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Microviscosity of cucumber (*Cucumis sativus* L.) fruit protoplast membranes is altered by triacontanol and abscisic acid

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Abstract

Cucumber (*Cucumis sativus* L.) fruit protoplast membranes were probed with diphenylhexatriene (DPH) and pyrene, and also with two different plant growth regulators, triacontanol (TRIA) and abscisic acid (ABA). Fluorescence anisotropies of DPH and pyrene were measured after incorporating them into the membranes. The fluorescence lifetime of membrane-bound pyrene was also measured by using neodymium-doped yttrium aluminium garnet (Nd:YAG) laser of 35 ps pulses. The microviscosities of the membranes were calculated using the values of fluorescence anisotropy and lifetime. In the presence of TRIA and ABA, there was a sharp decrease in the fluorescence lifetime of pyrene. Similarly, there was also a decrease in the microviscosities of the membranes and increase in the rate of rotation of membrane-bound fluorophore, induced by the plant growth regulators. Furthermore, TRIA or TRIA + ABA could reduce the fluorescence anisotropy of both the fluorophores whereas, ABA decreased the anisotropy of only pyrene. This property of ABA may be due to its confinement to a specific spacial facet in the membrane. Fatty acid analysis indicated that membrane microviscosity fluctuations were not due to altered fatty acid composition alone as it is known that change in lipid–protein interaction would also alter the physical status of the membrane.

Keywords: Triacontanol; Abscisic acid; Membrane lipid; Membrane microviscosity; Membrane phase transition; Protoplast

1. Introduction

Triacontanol (TRIA), a long chain aliphatic alcohol ($C_{30}H_{61}OH$) has been known to be a potent plant growth promoting substance of many agronomic and horticultural crops [1]. This compound has also been shown to have an inhibitory effect on some of the cells responsible for inflammation in animals [2]. TRIA is known to increase dry weight, reducing sugars, soluble proteins, and free amino acids in plants [1], and it also stimulates the membrane-bound ATPases [3,4]. Further, we have also observed that TRIA would enhance the glycolipid levels in cotton (*Gossypium hirsutum* L.) leaves [5]. It has been suggested that TRIA may stimulate plant metabolism and consequently the growth [1]. Although a second messenger concept has been elucidated for TRIA action in plants, a clear picture of the molecular mechanism of action of this newly discovered plant

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growth regulator is yet to emerge [6]. Another plant growth regulator, abscisic acid (ABA), is a partially hydrophobic molecule, and this hormone is known to be involved in stress-related functions of plants [7]. Further, it has been suggested that the beneficial effects of ABA on chill tolerance of plants is manifested through a modification of the physical status of the membrane [8].

Cell membranes are considered to be the primary targets of hormonal action in plants [9]. Biomembranes, in general, contain a great variety of lipids and proteins which are distributed asymmetrically within the lipid bilayer [10]. It has been considered that hormone responsive system includes at least three essential elements: (i) a membrane receptor site, (ii) a transduction mechanism to recognize changes in the receptor site and, (iii) an amplifier which translates receptor changes into intercellular response [11]. In addition to this, the physical parameters regarding the membrane lipid assembly and the conformational change in the receptor site, perhaps, would ensure a valuable information in understanding the molecular mechanism of action of growth regulators. In fact, drugs and pesticides influence protein function via modulation of lipid environment in the neighborhood of protein molecules in the membranes [12]. Merril and Schroeder [13] have described that lipids modulate cell behavior through subtle alterations in the membrane fluidity. Although the physical status of membrane lipids in the process of adaptation of living organisms to various environmental factors has been described at greater length [14], information on this property of membrane lipids in hormonal signal recognition and transduction seems to be void till date.

