Relationship of Bacterial Cell Surface Hydrophobicity and Charge to Pathogenicity, Physiologic Race, and Immobilization in Attached Soybean Leaves

ABSTRACT

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Cell surface hydrophobicity and charge were compared for pathogenic and nonpathogenic strains of *Xanthomonas campestris* pv. *glycines*, strains representing three physiologic races of *Pseudomonas syringae* pv. *glycinea*, and strains of several other phytopathogenic bacteria previously examined (electron microscopy) for active immobilization in soybean leaves. Three methods were utilized to determine cell surface hydrophobicity and one method for cell surface charge. Based on hydrophobic interaction chromatography, bacterial strains that were actively immobilized in attached soybean leaf intercellular spaces had lower cell surface hydrophobicity than those which were not immobilized. Two additional assays of cell surface hydrophobicity gave conflicting results. Variation in relative cell surface hydrophobicities of bacterial strains was related to method. However, all three methods gave similar results when strains of *Salmonella* with known relative surface hydrophobicities were tested. Bacterial cell surface charge was not related to in planta immobilization.

Adherence of pathogenic bacteria to the surface of animal host tissues is a prerequisite for pathogenesis (21); bacterial cell surface hydrophobicity and charge are important factors in determining if bacterial adherence and colonization will occur. A hydrophobic bacterial cell surface may allow bacteria to overcome the repulsive forces between the negatively charged bacterial and eukaryotic cell surfaces, thereby permitting specific ligand-receptor interactions (21).

Adherence of bacteria and fungi to plant cell surfaces also occurs (14,15,23). Adherence of plant pathogenic or symbiotic bacteria appears to be a prerequisite for successful invasion by *Agrobacterium* and *Rhizobium* species and also for induction of plant defense mechanisms by other bacteria including *Pseudomonas solanacearum* (28). Initially, most attention was directed toward the involvement of specific ligand-receptor interactions. More recently, other less specific interactions such as ionic or hydrophobic interactions have been proposed as being important first steps leading to irreversible attachment due to lectin-ligand binding (25,28). The recent finding of hydrophobic binding sites in addition to carbohydrate binding sites for several legume lectins (26) may have significance in this interaction.

There has been a paucity of research on the importance of bacterial cell surface charge and hydrophobicity for pathogenicity toward plants compared to that which has been reported for animal systems. Previously, we reported on the interaction of numerous compatible, incompatible, and saprophytic bacteria with soybean leaves at the ultrastructural level (6,7,10). These studies indicated that bacterial cell immobilization by electron-dense material of unknown composition in soybean leaves is an active defense response of soybean towards nonpathogenic strains of the soybean pathogen *Xanthomonas campestris* pv. *glycines* and incompatible strains of the soybean nonpathogens *X. c.* pv. *campestris* and *Erwinia carotovora* subsp. *atroseptica*. Active immobilization may also play a role in the restricted growth of *Corynebacterium flaccumfaciens* pv. *flaccumfaciens* and the saprophyte, *Bacillus cereus* (7). Active bacterial immobilization is not a defense response of soybean towards incompatible races of the soybean pathogen *Pseudomonas syringae* pv. *glycinea* and incompatible strains of the soybean nonpathogens *P. s.* pv. *phaseolicola* and *P. s.* pv. *syringae* where early passive envelopment by electron-dense material does occur (6). Passive envelopment can be influenced by the bacterial cell surface hydrophobicity (1).

The purpose of this study was to determine if a relationship exists between cell surface hydrophobicity and charge of bacterial strains that are actively immobilized in soybean leaves. Also, we determined if physiologic races of *P. s.* pv. *glycinea* or pathogenic and nonpathogenic strains of *X. c.* pv. *glycines* differed in cell surface hydrophobicity or charge.

MATERIALS AND METHODS

Bacterial cultures. Bacterial strains used in this study are listed in Table 1. Strain 1137 of *P. s.* pv. *phaseolicola* was originally thought to be *P. s.* pv. *glycinea*, but this was recently shown to be incorrect (19). Strains of *Salmonella minnesota* are of different lipopolysaccharide (LPS) chemotype. Strains SF1111 and SF1114 have smooth (O-antigen present) LPS. Strain SF1111 is the parent strain of R60/SF1112 (hereafter referred to as SF1112) which has LPS of the Ra chemotype (shallow rough). Strain SF1114 is the parent strain of R5/SF1119 and R595/SF1167 (hereafter referred to as SF1119 and SF1167, respectively) which have LPS of the Rc (moderate rough) and Re (deep rough) chemotypes, respectively. All three rough strains lack O-antigen and, in the case of Re and Rc chemotypes, part of the core region. The structures of Ra to Re LPS of *Salmonella* are described in detail elsewhere (30).

