b
	Exudate	Buffer	73c	21c	77a	18d	76b	49d	58b	30d
	Buffer	Exudate	29d	70b	27d	69c	28d	66c	20c	49c
	Buffer	Buffer	2e	1d	2e	2e	2e	1e	2d	1e
Macrophomina	Exudate	Sclerotia	86b	145a	51c	139a	106a	159a	69b	172a
phaseolina	Buffer	Sclerotia	69c	143a	98b	120b	42c	123b	47c	141b
	Exudate	Buffer	127a	34c	87a	22d	72b	19d	72a	20d
	Buffer	exudate	50d	109b	29d	89c	66b	99c	52c	107c
	Buffer	Buffer	2e	2e	2e	1e	3d	2e	2d	1e
Phytophthora	Exudate	Oospores	37a	59a	34a	58a	46a	69a	53a	72a
drechsleri	Buffer	Oospores	14c	43b	17b	40b	10c	37b	20b	49b
f. sp. *cajani*	Exudate	Buffer	25b	10c	21b	6d	24b	8d	18d	7d
	Buffer	exudate	9d	15c	10c	23c	8c	25c	12b	25c
	Buffer	Buffer	2e	1a	2a	1e	2a	1e	2e	1e

x=Accumulation after 90 min.
y=Exudate was prepared from 1×10^6 propagules/ml.
I=Accumulation in capillary; II=Accumulation in chamber.
z=Data are means of four replicates; means in a column followed by same letters are not significantly different ($P=0.05$) using Duncan's multiple new range test (Separate statistical analysis was done for different types of fungal propagules in each column).

were accumulated in chambers containing conidial suspension whereas, 80×10^3 *Ar* cells per ml were accumulated in chambers containing exudate of *Cs* conidia.

Discussion

The results clearly demonstrate that motile bacteria can move towards propagules and their exudates. The differential chemotactic response of bacteria to the different fungal propagules and their exudates suggests differences in chemical stimuli and signals for each of the spore types. Similarly, other workers have also observed differential bacterial chemotaxis towards different types of fungal propagules (Arora *et al.*, 1983; Lim and Lockwood, 1988). It is also evident that chemotactic response was markedly affected by temperature, pH, incubation time and concentration of propagules or exudates. These factors influence

the tumbling frequency, swimming pattern and velocity of motile bacteria (Maeda *et al.*, 1970; Moench and Konetzka, 1978). Bacterial cells also responded to the variation in attractant concentration by modulating the swimming pattern.

In this study, greater chemotaxis was observed towards fungal exudates prepared from 10^6 propagules/m*l* than 10^4 propagules/m*l* (Fig. 1). However, no significant ($P=0.05$) difference between chemotactic response towards exudates prepared from 10^6 and 10^8 propagules/m*l* was observed which clearly indicate that after a saturating concentration of attractant, the chemotactic response does not increase. In fact, it has been observed that higher concentration of attractant act as repellent. For example, *Pseudomonas aeruginosa* showed maximum attraction towards 10^{-3} *M* serine, α-methyl asparate and glucose but higher concentration of these chemicals were unable to attract more bacteria (Moulton and Montie, 1979). Thus, concentration of attractant plays a significant role in the bacterial chemotaxis. It seems that chemotaxis operates over a limited range of concentration of attractants (response range). At the low extremes is the 'threshold' concentration that gives a detectable response and high concentration is the 'saturating' concentration above which bacteria cannot detect and move towards it (Adler and Templeton, 1967).

Chemotaxis of bacteria towards fungal propagules was greatly affected by temperature (Fig. 3). The optimum temperature for maximum chemotaxis towards fungal propagules ranged from 25 to 35℃. Thus, bacterial chemotaxis operates over a broad temperature range, indicating that temperature ranging between 20 to 35℃ is not a limiting factor for chemotaxis. Microscopic observations also revealed that swimming speed and tumbling frequency of the bacteria, which are most important for the tactic response, were affected by the extreme fluctuation in the temperature (15 and 40℃). In the case of *Bs* the swimming velocity at various temperatures, i.e. 20, 25, 30 and 35℃ was found in increasing order, but tumbling of *Bs* cells had a peak at 35 C and was very low at 15 and 40℃. At 30℃, most of the test bacteria behaved like a nonchemotactic swimming mutant. Thus, it seems that motility itself has greater dependence on temperature than chemotaxis.

Variation in the pH of fungal exudates significantly influenced the bacterial chemotaxis (Fig. 4). Other workers also reported inhibition of chemotactic response of many bacteria and fungal zoospores at extreme pH i.e. 1 to 3 and 10 to 14 (Seymour and Doetsch, 1972; Orpin and Bountiff, 1978; Klopmeyer and ries, 1987). In general, motility and chemotaxis was reduced at pH values above or below neutrality. Interestingly, *Bs* exhibited chemotaxis to a broader range of pH. The reduction in taxis of the test bacteria below pH 5 reflects a corresponding reduction in motility, as judged by microscopic observations; at pH 3 to 4 bacteria were nonmotile in the fungal exudates (though they were viable). In the exudates maintained at pH 9 bacteria were motile but at pH more than 9 motility was considerably reduced.

The effect of pH on bacterial chemotaxis was found to be almost similar in all the attractants though exudates of different fungal propagules contained different amino acids and sugars, but almost all exudates consisted of glucose, galactose and sucrose. Therefore, it may be possible that other chemicals present in the fungal exudates in a very minor amount could not be detected by the different chemoreceptors.

The results clearly demonstrate that fungal propagules may act as attractants to motile bacteria. Thus, these bacteria via chemotaxis may establish themselves in and around the sporosphere of the fungal propagules in soil and can impose energy (nutrient) stress which in turn may cause acceleration in loss of endogenous carbon compounds leading to loss of viability and decreased pathogenic aggressiveness. Further work is needed to ascertain the role of bacterial chemotaxis towards fungal propagule in microbial community ecology.

摘 要

운동성을 띠는 5가지 부생균과 1종류의 식물병원세균으로 *Agrobacterium radiobacter, Bacillus su-*

btilis, *B. polymyxa*, *Pseudomonas aeruginosa*, *P. fluorescens* 및 *Xanthomonas malvacearum*을 공시하여 이들 세균이 *Cochliobolus sativus* 분생포자, *Fusarium oxysporum f. sp. ciceri*의 후막포자, *Macrophomina phaseolina*의 균핵 및 *Phytophthora drechsleri* f. sp. *cajani* 난포자의 분비물에 미치는 화학주성을 서로 다른 무생물적 조건하에서 실험하였다. 균의 분비물의 농도와 세균세포의 끌리는 유인사이에는 정상관(r=0.76~0.89)이 있었다. 유사한 결과로서 화학주성반응과 배양간격에서도 통계적인 정상관이 있다(r=0.82~0.95). 세균의 화학주성반응은 온도와 pH에 크게 영향을 받는다. 최적 화학주성을 나타내는 온도로서 *A. radiobacter*는 25℃, *B. polymyxa*, *P. aeruginosa*, *P. fluorescens*와 *X. madracearum*은 30℃이었고, *B. subtilis*는 35℃가 최적이었다. 최대 화학주성을 나타내는 분비물의 주성은 pH 7이고 pH 3과 11에서는 buffer와 같았다. 시험용 모세관에 유인물질이나 buffer 중 어느 종류가 채워져도 유인물질이 있는 시험관에 연결되면 세균은 주성이 나타난다는 것이 증명되었다.

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