Fluorescence spectroscopy provides useful information on the physical properties (e.g., mobility, distribution and interaction) of lipids in membranes [15,16]. The use of fluorescent probes to monitor molecular order and motion within the lipid bilayer of liposomes has been recently reviewed by Lentz [17]. Hitherto, such studies on the effects of plant growth regulators and xenobiotics on the physical properties of membrane lipids have been done mostly on artificial lipid bilayer system (liposomes) [18–23], but to a lesser extent on cell organellar membranes [24–28]. Such studies on natural membranes associated with living cell are very few [29–31]. However, a vast literature is available on the physical properties of artificial lipid bilayers as affected by various physical and chemical factors [32–39]. On the other hand, only a few such observations are available regarding the action of plant growth regulators on membrane fluidity [21–23,40]. Further, there appears to be only one instance where the action of a plant growth regulator (ABA) has been studied on the physical properties of membranes associated with live protoplast [30], and no such studies have been yet attempted on the effect of TRIA on any of the membrane systems. In the present investigation, we have discovered that protoplasts from cucumber (*Cucumis sativus* L.) fruits offer an excellent system for studies on membrane dynamics in 'live' condition (membranes associated with the protoplasm). In addition to the study on the effects of TRIA and ABA on membrane microviscosity, the interaction of the plant growth regulators on membrane dynamics described here has been the first report.

2. Materials and methods

2.1. Protoplast isolation

Protoplasts were isolated mainly according to the method described by Wilson et al. [29]. The flesh of cucumber (*Cucumis sativus* L.) fruit was cut into small pieces and digested for 5–6 h at 35°C in a medium containing 25 mM citrate buffer (pH 4.6), 0.43 M mannitol, 10 mM $CaCl_2$, 4.3 g/l Murashige–Skoog salts, 1% gelatin, 1% cellulase (Celluclast, Novo Nordisk, Denmark), and 1% pectinase (Pectinex, Novo Nordisk, Denmark). Protoplasts were purified according to Niedz et al. [41], with a little modification. The digested tissue was filtered through 80 μm nylon mesh, and the filtrate was centrifuged at $35 \times g$ for 10 min. The pellet was suspended in a washing medium containing 10 mM citrate buffer (pH 5.6), 0.43 M mannitol, and 10 mM $CaCl_2$. The protoplasts were then washed 2–3 times in the washing medium, and finally layered on to a floatation medium (17.1% sucrose in 10 mM citrate buffer at pH 5.6), and centrifuged at $100 \times g$ for 12 min. The purified protoplasts were collected from the inter phase and resuspended in the washing medium, and this preparation of protoplasts was used for all the studies.

2.2. Incorporation of fluorophores and plant growth regulators

Laser grade pyrene was obtained from Exciton Chemical Co. Inc., OH, USA, and diphenylhexatriene (DPH) was a gift from Dr. P. Balaram, II Sc, Bangalore, India. Triacontanol (TRIA) was a gift from J.K. Internationals, Hubli, India, and abscisic acid (ABA) was purchased from SRL, Bombay, India. The protoplasts were labelled with fluorophores by adding the probes dissolved in a small quantity of acetonitrile [29]. The fluorescent labels were kept at one label per 200 phospholipids approximately. To achieve this, 15 μl of 4.3 mM fluorophore in acetonitrile was added per ml of protoplasts (100 000 protoplasts/ml). The protoplasts were counted using a hemocytometer. The final concentrations of TRIA and ABA in the protoplast suspension were maintained at 0.0096 mM and 0.95 mM respectively. TRIA was incorporated in the form of micellar dispersion in water, and ABA was dissolved in acetonitrile along with required quantity of the probe molecule. The overall proportions of lipid:fluorophore:TRIA:ABA were approx. 2000:10:2:200 respectively.