P. s. pv. *glycinea* strains 2159 and K1 represent physiologic race 1; A-29-2 and K4, race 4; and J3-17-2, race 5 (9).

Strains XP175 and A of *X. c.* pv. *glycines* are pathogenic on susceptible soybean cultivars, while strains S-9-8, 1136, and 1716 are naturally occurring nonpathogenic mutants (5). Bacterial suspensions were prepared in 20 × 150-mm glass culture tubes and optical density readings were made with a Bausch & Lomb Spectronic 88 spectrophotometer. Quartz cuvettes (1 cm) were used for final turbidity readings for all assays.

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Hydrophobic interaction chromatography. *Corynebacterium, Erwinia*, and *Salmonella* were grown on nutrient-dextrose agar (NDA) (Difco nutrient agar plus 10 g of Difco glucose and 5 g of Difco yeast extract per liter), *Xanthomonas* on Difco nutrient agar (NA), and *Pseudomonas* on King's medium B agar (KB) (11). *Salmonella* was grown overnight at 37 C and the others at 28 C. Washed (once with saline, pH 7.0) cells were suspended in saline (pH 7.0) and the turbidity was adjusted to $OD_{600\ nm} = 2.0$. The assay was performed as described by Faris et al (4) with only slight modifications. Two hundred microliters of bacterial suspension was carefully layered onto the surface of 1-ml columns of phenyl-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden) in glass wool-plugged Pasteur pipettes. After the bacterial suspension had entered the column bed, columns were carefully washed with 2 ml of 0.02 M sodium phosphate buffer, pH 6.8, containing 1 M $(NH_4)_2SO_4$. The eluate was collected and the $OD_{600\ nm}$ was determined. Each bacterial strain was tested on three columns per experiment. For comparison, 200 μl of the bacterial suspension was added directly to 1.8 ml of buffer and the $OD_{600\ nm}$ was determined.

Adherence to nitrocellulose filters. The procedure of Lachica and Zink (13) was followed. Bacteria were grown overnight on agar media as described above. Cells were washed once with saline (pH 7.0), suspended in saline (pH 7.0), and the turbidity was adjusted to $OD_{600\ nm} = 1.0$. The bacterial suspensions were passed dropwise (approximately one drop per second) through 13-mm-diameter nitrocellulose filters with 8.0-μm pore size (type SC; Millipore, Bedford, MA). Approximately 1 ml of eluate was collected, transferred to a cuvette, and the $OD_{600\ nm}$ was determined. Controls consisted of bacterial suspensions not passed through the filters.

Xylene adherence assay. Adherence of bacteria to xylene was determined as described by Rosenberg et al (27). Bacteria were grown overnight on agar media as described above. Alternatively, bacteria were grown to mid-log or stationary phase in nutrient dextrose broth (composition the same as NDA except Difco nutrient broth was substituted for NA) in a shaking water bath maintained at 37 C (*Salmonella*) or 28 C (others). Bacterial cells were harvested by centrifugation, washed once with saline (pH 7.0), and suspended in PUM buffer, pH 7.1 (27). Turbidity was adjusted to $OD_{600\ nm} = 1.5$. Bacterial cell suspension (2.4 ml) was added to acid-washed flat-bottomed Klett tubes (12 ml) and various volumes of xylenes (J. T. Baker Chemical Co., Phillipsburg, NJ) were added. Following a 10-min preincubation at 30 C, the tubes were agitated for 2 min with a Vortex-Genie mixer (Fisher Scientific Co., Pittsburgh, PA) at the #2 setting. The layers were allowed to separate for 15 min at room temperature. The aqueous phase was carefully removed, transferred to a cuvette, and the $OD_{600\ nm}$ was determined. Cell suspension with no xylenes added were used as controls.

