Indirect Determination of Cytoplasmic Conductivity and Membrane Capacitan of Plant Protoplasts by Electro-Rotation Method

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Abstract

520 Protoplasts of *Dendobium* and *Lilium longiflorum* perform only one rotation peak between 1 kHz and 15 MHz, which shifts towards a higher frequency when the solution conductivity (σ_s) is increased. The protoplasts rotate faster at higher field strengths. The second rotation peak for *Lilium longiflorum* protoplast appears at about 25 MHz, with a much faster rotation rate than at the first peak. Using a spherical single shell model, theoretical plots for the best fit of the second peak show that the faster increased rate reflects smaller cytoplasmic permittivity. The study reveals that in a low conducting solution, the cytoplasmic conductivity for protoplasts of *Dendobium* and *Lilium longiflorum* is 80-85 mS.m^{-1} and 320-800 mS.m^{-1}, respectively. The specific membrane capacitance is 6.4-22.0 mF.m^{-2} for *Dendobium* protoplast and 3.3-4.9 mF.m^{-2} for *Lilium longiflorum* protoplast.

Key Words: Electro-rotation, plant protoplast, membrane capacitance, cytoplasmic conductivity

Introduction

Electro-rotation is a non-invasive method for determining dielectric parameters such as permittivity (ε) and conductivity (σ) of membranes and the interior of cells. It utilizes simple Physics tools to describe complex properties of biological materials. Theory used to explain cell behavior in an electric field has been developed extensively from a single shell to double shell and multi-shell models so that it can represent to a reality. Studies have been made by measuring spin speed of a cell bathing in a low conductivity solution at various field frequencies. Dielectric parameters in many biological systems such as human cells, plant protoplasts, pollen grains, and yeast$^{(1-6)}$ have been reported, which is of interest to biophysical scientists and bio-technologists. Its sensitivity in detecting some abnormality of the investigated cells by means of electrical parameters brings about a new approach for today technological world. An example is shown as determination of the effect from surface charges on ion exchange polymer beads[7].

Cell rotation can be induced using two-parallel electrodes[1]. Two spherical cells are needed for a secondary electric induction, leading to a rotation of one cell at the contact point on the other which is attached to an electrode. When a four-electrode system is used[2-6], a selected cell is placed between two pairs of parallel electrodes with two applied fields with a $\pi/2$ phase shift. When a three-electrode system is used to investigate surface charge effect on latex particles[7], signals applied to the electrodes have to come from three generators. With all these methods, the net induced dipole moment tends to align itself along the direction of the applied field, leading to a torque and cell rotation. Through the frequency spectrum, most cells show two-characteristic rotation peaks. Very few show three peaks, particularly in a high conductivity solution[5]. Normally, when a system with more than one barrier-such as plant cells or protoplasts, is studied a single shell model has still been used for simplification. Using both the single shell and the multi-shell model, Sukhorukov *et. al.*[5] did not find any discrepancy when fitting theoretical curves of the single shell model to

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experimental data of generative cells of *Lilium longiflorum*, provided the solution conductivity (σ_s) is low. In their case with σ_s varying from 3 mS.m^{-1} to 460 mS.m^{-1}, the spin speed of the cells at low frequencies differed from that at higher ones.

In our previous study[8], estimations for dielectric constants of mesophyll protoplasts were made by a dielectrophoretic method, and a large variation of the constants was evident. It was deduced that part of the variation arises from an approximation of the real part, Re[f(ω)], of the complex dielectric constants of the cells, through the speed of cell translation towards an electrode which is not uniform. This allowed the fitting of experimental data by the iterative method varied within a certain range. The electro-rotation technique offers more restriction to the guessed values, which are governed by both the Re[f(ω)] and the imaginary part-Im[f(ω)] of the complex dielectric functions. Theory described the relation between the dielectric constants with Re[f(ω)] and Im[f(ω)] functions has been described in details by Mahaworasilpa et. al[1]. It is, therefore, anticipated that the values estimated by the electro-rotation method would be limited to a narrower range than those obtained by the pervious method. This study reveals values of membrane and cytoplasmic conductivity, and membrane specific capacitance of two mesophyll protoplasts and compares the values with those obtained from other laboratories.

Theoretical concepts

In this study, a parallel electrode system of nickel alloy is again used to attract two cells lining along the field direction as shown in Picture 1a. The cell spin speed (Ω), as described[1], is

$$\lambda = \frac{3\varepsilon_S}{8\eta} E_m^2 Sin2\theta \left(\frac{\mathrm{Im}^2\left[f(\omega)\right]}{4 - \mathrm{Re}\left[f(\omega)\right]} \right) \tag{1}$$

where ε_s, η, E_m, and θ represent solution permittivity, viscosity, amplitude of the applied field, and the angle between the rotating protoplast and the field E (Picture 1c), respectively. As in (1), the imaginary part-Im[f(ω)] and the real part-Re[f(ω)] are frequency dependent and their values depend on complex dielectric parameters of the cells interior, the membrane, and the external solution. Other parameters in the equation can be obtained from the experimental conditions. The ε_s is assumed to be $80\varepsilon_0$ throughout the study.