2.3. Measurement of fluorescence anisotropy

The optical anisotropy measurements were done essentially according to the method described by Lakowicz [15]. The output from a 450 W high pressure Hg lamp (HBO 200, Carl Zeiss, Jena, Germany) was used as the light source. The light beam was made to pass through a 0.125 m grating monochromater (Oriel, USA) to separate out the emission of Hg near 361 nm. The light beam was then polarized using a polarizer (Melles Griot, USA) and made incident on the sample cuvette ($10 \times 10 \times 45$ mm^3, QS 10, laser grade, Hellma, Switzerland) housed in a variable temperature unit (DK 2A, Beckman Inc., USA) after suitable modifications. The fluorescence was collected and detected at 90° to the incident beam after passing through a glass filter (OY18 Pilinkington, Great Britain), and an analyzer (second polarizer) using a fiber optic coupling connected to a photomultiplier tube (PMT) (R 955, Hamamatsu, Japan) assembly. The PMT was biased at -850 V, and the signal from the PMT was monitored and measured on an electrometer amplifier (EA 815, ECIL, Hyderabad, India). The measurement temperatures were accurate to ± 0.25°C. The fluorescence intensities were measured parallel and perpendicular to the vertically polarized incident beam. The polarizers were fixed on a graduated (angles in degrees) mounts (Melles Griot, USA). The G factor (equivalent to the grating factor when an emission monochromater is used) was determined before studying each sample. This was necessary to ensure reliable data as any optical component could bring about its own polarization effects [15]. This factor was found to be close to unity. Anisotropy was calculated using the following relationship [15]:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

in which $G = I_{HV}/I_{HH}$

where I_{VV} represents fluorescence intensity parallel to vertically polarized excitation beam, while I_{VH} is that of perpendicular component. I_{HV} and I_{HH} are perpendicular and parallel intensities when the incident light is horizontally polarized.

2.4. Measurement of fluorescence lifetime

Pulse sampling technique [15] was employed for the measurement of excited state fluorescence lifetime of pyrene in the protoplast membrane. A pulsed (35 ps duration) Nd-YAG laser (YC 510C, Continuum, USA) available at our laser facility was used. The frequency tripled output (355 nm, 35 ps, 10 Hz) of the Nd-YAG laser was linearly polarized. The beam was further attenuated using a beam attenuater. An iris diaphragm was used as a spatial filter (1 mm diameter). The energy per pulse of the laser after iris diaphragm was about 100 μJ. A lens (f = 100 mm) focused the beam onto the sample contained in a quartz cuvette ($10 \times 10 \times 45$ mm^3, QS 10, laser grade, Hellma, Switzerland). The quartz cuvette was housed in the variable temperature unit. This set-up was in turn placed in a light-proof sample chamber to facilitate stray-light-free fluorescence detection at right-angles to incident beam. A color glass filter (OY 18, Pilinkington, UK) was used to cut off the scattered incident light. The fluorescence from the sample was collected and detected using a fiber optic arrangement consisting of fiber coupler, and a PMT (R 955, Hamamatsu, Japan). The PMT was biased at -850 V. The output from the PMT was suitably

amplified and monitored on a Digitizing Signal Analyzer (DSA 601 A, Tektronix, USA) fitted with a preamplifier (11A52, Tektronix, USA; input impedance 50 ohms, 600 MHz bandwidth). The DSA 601 A was programmed to give the decay time directly. The sampling rate was 250 Ms/s, and 2048 events were sampled and averaged. A set of three such observations were made for each sample at each temperature.

2.5. Determination of membrane microviscosity

After getting the mean values of anisotropy (r) and lifetime (τ) for pyrene at various temperatures, the membrane microviscosity as experienced by the fluorophore was calculated according to Perrin equation [15,17]:

$$r = r_0/[1 + (\tau/\phi)]$$

in which the rotational correlation time, $\phi = V_0\eta/RT$, where r is the mean anisotropy, r_0, the limiting anisotropy, τ, the average lifetime, V_0, the molar volume of the probe, η, the viscosity, R, the gas constant and T, the absolute temperature. The rate of rotation (R_r) of the fluorophore was calculated by using the formula [33,42]:

$$R_r = [(r_0/r) - 1]/6\tau$$

The microviscosities of the protoplast membranes were also calculated by the 'graphic' interpolation method, using white oil viscosity as the reference system [15,33]. The viscosities of the white oil at different temperatures were determined according to the following relationship for DPH fluorescence properties in white oil, as given by B.R. Lentz (personal communication).