Adherence to hydroxylapatite. Bacterial cell surface charge was determined by the procedure of Lachica and Zink (13). Bacteria were grown to mid-log phase in NDB or overnight on agar media as described above. Bacterial cells were washed once with saline (pH 7.0), suspended in saline (pH 7.0), and the turbidity was adjusted to $OD_{600\ nm} = 1.0$. Hydroxylapatite (high-resolution; Calbiochem-Behring, LaJolla, CA) was added to acid-washed flat-bottomed Klett tubes (12 ml) at 100 mg per tube. Two milliliters of bacterial suspension was added to each tube and tubes were agitated with a Vortex-Genie mixer at the #2 setting for 2 min. The hydroxylapatite was allowed to settle out of suspension for 30 min at room temperature. One milliliter of the remaining cell suspension was transferred to a cuvette and the $OD_{600\ nm}$ was determined. Each bacterial strain was tested three times per experiment. One tube per bacterial strain had no hydroxylapatite added for use as a control.

Capsule visualization. The presence of capsules was determined by the wet-mount India ink procedure as described by Mort and Bauer (20).

RESULTS

Bacterial cell surface hydrophobicity. Hydrophobicity of the bacterial strains was first tested by using hydrophobic interaction chromatography which measures the adsorption of bacterial cells to phenyl-Sepharose beads which have a hydrophobic surface (4). For this assay, only washed cells from overnight agar cultures were used. As a preliminary check, strains of *S. minnesota* differing in lipopolysaccharide (LPS) chemotype were tested. As expected, strains of *S. minnesota* with deep rough (strain 1167) or moderately rough (strain 1119) LPS were significantly more hydrophobic than their smooth LPS parent strain 1114 (Table 2). The shallow rough strain 1112 did not differ in hydrophobicity as determined by this assay method from the smooth LPS parent strain 1111.

Based on hydrophobic interaction chromatography of phytopathogenic bacteria, strains which are partially or totally immobilized in soybean leaf intercellular spaces by 24–48 hr after inoculation (6,7,10) had relatively low cell surface hydrophobicity. Nonpathogenic strains of *X. c.* pv. *glycines* which are immobilized in leaves of soybean cultivar Clark had significantly lower cell surface hydrophobicity than two pathogenic strains of *X. c.* pv. *glycines* which are not immobilized in leaves of susceptible cultivar Clark or in leaves of the resistant cultivar Clark 63 (5). Cells of races 1 and 4 of *P. syringae* pv. *glycinea* did not differ significantly in surface hydrophobicity while the one race 5 strain tested (J3-17-2) was more hydrophobic than races 1 and 4.

Adherence to nitrocellulose filter assays confirmed the results of the hydrophobic interaction chromatography, demonstrating higher cell surface hydrophobicity for the strains of *S. minnesota* with rough LPS chemotypes (Table 2). However, there was no positive correlation between low bacterial cell surface hydrophobicity and in planta immobilization (Table 2). In contrast to the results with the hydrophobic interaction chromatography,

TABLE 1. Source of bacterial strains and their tendency to be actively immobilized in soybean leaves

Bacterium	Strain	Source	Actively immobilized during incompatible interaction with soybean[a]
Corynebacterium flaccumfaciens pv. *flaccumfaciens*		M. Schuster	±
Erwinia carotovora subsp. *atroseptica*	SR8	A. Kelman	+
Pseudomonas syringae pv. *glycinea*	2159	NCPPB[b]	−
	K1	B. Kennedy	−
	A-29-2	W. Fett	−
	K4	B. Kennedy	N.D.
	J3-17-2	W. Fett	−
pv. *phaseolicola*	1137	NCPPB	−
Xanthomonas campestris pv. *campestris*	Xc42	UWCC[c]	±
pv. *glycines*	XP175	M. Starr	−
	A	ERRC[d]	N.D.
	S-9-8	W. Fett	+
	1136	NCPPB	+
	1716	NCPPB	±
Salmonella minnesota	SF1114	S. Schlecht	N.D.
	R5/SF1119	S. Schlecht	N.D.
	R595/SF1167	S. Schlecht	N.D.
	SF1111	S. Schlecht	N.D.
	R60/SF1112	S. Schlecht	N.D.