The amplitude E_m is related to electrode geometry and varies along the z-position from the middle of the electrodes. Its magnitude is taken at the center of the rotating protoplast. Equation (2) provides the E_m for parallel cylindrical electrodes. Details can be found elsewhere[1].

$$E_m = \frac{Vd}{\left[2\ln\left(\frac{d-a}{a}\right)\right]\left[\frac{d^2}{4} - z^2\right]} \tag{2}$$

To assure an optimal fit between the theory and experimental data, Archer *et al* suggest a regression function (R_c) as given in the Equation (3)

$$R_c = 1 - \left[\frac{\sum_i (R_{exp} - R_{theory})^2}{\sum_i (R_{Exp})^2} \right] \tag{3}$$

The $R_{Exp.}$ and $R_{Theor.}$ are experimental and theoretical values of arbitrary *i* data. The best fit provides R_c, which is usually closed to unity.

Materials and Methods

Protoplast preparation

Leaves (3-4 cm in length) of *Dendobium* sp. and *Lilium longiflorum* sp. were preplasmolysed in 0.7 M. mannitol solution for 15 min, and cut into strips about 1-2 mm in width before being transferred to an enzyme solution. The enzyme for preparing *Dendobium* protoplasts contained 2% Cellulase Onozuka R-10, 1% Driselase and 1% Marcerozyme R-10, whereas the Cellulase and Driselase for preparing *Lilium longiflorum* protoplasts were 50% diluted. After 4-h incubation on a shaker, protoplasts were sieved through a 141 μm mesh stainless steel screen to remove cell debris. Both enzymes were dissolved in 0.7 M mannitol at pH 5.7. The filtered protoplasts were centrifuged at 40g for 5 min. The filtrate was removed using a Pasteur pipette and washed twice in 0.7 M mannitol. The protoplasts were washed and re-suspended in mannitol solution of 0.4 M and 0.5 M for *Dendobium* and 0.6 M for *Lilium longiflorum*. Solution conductivity was measured using a conductivity meter (Tetracon 325, LF 318), and solution viscosity (η) was measured using a capillary viscometer (Schott Gerate, model 516 10).

To reduce possible effects of the sticky surface of *Lilium longiflorum* protoplasts, later experiments (Figure 5b) were carried out by adding 0.3 % BSA by weight to the 0.6 M mannitol. This raised the viscosity of the solution from 1.37±0.02 to 1.42±0.05 mNs.m^{-2}. Note that concentrations of 0.4 M, 0.5 M and 0.6 M mannitol solution has no effect on the spherical shape of the mesophyll protoplasts for at least 5 hours.

Induced rotation

Two selected protoplasts of equal radius (R) were placed in an experimental well. They were firstly induced to form a pearl chain by a dielectrophoretic force (F_{DEP}) under electric field strengths between 5 and 17 kV.m^{-1} at 1MHz. It was found that the electric field greater than 30 kV.m^{-1} lead to an elongation of *Dendobium* protoplasts. To avoid possible release of internal organelle, the applied field was kept at 17 kV.m^{-1}, or less. After the chain attached to an electrode, the second protoplast was re-arranged so that a desired angle θ was obtained. Picture 1c shows rotation direction of the protoplast, at a fixed contact point between the two. A clockwise rotation was also observed on the opposite side of the $-y$ axis. Similarly, the two protoplasts can be attached to the other electrode and θ can be measured in another quadrant. For θ greater than 60°, the F_{DEP} becomes dominant and the protoplast is likely to be attracted directly to the electrode. Experiments were recorded by using a CCD camera so that the experimental data could be displayed whenever further analysis was required.