$$r_0/r = 8.2305 \times 10^{-3}X + 1.283; \text{ as } X > 100$$

where $X = T\tau/\eta\tau_{22}$, $r_0 = 0.393$, r = anisotropy, τ = fluorescence lifetime, τ_{22} = fluorescence lifetime of DPH at 22°C (10.4 ns), T = °K, and η = viscosity in poise.

A calibration curve of fluorescence anisotropy versus viscosity was prepared by plotting r_0/r against $T\tau/\eta$ for pyrene in white oil (USP 35) at different temperatures. The fluorescence anisotropy (r) of pyrene incorporated into the protoplast membrane interior was then measured experimentally, and the microviscosities of the membrane interior was calculated from the calibration curve by comparing the corresponding $T\tau/\eta$ values for r_0/r in the membrane at each temperature.

2.6. Lipid analysis

Lipid analysis of the protoplasts was done according to the methods described by Christie [43]. Prior to lipid extraction, the protoplasts were incubated for 3 h at 25°C with plant growth regulators at the concentrations mentioned above. The methyl esters of fatty acids were analyzed on a Shimadzu 9A gas chromatograph, equipped with a flame ionization detector and Shimadzu C-R2A chromatopac data processor. The analysis was conducted on a packed column (15% DEGS on chromosorb, 80–100 mesh) with the oven temperature of 180°C, and the injector temperature of 220°C. Heptadecanoic acid was used as the internal standard.

2.7. Statistical treatment

The results were subjected to the analysis of variance in a two-way randomized complete block design, and further to the *t*-test of paired comparison of non-independent variables for significant difference [44].

3. Results

The effects of the plant growth regulators, TRIA and ABA, and the combination of these two compounds on the interior membrane fluidity of cucumber (*Cucumis sativus* L.) fruit protoplast membranes were probed with two structurally different fluorophores, diphenylhexatriene (DPH) and pyrene. Table 1 lists the fluorescence anisotropies of DPH under different treatments and at various temperatures. TRIA treatment resulted in a marked decrease in the anisotropies at all temperatures measured. On the contrary, treatment with ABA did not bring about any statistically significant change (by paired comparison test) in the anisotropies compared to those of control at different temperatures. However, treatment with TRIA and ABA together induced changes in the anisotropies (Table 1). The values of fluorescence

Table 1
Fluorescence anisotropy of DPH in cucumber (*Cucumis sativus* L.) fruit protoplast membranes

Temperature [C]	Treatment			
	Control	TRIA	ABA	TRIA + ABA
10	0.379 ± 0.013	0.313 ± 0.007	0.365 ± 0.012	0.362 ± 0.007
15	0.353 ± 0.012	0.300 ± 0.006	0.342 ± 0.011	0.303 ± 0.007
20	0.327 ± 0.008	0.247 ± 0.004	0.297 ± 0.011	0.244 ± 0.006
22	0.289 ± 0.006	0.225	0.265 ± 0.010	0.210 ± 0.006
25	0.245	0.189 ± 0.003	0.265 ± 0.010	0.210 ± 0.006
27	0.178 ± 0.003	0.154 ± 0.005	0.179 ± 0.010	0.174 ± 0.006
30	0.144 ± 0.003	0.124 ± 0.005	0.163 ± 0.009	0.182 ± 0.005
32	0.132 ± 0.002	0.135 ± 0.005	0.149 ± 0.008	0.102 ± 0.005
35	0.136 ± 0.002	0.130 ± 0.004	0.126 ± 0.008	0.117 ± 0.004
40	0.136 ± 0.002	0.130	0.137 ± 0.008	0.098 ± 0.003

Note: ± Represents standard deviation. Changes in anisotropy values for ABA-treated protoplasts are not significant compared to control in the paired comparison test. Others (TRIA and TRIA + ABA) are significant at $t = 1\%$ compared to control values.

anisotropy of pyrene in protoplast membranes are given in Table 2. In this case, both TRIA and ABA were able to significantly decrease the anisotropies at all the temperatures tested. Further, there was a consistent decrease in anisotropies with increase in temperatures in all the cases (Tables 1 and 2).