[a] Based on previous ultrastructural studies with soybean leaves (6,7,10). + = total or almost total bacterial population immobilized; ± = part of population immobilized but many free cells observed; − = no cells immobilized; and N.D. = not determined.

[b] NCPPB = National Collection of Plant Pathogenic Bacteria, Hatching Green, England.

[c] UWCC = University of Wisconsin Culture Collection, Department of Plant Pathology, University of Wisconsin, Madison.

[d] ERRC = Eastern Regional Research Center.

nonpathogenic strains of *X. c.* pv. *glycines* were as hydrophobic or more hydrophobic than pathogenic strains of *X. c.* pv. *glycines* based on adherence to nitrocellulose filters. The nitrocellulose filter assay did show a higher cell surface hydrophobicity for strain J3-17-2 of *P. s.* pv. *glycinea* race 5 than for the race 1 and 4 strains, agreeing with the hydrophobic interaction chromatography assay, although the results for strain J3-17-2 were not significantly different statistically from the race 4 strain K4.

Based on adherence to xylenes, higher cell surface hydrophobicity was associated with strains of *S. minnesota* with rough LPS chemotypes (Table 2). There was no relationship between relatively low cell surface hydrophobicity of the phytopathogenic bacteria and in planta immobilization. By this assay, the pathogenic strains of *X. c.* pv. *glycines* grown to mid-log phase in liquid culture had higher cell surface hydrophobicity than the three nonpathogenic strains of *X. c.* pv. *glycines*, but differences were not significant. Results with cells from stationary phase cultures for strains of *X. c.* pv. *glycines* 1716 and S-9-8 were similar to the results obtained by using cells from mid-log phase cultures. Cells of strain XP175 of *X. c.* pv. *glycines* from stationary phase cultures were slightly less hydrophobic than cells from log-phase cultures (*unpublished*). There was no significant difference in hydrophobicity between pathogenic and nonpathogenic strains of *X. c.* pv. *glycines* grown on agar media or between physiologic races of *P. s.* pv. *glycinea* grown in liquid media or on agar media. Values for cells grown on agar or in liquid media differed greatly for some bacterial strains.

Bacterial cell surface charge. Only one assay was used to determine bacterial cell surface charge. This assay is based on an electrostatic interaction between a negatively charged bacterial cell surface and the positively charged calcium site on the hydroxylapatite surface (13).

The strains of *S. minnesota* with rough LPS chemotypes (strains 1112, 1167, and 1119) all had significantly higher cell surface charge than the smooth LPS type parent strains (1114 and 1111) (Table 2). There was no relationship between bacterial cell surface charge with in planta immobilization or with pathogenicity of strains of *X. c.* pv. *glycines* using cells grown on agar or in liquid media. When cells grown in liquid media were used, strain J3-17-2 of *P. s.* pv. *glycinea* race 5 was significantly less negatively charged than the race 1 and race 4 strains. However, this was not the case for cells grown on agar media. As with the xylene adherence assay, values for the same strain grown on agar or in liquid media sometimes differed greatly.

Presence of capsules. Bacterial strains were examined for capsule formation after growth for up to 3 days on agar media or growth to mid-log and stationary phase in NDB. Small capsules (extending up to 2 μm from the cell wall) were evident only for strains K1, K4, A-29-2, and J3-17-2 of *P. s.* pv. *glycines* grown for 24 hr on KB agar. Less than 10% of the cells had capsules and capsules appeared to be removed by washing the cells once with saline (pH 7.0).

DISCUSSION

The three assays for bacterial cell surface hydrophobicity and the

TABLE 2. Cell surface hydrophobicity and charge of selected phytopathogenic and nonpathogenic bacteria