Results

Preliminary study

In the beginning of this study it was observed that when protoplast B was rotating on the one attached to the electrode at frequencies in the kHz range, this one was also rotating along the electrode length. This indicates a near zero DEP force[9] acting on A. The rotation of A could, thus, be due to some force caused by the rotating protoplast. In some experiments, a protoplast rotated and translated towards an electrode at a frequency about 15 MHz. However when two protoplasts were attached to each other between the electrodes, rotation at this high frequency was not observed. This implies an increase in the DEP force due to a resultant of two polarized protoplasts. At an arranged θ angle, if the axis of rotation was not perpendicular to the observing plane, the evidence indicated a non-parallel electrode pair. When this was the case, the electrodes were re-adjusted. The "mysterious" circular pockets appearing in the protoplasts (Picture 1a or 1b) could affect the induced dipole to some extent since angle θ I (Picture 1c) was often altered to either direction when the field frequency was changed. Picture 1d shows that the protoplast A is going to burst under 6.7 kV.m^{-1} field strength and at the low frequency of about 7 kHz. This happens as soon as the protoplast B is repelled by a negative

F_{DEP}. It is interesting to point out that if the field frequency is changed too coarsely, the angle θ was affected strongly. Data shown in Table 1 demonstrates the described effect for two pairs of protoplasts. Note that when θ is close to 45°, the sin2θ in equation (1) corresponds to the maximum spin speed.

Effects of electric field strengths and the field frequencies

Two experiments were carried out in this study: one with a fixed frequency while increasing the field strength and the other with fixed field strength while varying the frequency. The former was simply done by increasing the applied voltage. Figure 1 shows spin speeds of a 45 μm protoplast on a 60 μm one. As seen, the speed increases with the field strength. The greater speed obtained at 35 kHz indicates that the frequency is close to the rotation peak (see Figure 2). The fluctuation of these data is due to a shift of θ from 30° to 60° for 35 kHz and from 30° to 40° for 55 kHz when changing the applied voltage. The relationship between the spin speed and E^2 is linear, as described by (1). As restricted by the theory[1], dielectric parameters estimated by this method must be deduced from protoplast of the same size. Fig. 2a and 2b show the spin speed of *Dendobium* protoplasts of 62 and 70μm size under 5.7 and 6.5 kv.m^{-1}, respectively. As before, θ for data shown in Fig. 2a varies from 6° to 10° when the frequency changes from 7.5 kHz to 26 kHz. In Fig. 2a, rotation peak is observed near 7.5 kHz and it reaches the peak at 15 kHz. When σ_s is decreased, rotation is observed at 4 kHz and it reaches the peak at frequency slightly greater than 6 kHz. Hence, the greater spin speed in Fig 2b might be explained as due to smaller σ_s and the higher field strength. In red blood cells[4], rotation peak shift towards a higher frequency was also reported as due to an increase in σ_s. Note that between 1 kHz to 15 MHz, only one rotation peak is observed for *Dendobium* protoplasts.

For *Lilium longiflorum*, a preliminary study revealed that the protoplast could stand a field strength of at least 85 kV.m^{-1}. When two protoplasts were induced for rotation, the spin speed was rather erratic, due to some friction-like spin manner. It was noted that the protoplast surface was rather sticky, and this could be the cause of the non-uniform spinning. In addition, some difficulties arose during the study due to less elasticity of the membrane, compared to *Dendobium*, leading often to membrane breakdown. The fact that the rotation normally occurs near zero dielectrophoretic force[9], the studied protoplasts often breakdown before a complete rotation spectrum could be successfully obtained. This is why only half of the spin speed data is plotted against rotation frequency in Fig. 3a. The peak of the rotation is likely close to 50 kHz, with θ equals to 6° with -y axis. The small spin speed on the right hand flank is near 100 kHz. In addition, a second rotation peak was not observed up to 15 MHz.

To reduce problem from the sticky surface, 0.6 M mannitol with an addition of 0.3 % BSA was used as a suspension medium. Moreover, in order to investigate further for the second rotation peak a new function generator with frequency up to 30 MHz was used. Experiments were carried out, arranging θ at 45° with respect to -y axis. Fig. 3 b shows two rotation peaks of the protoplast with a uniform spinning for two fields strengths. The first rotation peak occurs between 10 kHz and 40 kHz, whereas the second is at about 25 MHz. As shown, the measured spin speed of the second peak under 5.1 kV.m^{-1} field strength increases faster and is much greater in magnitude than at 4.2 kV.m^{-1}. The greater field also provides a greater spin speed, similar to the observation shown in Fig. 1, where it depends directly on the field strength. Moreover, the smaller σ_s also shifts the first rotation spectrum towards a lower frequency, similar to what was observed in *Dendobium* protoplasts and in animal cells[1].

Estimation of dielectric parameters

The Re[f(ω)] and the Im[f(ω)] in (1) are described by σ_m, σ_c, ε_s, ε_m, ε_c, and δ. These parameters are estimated so that spin speed can be calculated as a function of the frequency. The regression function (R_e) as in (3) is applied to find the best fit to the experimental data. For *Dendobium* protoplasts, σ_c varies from 80 to 85 mS.m^{-1} under σ_s between 5.6 and 30 mS.m^{-1}. The calculated lines fit well to the data values, as shown in Fig. 2a and 2b, when the single shell model is used. The estimated values for the parameters are much the same for both cases, despite of only exhibiting left hand flank. However, the greater ε_m in the higher conductivity solution is expected due to possible more ions surrounding the cell exterior. Taking the average between the two values, the specific membrane capacitance (ε_m/δ) of *Dendobium* protoplast comes at 14.2 mF.m^{-2}.

