J. Univ. Kuwait (Sci.) **17**, 1990

An ultrastructural study of two cotton lines with intermediate levels of resistance to *Xanthomonas campestris* pv. *malvacearum*

ALI H. AL-MOUSAWI

Department of Biology, College of Science, University of Kufa, Hilla, Iraq

ABSTRACT

Two cotton lines, OK 2.3 and OK 1.2, which have low and high levels, respectively, of resistance to bacterial blight were derived from a cross between a highly susceptible line, Ac 44, and an immune line, Im 216. Final bacterial population densities of *Xanthomonas campestris* pv. *malvacearum* in leaves of Ac 44, OK 2.3, OK 1.2, and Im 216 were inversely related to host resistance. Ultrastructural effects of the interactions between *X. campestris* pv. *malvacearum* and cotyledons and leaves of OK 2.3 and OK 1.2 were studied. Fibrillar materials and enveloping films appeared around bacteria in both lines within 4 h after inoculation. The average numbers of bacteria observed within each envelope increased with time in both lines. After 48 h, most enveloping films had ruptured in the less resistant line, OK 2.3, and bacteria had emerged from them. Most envelopes were ruptured within 72 h in OK 1.2. The results indicate that the enveloping structures do not prevent bacterial multiplication, but that persistence of envelopes is correlated with host resistance.

INTRODUCTION

In previous papers (Al-Mousawi *et al.* 1982b, 1983) live incompatible or saprophytic bacteria were infiltrated into cotton (*Gossypium hirsutum* L.) cotyledons. Fibrillar materials developed around the bacteria and enveloping films attached bacteria to host cell walls. Killed compatible or incompatible bacteria and starch grains were also enveloped, though without associated fibrillar materials. In a compatible cotton line, however, *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye (*X. malvacearum*) was not enveloped (Al-Mousawi *et al.* 1982a). Since a wide variety of particles were enveloped in cotton, envelopment may be the usual fate of bacteria and other hydrophilic particles, while live compatible bacteria somehow escape envelopment.

The enveloping materials in cotton appeared too fragile to restrict bacterial growth (Cason *et al.* 1978). In tobacco, enveloping structures are more electron-dense (Goodman *et al.* 1976; Sequeira *et al.* 1977; Politis & Goodman 1978) and it has been suggested that they do entrap incompatible bacteria and inhibit their growth (Goodman *et al.* 1976). In bean and soybean it appears unlikely that bacterial envelopment plays a major role in disease resistance, since films develop

around compatible as well as incompatible and saprophytic bacteria (Sigee & Epton 1976; Hildebrand *et al.* 1980; Daub & Hagedorn 1980; Fett & Jones 1982).

Previous ultrastructural studies of cotton-bacterial interactions (Cason *et al.* 1978; Al-Mousawi *et al.* 1982a,b, 1983) have involved extreme cases of compatibility (pv. *malvacearum* with the highly susceptible line Ac 44) and incompatibility (pv. *malvacearum* with the bacterial blight-immune line Im 216). In the compatible interaction, envelopment of bacteria was never observed (Al-Mousawi *et al.* 1982a, 1983) but in the incompatible interactions envelopes formed around all bacteria (Cason *et al.* 1978; Al-Mousawi *et al.* 1983). The present study employs two cotton lines, OK 2.3 and OK 1.2, which possess homozygous low and high levels, respectively, of resistance to bacterial blight. The work was undertaken to elucidate the role of envelopment in disease resistance in cotton.

