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Microviscosity of Plasmalemmas in Rose Petals as Affected by Age and Environmental Factors[1]

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ABSTRACT

The microviscosity of the plasmalemma of protoplasts isolated from rose (*Rosa* hyb. cv. Golden Wave) petals was measured by fluorescence depolarization. The plasmalemma's microviscosity was found to increase in petals which were allowed to age on cut flowers or after isolation as well as in isolated protoplasts aged in an aqueous medium. Increasing the temperature of the cut flowers or the isolated protoplasts enhanced the increase of the microviscosity of the protoplast plasmalemma. The mole ratio of free sterol to phospholipid was greater in protoplasts isolated from old flowers or in protoplasts aged after isolation than in protoplasts isolated from younger flowers. Microviscosity was greatest when protoplasts were aged at pH 4.4 and in the presence of Ca^{2+}. Artificial alterations of the sterol to phospholipid ratio in the protoplasts, induced by treatment with liposomes, caused similar changes in their measured microviscosity.

These findings strongly suggest that the increase in the petal plasmalemma microviscosity with age is associated with an increase in the sterol to phospholipid ratio which results, at least partially, from the activity of endogenous phospholipases.

Growing plant tissue is characterized by a sequence of metabolic and hormonal changes which terminate with the death of the tissue (18). This process is referred to as development and senescence of tissue. In the last few years development and senescence of flower petals were studied extensively. Recently attention was focused onto the plasma membranes as a possible location for these processes to occur and be regulated (1, 7, 15).

Because of the cell wall, the plasmalemma of plant cells, unlike the plasmalemma of animal cells, is not accessible to direct observation. This difficulty has been circumvented lately by methods which facilitate the preparation of plant cells free of cell wall, so-called protoplasts, by the action of specific enzymes on the plant tissue (5). We have reported (2) that the microviscosity in the lipid core of the protoplast plasmalemma from rose petals increases markedly with age and that it is much less sensitive to temperature than the microviscosity of mammalian membranes. A similar trend of microviscosity changes upon aging in plant microsomal fractions has been demonstrated recently (16).

The following study elaborates on the mechanism of changes in microviscosity of the lipid core in the plasmalemma.

MATERIALS AND METHODS

Plant Material and Environmental Conditions. Roses (*Rosa* hyb.) Golden Wave (Sin. Dr. Verhage) were grown in a greenhouse under standard cultural conditions. Flowers were either allowed to develop on the plant or were picked as tight buds (stage "A," ref. 2), cut to a uniform length of 40 cm, and placed with the stem base in deionized H_2O (10 cm) under controlled conditions at 55 ± 10% relative humidity and continuous illumination from cool white fluorescent light at a light energy flux density of 650 $\mu w/cm^2$. Temperature varied according to the specific experiments. For isolation of petals, flowers were cut at stage "A," three uniform petals were selected from the second whorl and placed individually in a test tube with their base in deionized H_2O under the same conditions as the cut flowers.

Isolation of Protoplasts. After preliminary screening, two enzyme solutions which gave similar preparations were selected, one containing 0.6 M mannitol (Merck) solution at pH 5.4 and 0.50% Cellulysin (Calbiochem) + 0.25 Driselase (Kyowa Hakko Koggo Co.), the other containing 0.6 M mannitol solution at pH 5.4 and 0.50% cellulase "Onozuka" R-10 (Kinki Yakult Co.) + 0.25% Driselase + 0.10% Macerozyme R-10 (Kinki Yakult Co.).

The maceration solutions were filtered through a Gelman filter unit with a 0.45-μm membrane into Petri dishes. The lower epidermis of three petals from the second whorl of the flower was peeled off and the remaining parts were placed in the maceration solutions in the dark at 21 ± 1 C for 14 to 17 hr without shaking. After maceration, the crude protoplast suspension was filtered through glass fibers and kept for 15 to 30 min to allow precipitation of the protoplasts. Then the protoplasts were washed twice with fresh 0.6 M mannitol solution and the yield was 5 to 10 × 10^6 protoplasts/g fresh wt of petals. Protoplasts were further purified on lymphoprep (Nyegaard A/S) as described by Larkin (10) giving a homogeneous protoplast suspension with about 70% recovery. The obtained protoplasts were relatively large (40–60 μm in diameter), and contained voluminous vacuoles and a few chromoplasts showing some cyclosis. The viability of the protoplasts and the integrity of their membranes were evaluated by their accumulation of neutral red (BDH) and fluorescein diacetate (FDA) (Pfaltz & Bauer Inc.) (10, 11). More than 70% of the protoplasts in all of the experiments showed dye accumulation.