For *Lilium longiflorum*, Fig. 3 a and 3b show that σ_c increases from 320 to 800 mS.m^{-1} when 0.3% BSA is added while ε_c is reduced from $105\varepsilon_0$ to $60\varepsilon_0$. The BSA might help sealing some leaks since the stickiness of the surface disappeared and, as a consequence, the σ_c increases. It should be pointed out that the theoretical line for the second rotation peak of *Lilium longiflorum* protoplast does not fit well with the experimental data. Fig. 4 shows that smaller ε_c corresponds with a faster increase in the rotation rate. Interestingly, this change has no effect on the best fit for the first peak at low frequencies. This implies that the rather high ε_c obtained from Fig.3a can be reduced to $60\varepsilon_0$ without affecting other dielectric values, although the R_c value would be somewhat reduced. Tests for other possible dielectric values using $\varepsilon_c=60\varepsilon_0$ were also made, as shown in Table 2. The best regression (R_c=0.95093) for this protoplast yields a specific membrane capacitance of 3.27 mF.m^{-2}.

Discussion

Of the peaks found in the rotation speed as functions of the field frequency, the first occurs near the lower boundary of DEP spectrum[9]: namely from 10 to 20 kHz for *Dendobium* and around 100 kHz for *Lilium longiflorum* protoplasts. The second has a greater spin speed and it is located at a much higher frequency, namely at about 25 MHz. Sukhorukov *et.al.*[5] reported about three characteristic peaks in protoplast of *Beta vulgaris* mesophyll and pollen grains, found by using a four-electrode system. The number of peaks seems torelate to number of interfaces, which corresponds with the number of shell of the protoplasts. Note that the third peak they found appears next to the first one only if high σ_s between 190 and 460 mS.m^{-1} is used. Interestingly, if the fast trough found in the estimated Re[f(ω)] values[8] implies dispersion due to the interfaces, then the two rotation peaks found in this study indicate two major shells in the protoplasts with some chloroplasts intervening between the two. This is similar to the finding that the theoretical plot for the single shell model does not seem to fit well at the second rotation peak. In other words, the well curve-fitting appearing at the first rotation peak explains mainly the estimated parameters for the first cell boundary which is, in this study, referred to as protoplast membrane.

An increase in solution viscosity due to an addition of 0.3% BSA has no effect on the first rotation peak, and its best fit is not affected by ε_c either. This seems to imply that the first rotation peak is adequate if one wishes to estimate dielectric constants of single cells with the two-electrode system. However, if one wishes to estimate ε_c, then the experimental data at high frequency should be carried out as well. The ε_c of protoplasts in this study, from $60\varepsilon_0$ to $70\varepsilon_0$, is close to that of kidney fibroblasts[3] ($75\ \varepsilon_0$).

Concerning the estimated conductivity of the two protoplasts, σ_c, for *Lilium longiflorum* is much greater than that for *Dendobium*. Moreover, the larger σ_m of the former implies that its membrane is more permeable. Although the addition of BSA increases the σ_c value, its effect on C_m is rather small. Note that a large σ_c for *Lilium longiflorum* protoplasts was also reported by Sukhorukov *et. al.*[5], using a four-electrode system. In their case, a rise of the speed near 26.5 MHz was reported and it was suggested to be due to dispersion of the internal organelle, such as lipid bodies and mitochondria. In our study, the high frequency in MHz range might have reduced membrane impedance, allowing field penetration readily to the cells interior. Inhomogeneous distribution of chloroplasts in the cytoplasm and the appearance of the circular pockets in the vacuole might cause the shift of protoplast alignment when the field frequency is changed. In other words, the θ is affected due to surface positioning of the rotating protoplast at the contact point between the two. A complete rotation peak at the higher frequency could not be obtained due to the limitation of the frequency generator.

This study finds that membrane thickness is close to 10 nm for both mesophyll protoplasts. Table 3 shows the estimated parameters obtained by dielectrophoresis[8] and by electro-rotation. Importantly, the regression function proposed by Archer *et. al.*[3] helps to narrow down possible values for these parameters to a satisfied range.