MATERIALS AND METHODS

Cotton lines OK 2.3 and OK 1.2 were derived from segregating generations of a cross between the blight-susceptible line Ac 44, which has no major genes for resistance to bacterial blight (Brinkerhoff 1970), and the blight-immune line Im 216, which has homozygous immunity to bacterial blight that is probably due to three major resistance genes B_2, B_3 and b_7 and a complex of minor genes (Brinkerhoff & Verhalen 1976; Barnes 1980). Each segregating generation from this cross was inoculated with pv. *malvacearum* Race I. Symptom expression was graded 2 weeks after inoculation on the basis of lesion size in foliage leaves and was recorded on a scale from 0·0 (no macroscopically visible lesions) to 4·0 (high susceptibility) (Barnes 1980). Im 216 was graded 0·0. OK 1.2 developed lesions from 0·9 to 1·2 mm long which were angled at leaf veins, and it was graded 1·2. OK 2.3 showed mesothetic or mixed reactions and was graded 2·3. The smaller lesions, which predominated, ranged from 1·5 to 3·1 mm long. The larger lesions averaged 4·6 mm in length. Ac 44 had lesions ranging from 5 to 8 mm in length, and it was graded 4·0. With increasing severity of symptoms in Ac 44 the lesions were still angular, but spread across progressively larger veins. Individual plants used in this study were the result of twelve generations of self-fertilization. Homozygosity, indicated by uniform infection grades, was achieved by the F6 generation in OK 1.2 and OK 2.3. Present data indicate that the low level of blight resistance in OK 2.3 is due to the gene B_2 and that the higher level of resistance in OK 1.2 is due to genes B_3 and b_7. (Barnes, Brinkerhoff & Johnson, unpublished)

Plants of Ac 44, OK 2.3, OK 1.2, and Im 216 lines were grown in a greenhouse as described previously (Al-Mousawi *et al.* 1982a). Daily maximum temperature in the greenhouse was $34° \pm 2°C$, and minimum night temperature was $21° \pm 3°C$. Relative humidity was near 100% at night, and the average daily minimum was $40 \pm 15\%$ in the daytime. Cotyledons of 2 week-old plants or the third and fourth fully-expanded foliage leaves of 6 week-old plants were used. Race 3 pv *malvacearum* was cultured and inoculum suspensions of 5×10^7 bacteria/ml in sterile saturated calcium carbonate solution were prepared as reported by Essenberg *et al.* (1979). Cotyledons were inoculated by means of a syringe without a needle (Al-Mousawi *et al.* 1982a). Leaves were infiltrated with the inoculum suspension through stomata of their abaxial surfaces by means of an 8L hand-pumped sprayer (manufactured by

AG Chemical Equipment Co. Inc., (bp) 4900 Viking Drive, Minneapolis, Minnesota, 55435).

Samples for electron microscopy were taken from cotyledons and leaves of OK 1.2 and OK 2.3 at 4, 24, 48, 72 and 96 h after inoculation. Tissues were fixed, embedded, and observed by transmission electron microscopy as described by Al-Mousawi *et al.* (1982a). Bacterial population densities were determined in a separate experiment by the plate count method (Essenberg *et al.* 1979) with the aid of a Spiral Plater (Spiral Systems Instruments, Inc., Bethesda, MD 20814) (Gilchrist *et al.* 1977).

RESULTS

BACTERIAL POPULATION TRENDS

The growth rate of pv. *malvacearum* in Im 216 was slower than that in the other three lines (Fig. 1). Growth rates in the other three lines were very similar for 2 days, after which growth in the highly resistant line OK 1.2 was slower. Bacterial growth in OK 2.3 was very similar to that in susceptible Ac 44 until the fifth day, after which growth stopped in OK 2.3 and continued in Ac 44.

ELECTRON MICROSCOPIC OBSERVATIONS

Four hours after inoculation all bacterial cells were associated with fibrillar materials and enveloped within films in both lines OK 2.3 and OK 1.2 (Pls 1, 2). In OK 2.3 most envelopes were intact at 24 h (Pl. 3), but a few were broken and had released bacteria (Pl. 4). At 48 h most envelopes were broken in OK 2.3 (Pl. 5) and at later sampling times all bacterial cells appeared to be free in the intercellular spaces (Pl. 6 and Table 1). In the more resistant line, OK 1.2, a similar sequence was observed (Pls 7–9), except that intact envelopes persisted about a day longer than in OK 2.3 (Table 1). Numbers of bacteria associated with each envelope increased with time in both OK 2.3 and OK 1.2 (Table 2).