			Cell surface hydrophobicity				Cell surface charge	
					XA[d] (Percent of initial turbidity)		HA[e] (Percent of initital turbidity)	
Bacterium	Strain	Pathogenicity of race[a]	HIC[b] (Absorption to gel [%])	NFA[c] (Percent of initial turbidity)	Log-phase cultures	Agar cultures	Log-phase cultures	Agar cultures
Phytopathogens:								
Xanthomonas								
campestris pv. *glycines*	A	P	66 a[f]	19 a	57 ab	38 a	97 j	77 c
	XP175	P	61 a	47 b	44 a	47 ab	73 g	76 c
	1716	NP	32 cd	10 a	71 bc	38 a	77 g	64 bc
	1136	NP	27 cd	20 a	64 bc	58 b	48 d	76 c
	S-9-8	NP	26 cd	13 a	60 ab	53 b	99 i	52 b
pv. *campestris*	Xc42	···	17 de	N.D.[g]	59 ab	N.D.	44 cd	N.D.
Pseudomonas								
syringae								
pv. *glycinea*	J3-17-2	5	59 a	42 b	59 ab	74 c	66 f	76 c
	2159	1	41 bc	75 d	67 bc	85 c	60 e	53 b
	A-29-2	4	37 bc	63 cd	64 bc	80 c	39 b	74 c
	K1	1	33 c	71 d	66 bc	80 c	42 bc	72 c
	K4	4	32 cd	53 bc	59 ab	81 c	41 bc	16 a
pv. *phaseolicola*	1137	···	51 ab	N.D.	68 bc	N.D.	13 a	N.D.
Erwinia								
carotovora								
subsp. *atroseptica*	SR8	···	5 e	N.D.	65 bc	N.D.	81 h	N.D.
Corynebacterium								
flaccumfaciens								
pv. *flaccumfaciens*	···	···	4 e	N.D.	99 d	N.D.	9 a	N.D.
Nonphytopathogens:								
Salmonella								
minnesota	1119	···	26 A	21 B	34 A	N.D.	N.D.	22 A
	1167	···	26 A	13 AB	53 B	N.D.	N.D.	22 A
	1111	···	16 AB	27 BC	69 BC	N.D.	N.D.	70 B
	1112	···	16 AB	3 A	57 B	N.D.	N.D.	15 A
	1114	···	13 B	36 C	85 C	N.D.	N.D.	57 B

[a] P = Pathogenic on susceptible soybean cultivars, NP = nonpathogenic on soybean; numbers 1, 4, and 5 refer to physiologic races distinguishable on differential soybean cultivars.
[b] HIC = Hydrophobic interaction chromatography. Values are means of data from three separate experiments with three repetitions per experiment.
[c] NFA = Adherence to nitrocellulose filters. Values are means of data from three separate experiments with two repetitions per experiment.
[d] XA = Adherence to xylenes. Values are means of combined data from two separate experiments for the 200- and 400-μl xylenes levels.
[e] HA = Adherence to hydroxylapatite. Values are means of data from three separate experiments with two repetitions per experiment.
[f] Means assigned different letters are significantly different ($P = 0.05$) as determined by the Banferroni *t*-test (18). Data for the phytopathogens or nonphytopathogens were analyzed separately.
[g] N.D. = Not determined.

one assay for cell surface charge gave the expected results for the strains of *S. minnesota* with known LPS chemotype. Strains of *Salmonella* with a rough LPS chemotype (lacking the O-antigen and having varying degrees of the core oligosaccharide) are known to have hydrophobic, negatively charged cell surfaces, whereas strains with smooth LPS chemotype (having their O-antigen and core oligosaccharide) are hydrophylic and uncharged (16).

Initial results obtained by using hydrophobic interaction chromatography to determine the cell surface hydrophobicity of the phytopathogenic bacteria indicated that a relationship may exist between bacterial cell surface hydrophobicity and immobilization in planta. The bacteria (*C. f.* pv. *flaccumfaciens, E. c.* subsp. *atroseptica, X. c.* pv. *campestris*, and nonpathogenic strains S-9-8, 1136, and 1716 of *X. c.* pv. *glycines*) which are immobilized to varying degrees during incompatible interaction in soybean leaves were, in general, less hydrophobic than bacterial strains (strains 2159, K1, J3-17-2, and A-29-2 of *P. s.* pv. *glycinea*; strain 1137 of *P. s.* pv. *phaseolicola*; and strains A and XP175 of *X. c.* pv. *glycines*) which were not immobilized during incompatible interaction in soybean leaves (6,7,10), although not significantly so in all cases. Pathogenic strains of *X. c.* pv. *glycines* were significantly more hydrophobic than nonpathogenic strains of this soybean pathogen, and strain J3-17-2 of *P. s.* pv. *glycinea* race 5 was significantly more hydrophobic than *P. s.* pv. *glycinea* races 1 and 4. However, these relationships were not supported by the nitrocellulose filter and xylene adherence assays.