Conclusion

This study shows that the protoplast spin speed depends on the external field. Rotation peaks are shifted to a greater frequency when the solution conductivity is increased. The effect of changes in solution viscosity, between 1.37 and 1.42 $mNs.m^{-2}$, on the speed is so small that it can be neglected. Only two dielectric dispersions of *Lilium longiflorum* protoplasts are evident in the frequency range 1 kHz to 30 MHz. The indirect determination from the first rotation peak found at low frequency is adequate for the estimation of the dielectric parameters, provided a regression function for the best fit is properly applied. So far, the single shell model predicts the first rotation peak of both protoplasts well. Cytoplasmic conductivity falls between 80 and 85 $mS.m^{-1}$ for *Dendobium* protoplasts and 320-800 $mS.m^{-1}$ for *Lilium longiflorum* protoplasts, respectively. Membrane specific capacitance of the former varies between 6.4-22 $mF.m^{-2}$ and of the latter between 3.3-4.9 $mF.m^{-2}$. To simplify the experimentation, this method should be further developed, possibly with a four-electrode system, before its applications can benefit technological usage.

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Table 1 The effect of changes in the field frequency on the spin speed of *Dendobium* Protoplasts in an 12 kV.m^{-1} field strength.

(a) 0.5 Mannitol				(b) 0.4 Mannitol			
A/B (µm)	**Hz**	**θ (degree)**	**Spin speed (rad.s^{-1})**	**A/B (µm)**	**Hz**	**θ (degree)**	**Spin speed (rad.s^{-1})**
70/70	3.0k	30	0.60	62/62	5k	60	0.67
	3.2k	44	0.72		10k	45	0.77
	3.3k	44	1.90		12k	45	0.92
	3.5k	48	0.80		14k	50	1.02
	3.8k	57	0.98		20k	40	0.84
	4.0k	60	1.07		23k	30	0.41

Table 2 Indirectly determined values of dielectric parameters for *Lilium longiflorum* protoplasts. The greatest R_c indicates the values for the best fit.

δ (nm)	ε_m	ε_c	σ_m (µS.m^{-1})	σ_c (mS.m^{-1})	R_c	C_m (mF.m^{-2})
7	$2.60\varepsilon_o$	$60\varepsilon_o$	1.75	800	0.95082	3.29
*9	$3.33\varepsilon_o$	$60\varepsilon_o$	**2.25**	800	**0.95093**	**3.27**
12	$4.46\varepsilon_o$	$60\varepsilon_o$	3.00	800	0.95081	3.29
15	$5.55\varepsilon_o$	$60\varepsilon_o$	3.70	800	0.95081	3.28

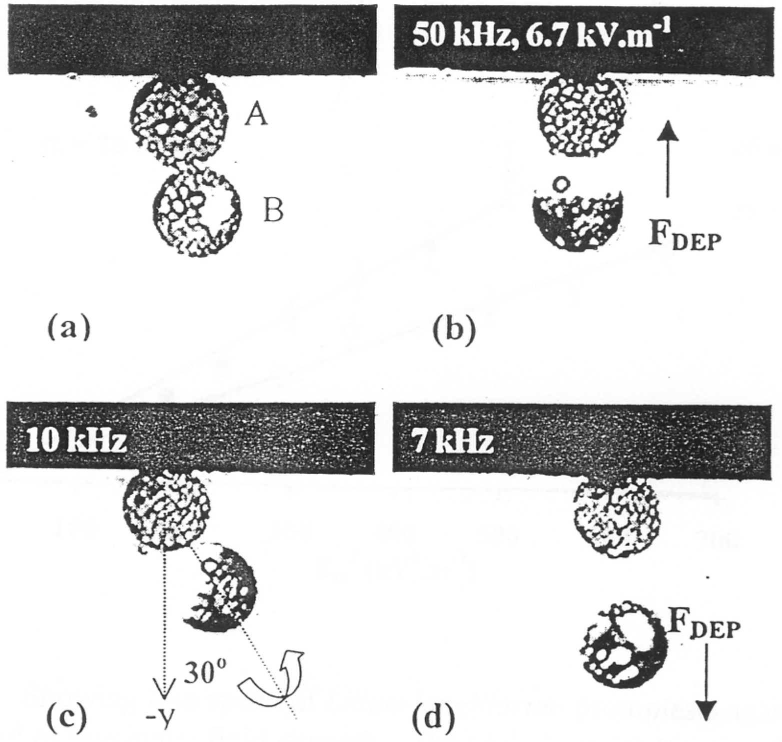
* the values used for theoretical plot in Figure 3b.

Table 3 Dielectric parameters of *Dendobium* and *L. longiflorum* protoplasts determined by means of dielectrophoresis and eletcro-rotation from several research laboratories. σ_s varies according to experimental condition of each laboratory.