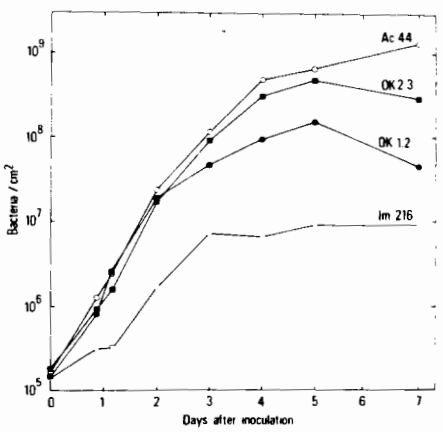

Fig. 1. Population densities of pv. *malvacearum* in leaves of four cotton lines following infiltration with 5×10^7 bacteria/ml. Six leaves, each on a different plant, were inoculated for each line at each sampling time. Each plotted point represents the mean bacterial population density of the six replicate discs.

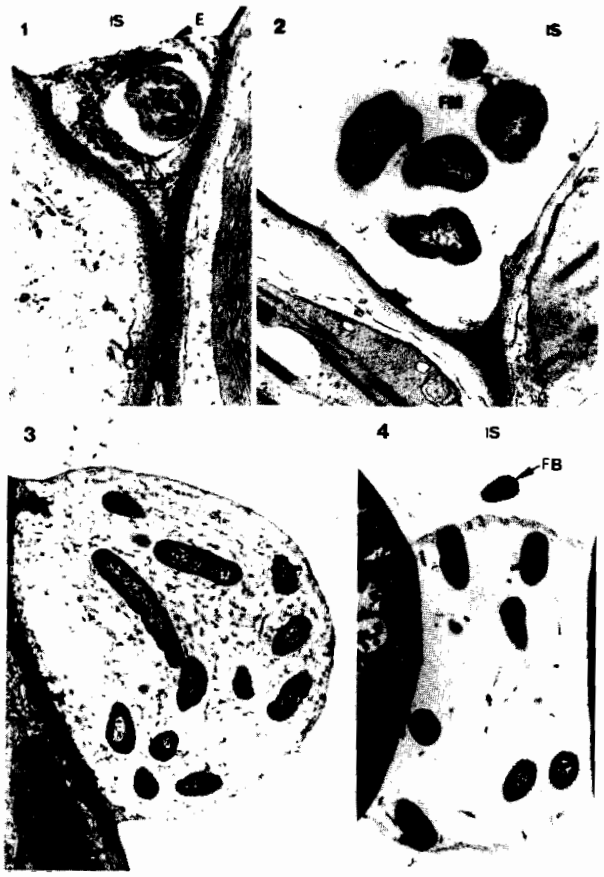

Plate 1. Mesophyll cell from leaf of OK 2.3, 4 h after inoculation with pv. *malvacearum*. A single bacterium is shown enveloped by fibrillar materials in intercellular space. × 58,000. B, bacterium; BE, broken envelope; E, envelope; FB, free bacterium; FM, fibrillar materials; IS, intercellular space.

Plate 2. Mesophyll cell from cotyledon of OK 1.2, 4 h after inoculation with pv. *malvacearum*. Five bacteria are enveloped by fibrillar materials. × 51,000. B, bacterium; BE, broken envelope; E, envelope; FB, free bacterium; FM, fibrillar materials; IS, intercellular space.

Plate 3. Mesophyll cell from leaf of OK 2.3, 24 h after inoculation with pv. *malvacearum*. Note enlarged envelope with divided bacteria. × 20,800. B, bacterium; BE, broken envelope; E, envelope; FB, free bacterium; FM, fibrillar materials; IS, intercellular space.