The effects of TRIA, ABA, and their combination on the excited state fluorescence lifetime of pyrene in protoplast membrane at four different temperatures

Table 2
Fluorescence anisotropy of pyrene in cucumber (*Cucumis sativus* L.) fruit protoplast membranes

Temperature [C]	Treatment			
	Control	TRIA	ABA	TRIA + ABA
10	0.365 ± 0.012	0.311 ± 0.010	0.333 ± 0.012	0.316 ± 0.008
15	0.340	0.302 ± 0.010	0.300 ± 0.013	0.284 ± 0.008
20	0.325	0.225	0.285 ± 0.010	0.205 ± 0.008
22	0.300	0.202 ± 0.010	0.259 ± 0.011	0.194 ± 0.007
25	0.276 ± 0.013	0.202 ± 0.010	0.222 ± 0.010	0.175 ± 0.007
27	0.242 ± 0.012	0.133 ± 0.008	0.205 ± 0.006	0.165
30	0.182 ± 0.012	0.129 ± 0.006	0.149 ± 0.009	0.145 ± 0.007
32	0.153 ± 0.012	0.099 ± 0.005	0.137 ± 0.008	0.140 ± 0.007
35	0.130 ± 0.011	0.099 ± 0.005	0.120 ± 0.007	0.100 ± 0.006
40	0.130 ± 0.011	0.109 ± 0.005	0.125 ± 0.007	0.100 ± 0.006

Note: ± Represents standard deviation. Changes in anisotropy values are significant compared to control in all the treatments at $t = 1\%$ level in the paired comparison test.

Table 3
Fluorescence lifetime of pyrene in cucumber (*Cucumis sativus* L.) fruit protoplast membranes (in nanoseconds)

Treatment	Lifetime 'τ' at temperature [C]			
	18	22	25	32
Control	359.0 ± 0.4	307.9 ± 2.0	290.5 ± 0.8	226.7 ± 1.5
TRIA	257.4 ± 0.7	214.0 ± 0.2	179.9 ± 0.5	118.1 ± 0.7
ABA	317.0 ± 1.0	271.9 ± 0.4	237.8 ± 1.6	163.1 ± 0.8
TRIA + ABA	243.3 ± 0.4	204.7 ± 0.5	182.1 ± 0.9	117.6 ± 0.3

Note: ± Represents standard deviation. The changes in fluorescence lifetime (τ) values are significant at $t = 1\%$ level compared to control values (paired comparison).

are given in Table 3. Treatment with TRIA and ABA individually, or in combination significantly decreased the fluorescence lifetime of pyrene by about 28–48% for TRIA, 12–28% for ABA, and 32–48% for TRIA + ABA with respect to control between 18 and 32°C. The temperature dependence of fluorescence lifetime of pyrene was found to be inversely related to temperature (Table 3).

The values of microviscosities of protoplast membranes are tabulated in Table 4. The comparison of derived microviscosities by two different methods (described in Section 2) revealed that though there was a small difference in values obtained by these two different methods, both the methods reflect con-