Dielectric parameters	**Dielectrophoresis(8)**		**Electrorotation ***		**Other laboratories**
	Dendobium	***Lilium longiflorum***	***Dendobium***	***Lilium longiflorum***	
$\varepsilon_s/\varepsilon_o$	80	80	80	80	
$\varepsilon_m/\varepsilon_o$	4 -25	35	6.5-20	3.3-5.0	
$\varepsilon_c/\varepsilon_o$	83-90	105	70	60-105	75(3)
σ_s (mS.m^{-1})	1-7	20	5.6-30	3.7-20	
σ_m (µS.m^{-1})	2-30	0.1	0.2-0.3	1.2-2.2	
σ_c (mS.m^{-1})	1.1-7.3	20	80-85	320-800	200-300(1), 300-420(3), 500-700(5)
δ (nm)	15	10	8-9	9	10(1)
C_m (mF.m^{-2})	9.4-14.7	31	6.4-22.0	3.3-4.9	2.7-9.8(1), 10.5-15.3(2), 20.0(3), 7-10(5)

* the present study, and numbers in the superscript refer to references.

Protoplasts of *Lilium longiflorum*



Picture 1

Electro-rotation of protoplast B due to a secondary induction on protoplast A. of which the dipole is caused by primary induction.

(a) and (b) showing circular pockets in two protoplast pairs

(c) rotation of B anti-clockwise at 30° with –y axis

(d) protoplast B is repelled from A due to negative DEP force and the membrane of protoplast A starts to break down.

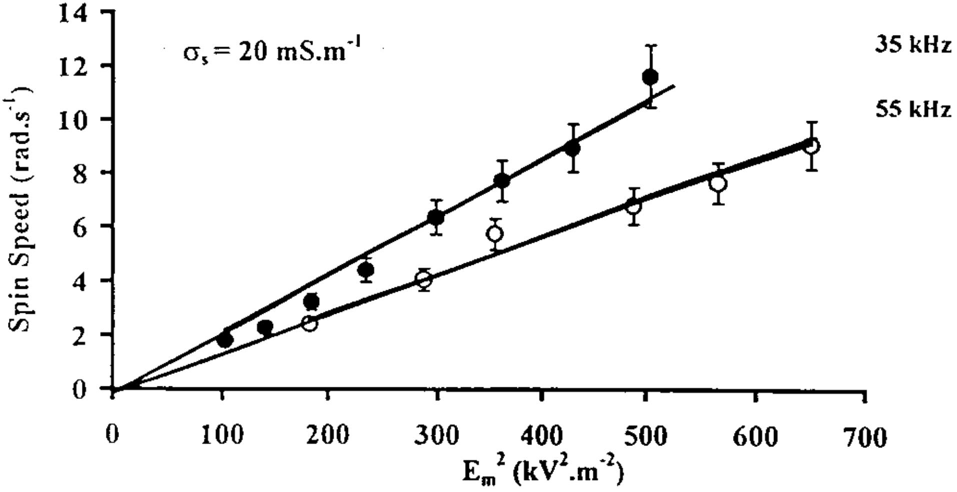


Fig. 1

Showing spin speed of *Lilium longiflorum* protoplasts against the squared of maximum field strength. The angle θ varies between 25° and 60°.