Plate 4. Mesophyll cell from leaf of OK 2.3, 24 h after inoculation with pv. *malvacearum*. A group of bacteria is enveloped and one bacterium is free in the intercellular space. × 23,700. B, bacterium; BE, broken envelope; E, envelope; FB, free bacterium; FM, fibrillar materials; IS, intercellular space.

DISCUSSION

Final bacterial population densities in the four lines studied were positively correlated with bacterial blight susceptibilities of the lines. Susceptibility is graded on the basis of lesion size. Im 216 is given a disease rating of 0·0, but it does develop necrotic lesions, too small to be seen with the unaided eye. These lesions are adjacent to single-cell colonies of pv. *malvacearum* (Essenberg *et al.* 1979).

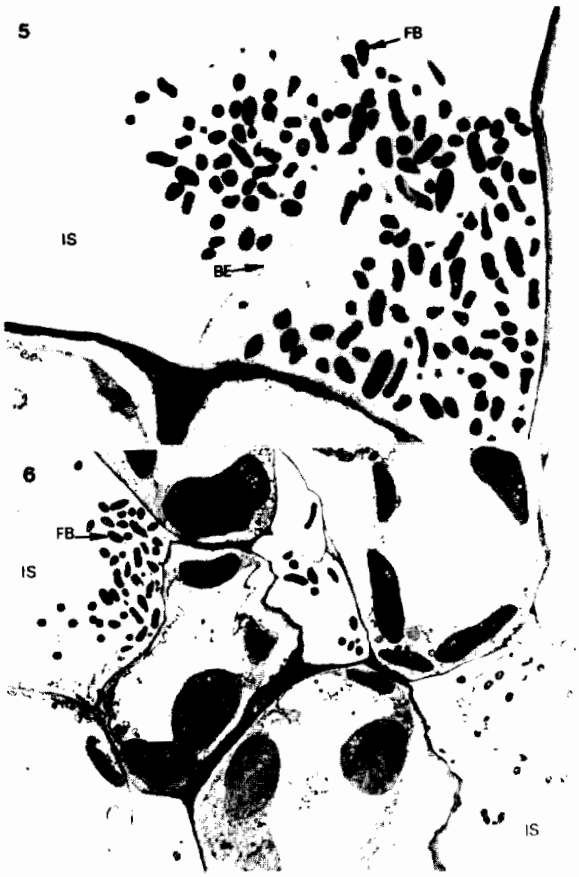

Plate 5. Mesophyll cell from cotyledon of OK 2.3, 48 h after inoculation with pv. *malvacearum*. Envelope appears to have been ruptured by increased number of bacteria. × 11,500. B, bacterium; BE, broken envelope; E, envelope; FB, free bacterium; FM, fibrillar materials; IS, intercellular space.

Plate 6. Mesophyll cell from leaf of OK 2.3, 72 h after inoculation with pv. *malvacearum*. No envelopes are visible and bacteria are free. × 5,900. B, bacterium; BE, broken envelope; E, envelope; FB, free bacterium; FM, fibrillar materials; IS, intercellular space.

Among the four lines studied a correlation has been observed between resistance to bacterial blight and persistence of enveloping films. No envelopment has been observed in Ac 44, the susceptible line (Al-Mousawi *et al.* 1982a, 1983). Films formed in the partially resistant lines OK 2.3 and OK 1.2 soon after inoculation, but most were disrupted within 2 and 3 days, respectively, persisting longer in the more highly resistant line (Table 1). Fairly small intact envelopes containing few bacteria have been found in Im 216 10 and 24 h after inoculation with high bacterial levels ($1–5 \times 10^8$ bacteria/ml) (Cason *et al.* 1978; Al-Mousawi *et al.* 1983). It could not be determined whether the envelopes persisted beyond those times in Im 216, as the cotyledons and leaves underwent confluent necrosis (Cason *et al.* 1978; Al-Mousawi *et al.* 1983). However, ultrastructural observations have been made of Im 216 5 and 6 days after low-level inoculation (5×10^5 bacteria/ml) (Al-Mousawi *et al.* 1982b).