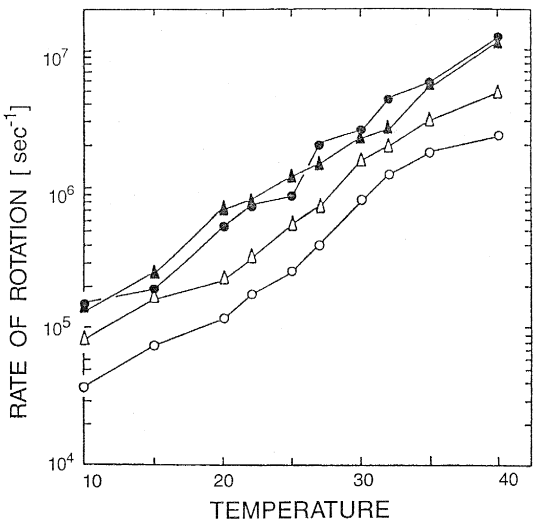

Fig. 1. Rotational rate of pyrene incorporated into cucumber (Cucumis sativus L.) fruit protoplast membranes. Control, empty circle; TRIA, filled circle, ABA, empty triangle; TRIA + ABA, filled triangle.

Table 4
Comparison of microviscosities of cucumber (*Cucumis sativus* L.) fruit protoplast membranes calculated by two different methods: (I) in situ analytical method, and (II) the graphic interpolation method with the white oil visosity. The figures given in the table indicate the viscosities in poise (P)

Temperature [C]	Treatment							
	Control		TRIA		ABA		TRIA + ABA	
	I	II	I	II	I	II	I	II
10	6.58	6.58	1.72	2.29	2.97	3.23	1.75	2.46
15	3.27	3.30	1.32	1.81	1.58	1.77	0.99	1.45
20	1.54	2.23	0.45	0.67	1.12	1.29	0.36	0.55
22	1.20	1.47	0.34	0.51	0.77	0.91	0.30	0.47
25	1.00	1.00	0.29	0.46	0.46	0.56	0.22	0.35
27	0.65	0.64	0.13	0.21	0.36	0.44	0.16	0.30
30	0.32	0.31	0.10	0.18	0.17	0.22	0.12	0.21
32	0.22	0.21	0.06	0.11	0.14	0.18	0.10	0.19
35	0.15	0.14	0.05	0.10	0.09	0.13	0.05	0.10
40	0.12	0.10	0.02	0.08	0.06	0.10	0.03	0.07

sistent and proportional changes in microviscosities upon growth regulator treatment as well as temperature increment (Table 4). A high degree of decrease in the microviscosity of the membrane interior was evident after treatment with TRIA, ABA, and TRIA + ABA (Table 4).

The decrease in the microviscosity of the membrane with increase in temperature and/or growth regulator treatment is also consistent with the enhanced rate of rotation of the fluorophore (Fig. 1). The rate of rotation of the fluorophore in control and ABA-treated protoplasts was of the order of 10^4–10^6 s^{-1}, whereas in TRIA-treated ones it was 10^5–10^7 s^{-1} (Fig. 1).

The fatty acid composition of the protoplast lipids is given in Table 5. TRIA treatment resulted in an increase of 18:3 fatty acid level, whereas ABA decreased 18:3 fatty acid, and increased 18:0 and 18:1 fatty acid levels. On the other hand, the treatment of protoplasts with a combination of TRIA and ABA resulted in an increase in 16:0 and 18:1 fatty acids and decrease in 18:2 and 18:3 4 fatty acids.

4. Discussion

Normally, phospholipid or glycolipid liposomes are used as the model membrane systems for studies on lipid dynamics [17,32,35]. In addition, natural membranes have also been used in some cases for such studies [24–28]. However, the major problems in using natural membrane in 'live' condition (membranes associated with the protoplasm) have been the light scattering and interference of biological molecules in fluorescence yield. In this regard, the flesh of cucumber (*Cucumis sativus* L.) fruit offers an excellent model for the study of membrane lipid biodynamics. This tissue is highly watery, and the protoplast obtained has a very large vacuole with cytoplasm being confined as a very thin and transparent layer between the plasma membrane and the vacuole (picture not shown). This property of the protoplast would reduce to a great extent the problem of light scattering. Further, the cells do not contain chlorophyll or any other pigments in detectable quantity, and they are also devoid of storage lipids and starch grains. These properties are of great value as these compounds are known to interfere in fluorescence yield [15]. In addition, the cells in this tissue are structurally and ontogenetically similar and are of same age. This would ensure homogeneity in lipid composition of the membranes. In fact, the cucumber fruit protoplast can be readily compared to a multilamellar liposome with all the advantages of using a 'live' and functional natural membrane for the experimental study.