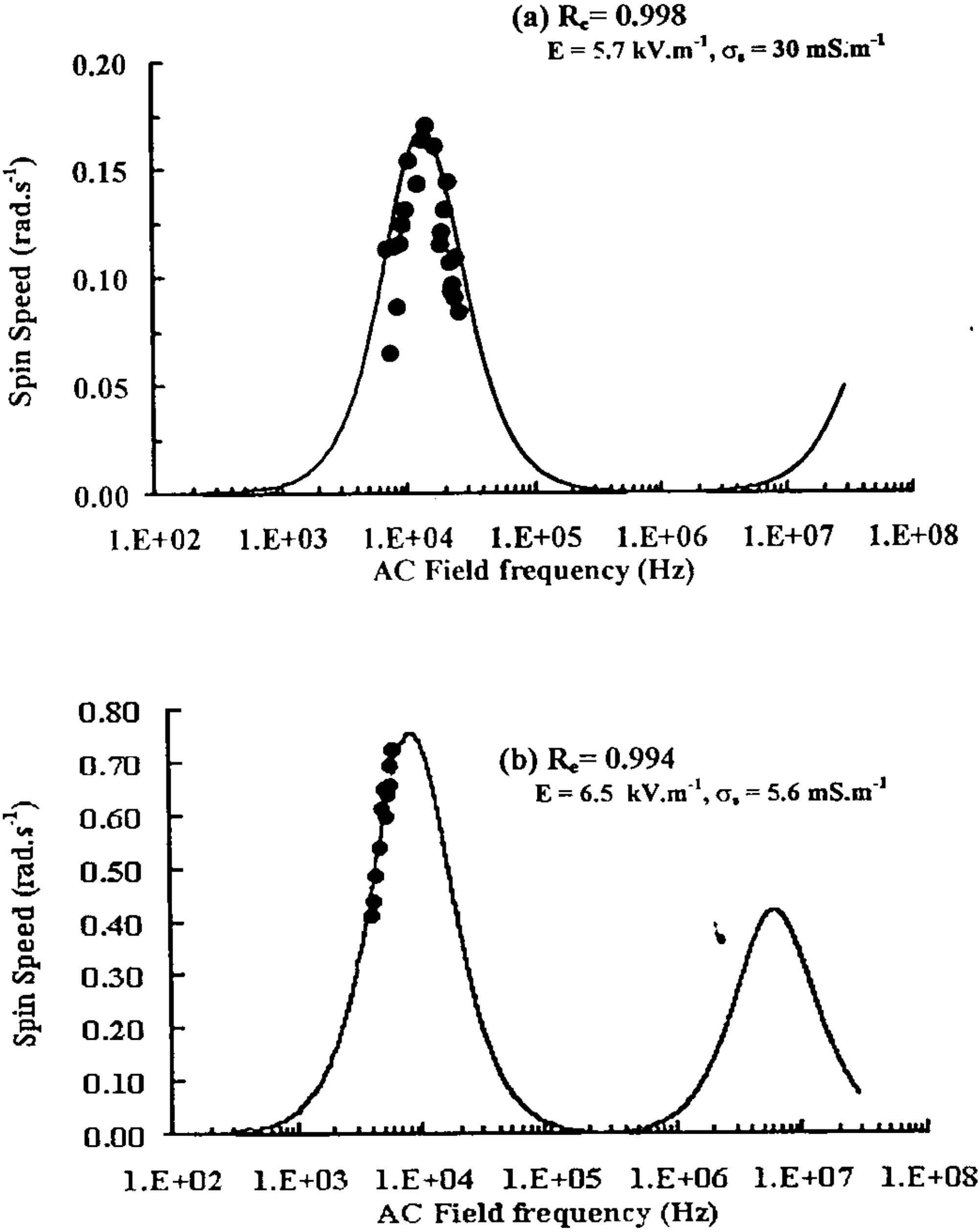


Fig. 2

Spin speed is plotted experimentally (dots) and theoretically (line) for *Dendobium* protoplasts. Estimate values for the best fits are;

(a) $\varepsilon_m = 20\varepsilon_o$, $\varepsilon_c = 70\varepsilon_o$, σ_m= 0.2 μS m^{-1} , σ_C = 85 mS.m^{-1} , and δ = 8 nm
(b) $\varepsilon_m = 6.5\varepsilon_o$, $\varepsilon_c = 70\varepsilon_o$, σ_m= 0.3 μS m^{-1} , σ_C = 80 mS.m^{-1} , and δ = 9 nm