Differences in results between the two-phase (hydrocarbon-water) separation assay and hydrophobic interaction chromatography were previously reported by Kjelleberg and Hermansson (12) for other bacteria. Hydrophobic interaction chromatography and two-phase separations appear to measure different aspects of cell surface hydrophobicity and possibly different surface components (12). Hydrophobic interaction chromatography is more likely to demonstrate differences between more hydrophilic bacteria, while two-phase separation techniques are better suited to differentiate between relatively hydrophobic bacteria. Compared to the results of hydrophobic interaction chromatography and xylene adherence assays for other bacteria (4,27), those tested in this study are relatively hydrophilic. Thus, hydrophobic interaction chromatography, which showed a relationship between relative hydrophobicity and in planta immobilization of the bacteria used in the present study, may have been the most appropriate assay for cell surface hydrophobicity of the three different assays employed.

There was no relationship between bacterial cell surface charge with in planta immobilization or with pathogenicity of strains of *X. c.* pv. *glycines*. Also, there was no relationship between cell surface charge and physiologic race of *P. s.* pv. *glycinea*.

For some bacterial strains, values obtained with the xylene adherence and the hydroxylapatite adherence assays of cells grown overnight on agar media or to mid-log phase in liquid medium differed greatly. Small capsules were detected only for strains of *P. s.* pv. *glycinea* grown on an agar medium and these capsules appeared to be removed by washing once with saline (pH 7.0), a standard procedure followed for all assays. Thus, differential capsule formation does not appear to be responsible for the differences between cultures grown on agar or in liquid media. The possibility exists that agar and broth cultures may have differed in the amount of residual exopolysaccharide slime layers which may not have been totally removed by the washing procedure or the composition of any slime layer or of the outer membrane may also have differed. Another possibility for these differences could be differential fimbriae (or pili) production (4); however, aerobic growth on agar media and growth to mid-log phase in liquid shake cultures generally does not favor fimbriae production (22).

The only Gram-positive bacterium included in our study, *C. f.* pv. *flaccumfaciens*, had the least hydrophobic and most negatively charged surface of any of the bacteria studied. These properties could be conveyed by the presence of techoic or lipotechoic acids (32).

McEvoy and Chatterjee (17), who used some of the same assays used in our study, reported that pathogenic exopolysaccharide-producing strain K60 of *P. solanacearum* is not hydrophobic, while nonpathogenic exopolysaccharide-negative mutant strain B1 is hydrophobic. Strain B1 is of a rough LPS chemotype and is immobilized in tobacco leaves while strain K60 is of smooth LPS chemotype and not immobilized in tobacco leaves (28,31). The nonpathogenic strains of *X. c.* pv. *glycines* used in this study do not differ from pathogenic strains in ability to produce exopolysaccharide in vitro or in the sugar composition of the exopolysaccharide produced (8). In addition, the nonpathogenic strains of *X. c.* pv. *glycines* do not appear to have a rough form of LPS (W. F. Fett, S. F. Osman, and T. S. Seibles, *unpublished*). Tobacco cell suspension cultures have been successfully used to study adherence of strains K60 and B1 of *P. solanacearum* (3). Unfortunately, nonpathogenic strains of *X. c.* pv. *glycines* do not adhere to soybean cell suspension cultures (R. M. Zacharius and W. F. Fett, *unpublished*).

In vitro assays of the type used here may not accurately reflect the state of the bacterial surface in leaf intercellular spaces. Nutrient availability can profoundly affect the composition of the bacterial envelope including the glycocalyx and the outer membrane (2) and does affect cell surface hydrophobicity of some bacteria (12). Possibly, assays for hydrophobicity and charge of bacterial cells immediately after removal from the intercellular space environment may gave a clearer picture of a role for cell surface charge or hydrophobicity in bacterial cell recognition leading to the immobilization defense response in soybean. Another difficulty in assessing the role of bacterial cell surface charge and hydrophobicity in adherence to plant cell walls is that the surface of plant cell walls exposed to the air in intracellular leaf spaces is poorly defined. There may be a layer of cutin or suberin over the exposed plant cell walls (24,29), but this has recently been questioned (33). A layer over the cell wall would mask the possible negative charge imparted by the presence of pectic substances and impart a hydrophobic nature to the plant cell surface.

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