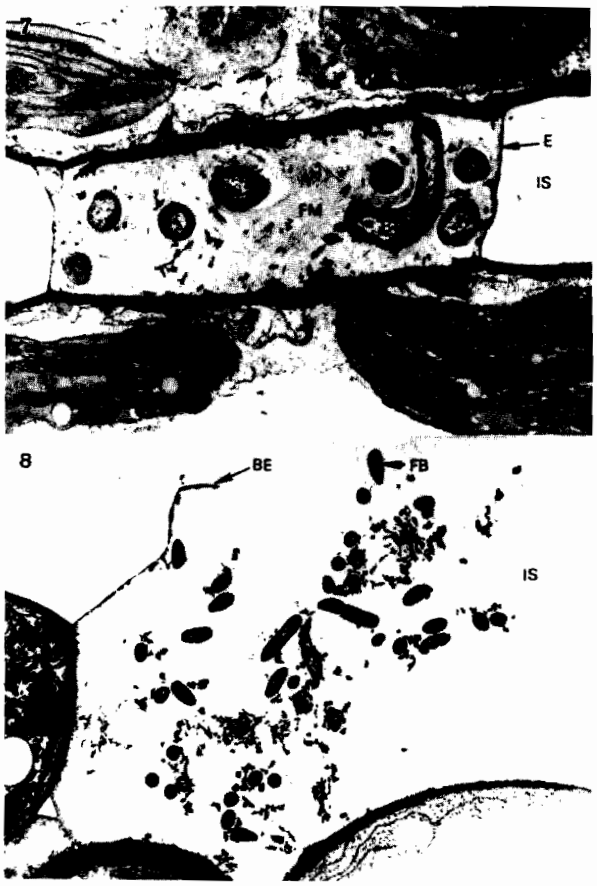

Plate 7. Mesophyll cell from leaf of OK 1.2, 24 h after inoculation with pv. *malvacearum*. Note divided bacterium within large envelope. × 30,100. B, bacterium; BE, broken envelope; E, envelope; FB, free bacterium; FM, fibrillar materials; IS, intercellular space.

Plate 8. Mesophyll cell from leaf of OK 1.2, 72 h after inoculation with pv. *malvacearum*. Envelope is ruptured and the bacteria are free in intercellular space. × 12,100. B, bacterium; BE, broken envelope; E, envelope; FB, free bacterium; FM, fibrillar materials; IS, intercellular space.

At 5 to 6 days, following several thousand-fold multiplication and subsequent inhibition, the bacterial colonies were surrounded by sparse enveloping material and individual peripheral bacteria did not appear to be confined (Al-Mousawi *et al.* 1982b). Light micrographs of similarly inoculated Im 216 leaves revealed a few enveloped, but mostly unenveloped bacteria 7 days after inoculation (Essenberg *et al.* 1979). Thus cells of pv. *malvacearum* are initially enveloped in the two resistant lines and in the immune line, but the envelopes appear not to persist intact.

Since numbers of bacteria within envelopes increased early after inoculation in OK 2.3 and 1.2 (Table 2), and subsequently emerged from the broken envelopes (Pls 5 and 8), it appears likely that the enveloping films ruptured as a result of bacterial population increases. Envelopment was not directly responsible for inhibiting bacterial growth in OK 2.3, as has been suggested to occur in tobacco (Goodman *et al.*

Plate 9. Mesophyll cell from leaf of OK 1.2, 96 h after inoculation with pv. *malvacearum*. No envelopes are visible around the bacteria. × 3,800. B, bacterium; BE, broken envelope; E, envelope; FB, free bacterium; FM, fibrillar materials; IS, intercellular space.

1976), since the envelopes broke and bacteria were released into the intercellular spaces within 2 days and did not stop multiplying until after 5 days (Fig. 1). It is also unlikely that envelopment restricted bacterial growth in OK 1.2, since 75% of the envelopes ruptured between 24 and 48 h before bacterial growth inhibition.