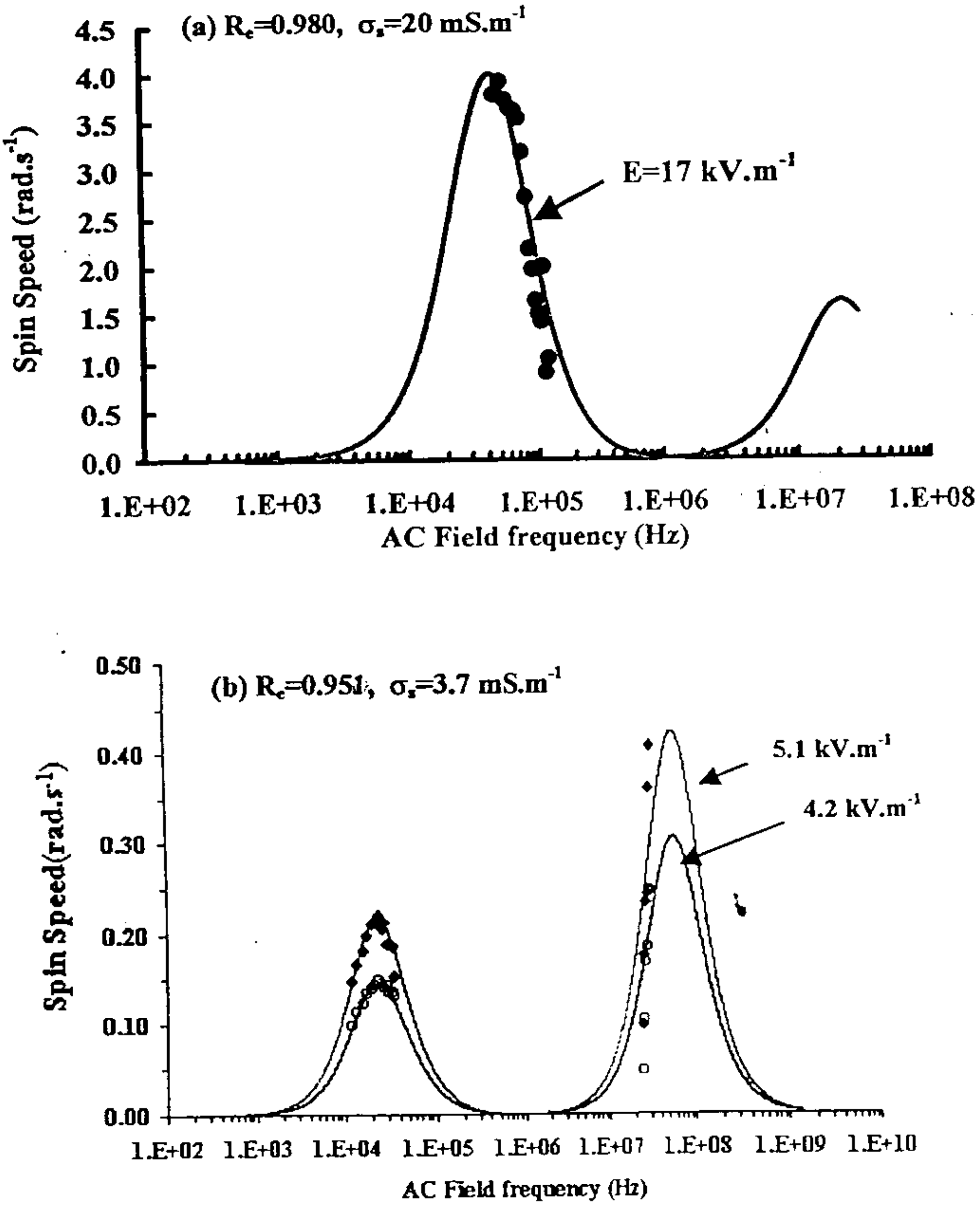


Fig. 3

The best fits by iterative method to estimate dielectric values of *Lilium longiflorum* protoplasts.

(a) $\varepsilon_m = 5\varepsilon_o$, $\varepsilon_C = 105\varepsilon_o$, $\sigma_m = 1.2$ µS/m , $\sigma_C = 320$ mS.m^{-1} , and . $\delta = 9$ nm ,

(b) $\varepsilon_m = 3.3\varepsilon_o$, $\varepsilon_C = 60\varepsilon_o$, $\sigma_m = 2.2$ µS/m , $\sigma_C = 800$ mS.m^{-1} , and . $\delta = 9$ nm

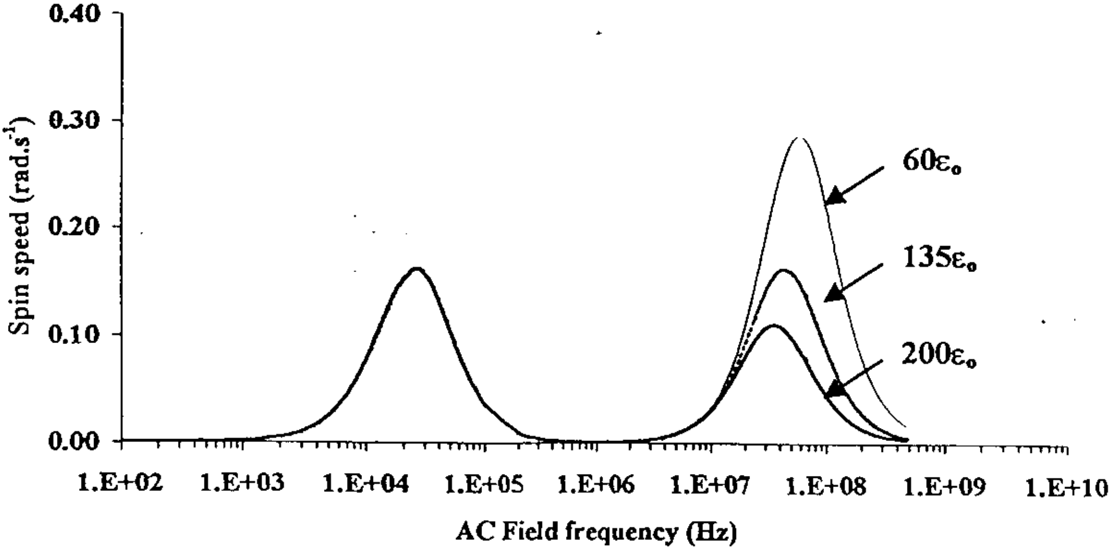


Fig. 4

Theoretical plots of protoplast spin speed with variation of ε_c, while other dielectric values are kept constant. The second rotation peak is either greater or smaller than the first one, depending on the ε_c value.