Normally, for the measurement of apparent microviscosity of the biological membranes, at first a calibration curve including the measured values of fluorescence anisotropy and lifetime of the probe in an isotropic solvent of known viscosity is made. Fluorescence anisotropies are then measured for the same probe partitioned into the lipid domains of the sample membrane system. By comparison with the calibration curve of the reference isotropic solvent (white oil), the microviscosity of the membrane interior is determined [15,33]. However, the validity of the assumption made in this method that the depolarizing motions of the fluorophore are identical in these diverse environments is seriously questioned [15]. Another method that we have adopted in this study is the direct evaluation of the microviscosity by measuring both fluorescence anisotropy and lifetime of the fluorophore in the sample membrane system itself. In this in situ procedure of measurement of fluorescence lifetime, the same probe would experience the environments of different lipid domains, and therefore, it

may exhibit a range of excited state fluorescence lifetimes [27]. However, it is also stated that the value of fluorescence lifetime yields information on the average polarity of the entire fluorophore environment (bulk lipid, and lipid/protein interfacial environment) [27]. In view of this, we have employed both the methods to assess the effect of TRIA, ABA, and TRIA + ABA on the microviscosities of the protoplast membranes (Table 4). Although there has been some minor difference in the values obtained from the two different methods, the overall effects of these growth regulators in decreasing the microviscosity of the protoplast membrane have remained unchanged (Table 4).

In the present fluorescence anisotropy study, two different fluorophores (DPH and pyrene) have been used, and these fluorophores simulate a rotating rod and a rotating disc respectively [17,39]. Since the probe structure and geometry would affect the derived properties of the system [17,34,39] the fluorophores of different size and shape have been found essential to monitor the changes in the physical status of the membrane. In addition, this would also offer an advantage of probing the bilayer at different depths [45].

The excited state fluorescence lifetime of various fluorophores at different temperatures decreases linearly between 10–50°C [39,42], and this has also been found to be true for pyrene at different temperatures tested within that range (Table 3). Therefore, the lifetime values at other temperatures have been derived from a plot of change in measured fluorescence lifetime versus temperature for calculating microviscosity of the membrane (Table 4) and the rotational rate of the fluorophore (Fig. 1) at different temperatures.

Decrease in the excited state fluorescence lifetime of a fluorescent probe (e.g., DPH) in an isotropic environment has been ascribed to the unhindered rotation of the fluorophore, and this is due to the retrenchment of the angular restriction by the surrounding microenvironment [17,46]. The increase in rotational rate of fluorophore in the presence of TRIA and ABA (Fig. 1) would indicate the action of these plant growth regulators in enhancing molecular mobility in the membrane. A long average lifetime and high static anisotropy have been recorded at low temperature gel phase lipid environment, and a shorter average lifetime and lower static anisotropy were noticed in the fluid liquid crystalline phase for many fluorophores [17,36,37,47]. In this connection, it is of considerable importance to note that TRIA has induced a significant decrease in static anisotropy of both DPH and pyrene (Tables 1 and 2). This also coincided with TRIA-induced decrease in average lifetime of pyrene (Table 3). Further, the observation implicates that TRIA may act as a modulating agent and bring about a change in the lipid phase around the fluorophore facilitating a less hindered rotation of pyrene along its axis. Membrane viscosity changes have been known to affect the behavior of membrane-bound enzymes and proteins [48]. Therefore, the change in membrane-bound ATPase activity induced by TRIA [3,4] and by pesticides [49,50], and the lipid modulation of membrane receptor function [19] could be due to altered microviscosity in the membrane.