Table 1. Average percentages of envelopes that appeared intact[a]

Hours post-inoculation	4	24	48	72	96
OK 2.3	100	86	5	0	0
OK 1.2	100	100	25	5	0

[a] At each sampling time electron micrographs of 22 envelopes or clusters of unenveloped bacteria were made from each cotton line.

Table 2. Average numbers of bacterial cells observed per envelope[a] in thin[b] sections

Hours post-inoculation	4	24	48	72	96
OK 2.3	2 ± 1·6[c]	11 ± 3·4	27 ± 17·6	[d]	[d]
OK 1.2	3 ± 1·8	7 ± 4·6	16 ± 9·4	33·6 ± 9·5	[d]

[a] Within apparently intact envelopes of near broken ones.
[b] Silver sections, approximately 60 nm thick.
[c] Standard deviation of the mean.
[d] All bacteria were free in intercellular spaces.

Envelopment may play a role in resistance by facilitating contact of bacterial elicitors of the resistance response with host receptors. If so, effectiveness of the resistance response probably derives from factors other than the envelope itself, such as the structures of elicitors. Two sesquiterpenoid phytoalexins have been isolated and identified from inoculated leaves of Im 216 (Essenberg *et al.* 1982). The contributions of these phytoalexins to the inhibition of pv. *malvacearum* which occurs in the resistant and immune lines of this study are currently under investigation.

REFERENCES

Al-Mousawi, A.H., Richardson, P.E., Essenberg, M. & Johnson, W.M. 1982a. Ultrastructural studies of a compatible interaction between *Xanthomonas campestris* pv. *malvacearum* and cotton. Phytopathology **72**: 1222–30.

Al-Mousawi, A.H., Richardson, P.E., Essenberg, M. & Johnson, W.M. 1982b. Cotyledon and leaf ultrastructure of a bacterial blight-immune cotton line inoculated with a low level of *Xanthomonas campestris* pv. *malvacearum*. Phytopathology **72**: 1230–34.

Al-Mousawi, A.H., Richardson, P.E., Essenberg, M. & Johnson, W.M. 1983. Specificity of the envelopment of bacteria and other particles in cotton cotyledons. Phytopathology **73**: 484–89.

Barnes, M. 1980. Evaluation of the concept of horizontal and vertical resistance by the cotton-*Xanthomonas malvacearum* host-pathogen system. M.Sc. thesis, Oklahoma State University, Stillwater, Oklahoma.

Brinkerhoff, L.A. 1970. Variation in *Xanthomonas malvacearum* and its relation to control. Annual Review of Phytopathology **8**: 85–110.

Brinkerhoff, L.A. & Verhalen, L.M. 1976. Inheritance of immunity to bacterial blight in an upland cotton cross. In: Proceeding of the Beltwide Cotton Production Research Conference. 5–7 January 1976, Las Vegas. National Cotton Council, Memphis, Tennessee.

Cason, E.T. Jr, Richardson, P.E., Essenberg, M.K., Brinkerhoff, L.A., Johnson, W.M. & Venere, R.J. 1978. Ultrastructural cell wall alterations in immune cotton leaves inoculated with *Xanthomonas malvacearum*. Phytopathology **68**: 1015–21.

Daub, M.E. & Hagedorn, D.J. 1980. Growth kinetics and interactions of *Pseudomonas syringae* with susceptible and resistant bean tissues. Phytopathology **70**: 429–36.

Essenberg, M., Cason, E.T. Jr, Hamilton, B., Brinkerhoff, L.A., Gholson, R.K. & Richardson, P.E. 1979. Single cell colonies of *Xanthomonas malvacearum* in susceptible and immune cotton leaves and the local resistant response to colonies in immune leaves. Physiological Plant Pathology **15**: 53–68.