Chemical Treatment. Whenever the concentrations of added chemical exceeded 10 mM, the concentration of the mannitol in the solution was reduced to give a final osmolarity of 0.6 M. Ethylenediaminetetraacetate (EDTA) (BDH) and ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetate (EGTA) (Sigma) were included in concentrations of 10 mM in the maceration of the incubation medium.

Buffers. Citrate buffer was used for pH 3.5 to 6 and phosphate buffer for pH 6 to 8. Both were included in the incubation medium at concentrations of 50 mM. There was no effect of the type of buffer on the results.

Microviscosity Determinations. Fluorescence polarization and

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intensity were measured with an instrument which has been previously described (20–22). A 365 nm band, generated from a 500-w mercury arc and passed through a polarizer, was used for excitation. The light emitted from the sample was detected in two independent cross-polarized channels, passing through a 2 M sodium nitrite solution which was used as a cut-off filter for wavelengths below 390 nm.

The emission intensities polarized parallel (I_{II}) and perpendicular ($I_{\perp}$) to the direction of polarization of the excitation beam were obtained by a simultaneous measurement of $I_{II}/I_{\perp}$ and $I_{\perp}$. These values related to the degree of fluorescence polarization, P, and to the total fluorescence intensity, F, by the following equations:

$$P = \frac{I_{II}/I_{\perp} - 1}{I_{II}/I_{\perp} + 1}; \quad F = I_{II} + 2\,I_{\perp}$$

With the aid of these values the microviscosity, $\bar{\eta}$, was determined (20–22).

Protoplasts were labeled with the fluorescence polarization probe 1,6-diphenyl 1,3,5-hexatriene (Fluka AG) as follows. An aliquot (50 μl) of 2 mM DPH[2] in tetrahydrofuran was injected into 50 ml of vigorously stirred 0.6 M mannitol solution. Such dispersion is virtually nonfluorescent. Three ml of the DPH dispersion was added to an equal volume of protoplast suspension of 10^5 protoplasts/ml. Labeling was performed at 37 C for 30 min, after which the protoplasts were washed twice and resuspended in the mannitol solution to form 10^5 protoplasts/ml.

Lipid Analysis. Samples of $2 \cdot 10^7$ protoplasts were extracted according to Renkonen *et al.* (17). The mole content of phospholipids was determined by phosphorus analysis after conversion to phosphoric acid with 70% $HClO_4$ at 190 C for 60 min by the method of Bottcher *et al.* (3). Free sterol in the lipid extracts was determined by the method of Chiamori and Henri (4) after digitonin precipitation.

Plasmalemma Lipid Modification. Alterations in the sterol to phospholipid ratio of the plasma membrane were carried out according to the method of Cooper *et al.* (6) as follows. Protoplasts were incubated for 6 hr at 37 C (pH 6) with 0.5 mg/ml liposomes prepared from a mixture of cholesterol (Sigma) and lecithin (Lipid Products) 1.2:1, M:M) or lecithin alone. The liposomes were made as outlined by Shinitzky and Inbar (22). After incubation the protoplasts were washed three times with 0.6 M mannitol and labeled with DPH for fluorescence polarization measurements.

All of the experiments described were repeated at least three times.

RESULTS AND DISCUSSION

The fluorescence polarization and intensity were monitored during the incubation of protoplasts with DPH dispersion at 37 C (Fig. 1). After 30 min the increase in fluorescence intensity was about 20-fold. After 12 min, a constant degree of fluorescence polarization was observed while DPH uptake continued (Fig. 1). Similar results were obtained for lymphocytes (21) labeled with DPH. The background fluorescence of the protoplasts amounted to less then 7% and allowed us to determine the microviscosity ($\bar{\eta}$) without applying any corrections. The labeled protoplasts viewed under fluorescence microscope showed an obvious glowing periphery. Attempts to photograph them failed bacause of rapid bleaching (20).

The microviscosity of the protoplast plasmalemma increased with aging in protoplasts from petals of cut flowers left to age in water, in protoplasts from separated petals kept in water, as well as in isolated protoplasts kept in a mannitol solution (Fig. 2). The observed increase of microviscosity with age is similar to that observed in protoplasts isolated from petals of intact flowers of different ages (2). The rate of increase in microviscosity was faster when either petals or isolated protoplasts were allowed to age.