Another important observation is that TRIA is able to bring about significant changes in the static anisotropies of both the probes, whereas ABA can only influence the fluorescence anisotropy of pyrene (Tables 1 and 2). This may be explained on the basis of the ability of the probe molecules to intercalate into different bilayer depths and domains [15,45]. Apart from this, it is known that ABA requires at least two component bilayers to be apparently effective [20,40] and ABA may act at the regions of membrane defects between two different phospholipids or between two different membrane phases [21]. Furthermore, Purohit et al. [30] have reported that ABA greatly affects the rotational correlation time of 5-doxylstearic acid incorporated into guard cell membranes. In this context, it may be possible that ABA being partially hydrophobic molecule may be restricted to a spatial facet in the protoplast membrane which probably could not affect the anisotropy behavior of DPH. Thus, it is probable that the ability of TRIA and ABA to couple to the dynamic membrane heterogeneity may provide some new clues to the understanding of the molecular mechanism of hormone action in plants.

Since the plant growth regulators are expected to change the lipid composition when applied to living cells [5,51] and sufficient time was required to conduct the experiments on fluorescence studies after hormone treatment, the analysis of fatty acid compo-

Table 5
Fatty acid composition of the total lipids obtained from cucumber (*Cucumis sativus* L.) fruit protoplasts after 3 h of treatment with plant growth regulators. Mean values and S.D. (±) of two determinations are given

Treatment	Fatty acids mol%				
	16:0	18:0	18:1	18:2	18:3
Control	36.28 ± 0.74	3.29 ± 0.09	1.45 ± 0.19	32.42 ± 0.83	26.58 ± 0.19
TRIA	34.68 ± 0.93	2.40 ± 0.62	1.20	31.31 ± 1.46	31.03 ± 1.18
ABA	37.03 ± 0.04	6.27 ± 0.01	7.25 ± 0.01	35.72 ± 0.04	13.85 ± 0.01
TRIA + ABA	53.24 ± 0.06	3.78 ± 0.16	7.77 ± 0.06	19.10 ± 0.03	16.13 ± 0.13

sition was mandatory. As there is no detectable storage lipid in the flesh of cucumber fruit, almost all the lipids extracted from the protoplasts are expected to be from the membranes. It has been shown that ABA increases the biosynthesis of monounsaturated long chain fatty acids [52], and it is evident here that ABA treatment increases 18:1 fatty acid level (Table 5). In addition, there is also an ABA-induced increase in 18:0 and a decrease in 18:3 fatty acids (Table 5). Since ABA has been shown to be a signal molecule for drought perception in plants [53], and plants subjected to drought are known to show a decreased level of 18:3 fatty acid and an increase in saturated fatty acids [54–58] the action of ABA in this case (Table 5) is in accordance with the plants subjected to drought. However, a combination of TRIA and ABA shows that the action of TRIA in increasing the level of 18:3 fatty acid is suppressed by ABA (Table 5). Interestingly, even though there occurs an increase in saturated fatty acids and decrease in polyunsaturated fatty acids in the presence of a combination of ABA and TRIA (Table 5), the membrane microviscosity in this case is still very much reduced when compared to both control and ABA-treated protoplasts (Table 4). In fact the fluidity of the biological membranes is regulated not only by the fatty acid making, as the lipid–protein interaction would also contribute to the physical status of the membrane [59]. Therefore, decrease in microviscosity of protoplast membranes treated with ABA and a combination of TRIA and ABA may be due to physical intercalation of these compounds into the membranes, and decrease in microviscosity caused by TRIA alone (Table 4) may not entirely be due to increase in 18:3 fatty acid (Table 5). As it is known that TRIA has an anti-inflammatory effect in animals [2], the current results may also provide a basis for further unravelling the mechanism of action of this compound in inflammatory responses in animal tissues.

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