Essenberg, M., Doherty, M.d'A., Hamilton, B.K., Henning, V.T., Cover, E.C., Mcfaul, S.J. & Johnson, W.M. 1982. Identification and effects on *Xanthomonas campestris* pv. *malvacearum* of two phytoalexins from leaves and cotyledons of resistant cotton. Phytopathology **72**: 1349–56.

Fett, N.F. & Jones, S.B. 1982. Role of bacterial immobilization in race-specific resistance of soybean to *Pseudomonas syringae* pv. *glycinea*. Phytopathology **72**: 488–92.

Gilchrist, J.E., Donnelly, C.B., Peeler, J.T. & Campbell, J.E. 1977. Collaborative study comparing the spiral plate and aerobic plate count methods. Journal of the Association of Official Agricultural Chemists **60**: 807–12.

Goodman, R.N., Huang, P.-Y. & White, J.A. 1976. Ultrastructural evidence for immobilization of an incompatible bacterium, *Pseudomonas pisi*, in tobacco leaf tissue. Phytopathology **66**: 754–64.

Hildebrand, D.C., Alosi, M.C. & Schroth, M.N. 1980. Physical entrapment of pseudomonads in bean leaves by films formed at air-water interfaces. Phytopathology **70**: 98–109.

Politis, D.J. & Goodman, R.N. 1978. Localized cell wall opposition: incompatibility response of tobacco leaf cells to *Pseudomonas pisi*. Phytopathology **68**: 309–16.

Sequeira, L., Gaard, G. & De Zoeten, G.A. 1977. Interaction of bacteria and host cell wall: its relation to mechanisms of induced resistance. Physiological Plant Pathology **10**: 43–50.

Sigee, D.C. & Epton, H.A.S. 1976. Ultrastructural changes in resistant and susceptible varieties of *Phaseolus vulgaris* following artificial inoculation with *Pseudomonas phaseolicola*. Physiological Plant Pathology **9**: 1–8.

(Received 16 January 1988, revised 5 December 1989)

دراسة التركيب الدقيق لسلالتين من نبات القطن ذواتي مقاومة متوسطة لبكتريا
Xanthomonas campestris pv. malvacearum.

على هاشم الموسوي
قسم علوم الحياة بكلية العلوم ، جامعة الكوفة ،
الحلة ، العراق

خلاصة

درست سلالتان من نبات القطن هما *OK 2.3* و *OK 1.2* لهما مقاومة ضعيفة وقوية على التوالي لمرض اللفحة البكتيرية . لقد تم الحصول على هاتين السلالتين من التزاوج بين سلالة غير مقاومة *Ac 44* وسلاسة ذات مقاومة كبيرة جدا *Im 216* .

أظهرت الدراسة أن الكثافة النهائية للبكتريا المسببة للمرض في أوراق نباتات السلالات *Ac 44* و *OK 2.3* و *OK 1.2* و *Im 216* تتناسب تناسبا عكسيا مع مقاومة المضيف . وعند دراسة نتيجة التفاعل بين هذه البكتريا وأوراق السلالتين *OK 2.3* و *OK 1.2* باستخدام المجهر الألكتروني تبين تكون مواد ليفية ورقائق مغلفة حول البكتريا بعد ٤ ساعات من حقنها في النباتات . وقد تكاثرت البكتريا وتزايد عددها في كلتا السلالتين بمرور الزمن . وبعد مضي ٤٨ ساعة من بدء الحقن بالبكتريا ، تمزقت معظم الرقائق مما أدى الى تحرر البكتريا في السلالة *OK 2.3* ذات المقاومة الضعيفة ، أما السلالة *OK 1.2* عالية المقاومة فقد تمزقت معظم الرقائق فيها بعد مضي ٧٢ ساعة .

تظهر هذه الدراسة أن الرقائق التي تغلف البكتريا لا تمنع إنقسام البكتريا وتضاعف أعدادها ، ولكن إستمرار بقاء هذه الرقائق له علاقة بمقاومة المضيف للبكتريا .