Since temperature is known to determine the rate of flower aging (13), we have further investigated the effect of the temperature at which the different preparations were kept, on the microviscosity of the plasmalemma.

Cut flowers were kept at different temperatures (15–29 C) (Fig. 3) and at several time intervals petals were taken for the isolation of protoplasts. The microviscosity was found to increase with time at all temperatures tested, and the increase was faster as the temperature was raised. Also, as expected, the rate of senescence

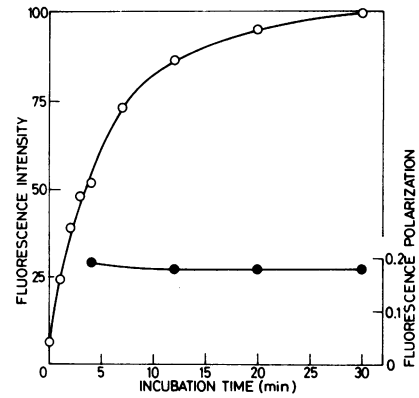

FIG. 1. Changes in fluorescence intensity (○) and degree of fluorescence polarization (●) during incubation of rose petal protoplasts in 1×10^{-6} M DPH solution at pH 6.5 and 37 C.

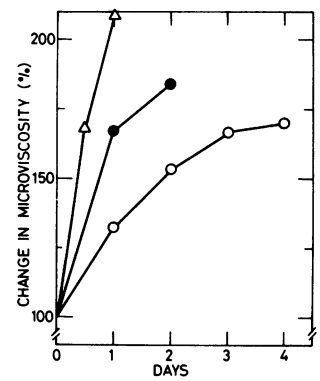

FIG. 2. Increase in microviscosity of rose petal protoplast plasmalemma isolated from cut flower (○) or from isolated petals (●) upon aging, and of protoplasts aged in aqueous solution (△). Aging and measurements were carried out at 22 C.

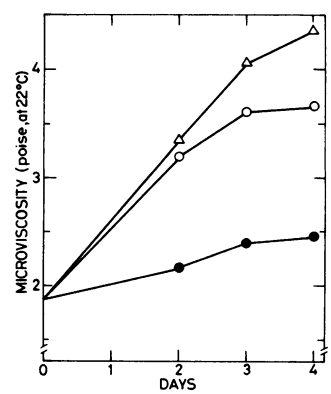

FIG. 3. Changes in microviscosity (22 C) of rose petal protoplast plasmalemma isolated from cut flowers kept at 15 C (●), 22 C (○), and 29 C (△) for various periods of time.

[2] Abbreviation: DPH: 1,6-diphenyl 1,3,5-hexatriene.

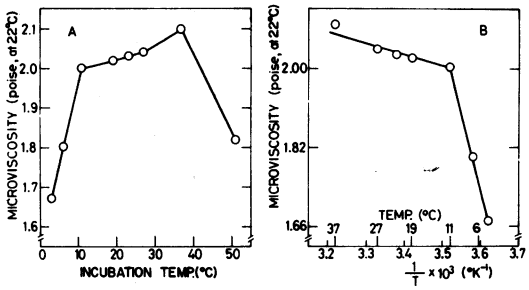

FIG. 4. Changes in microviscosity (22 C) of rose petal protoplast plasmalemma incubated for 10 hr at pH 4.4 at various temperatures. A: microviscosity *versus* temperature; B: log microviscosity versus 1/T.

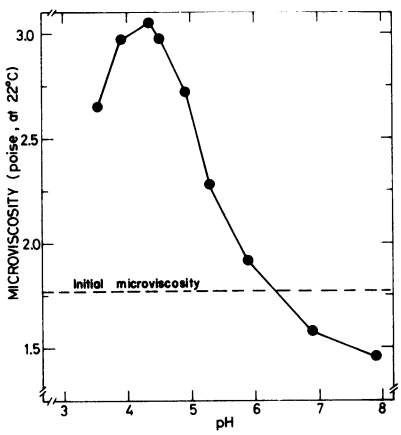

FIG. 5. Changes in microviscosity (22 C) of rose petal protoplast plasmalemma incubated for 10 hr at 22 C at various pH values.

of the flowers increased with temperature—at 15 C the longevity was 8 days while at 22 C it was 6 days and at 29 C it was only 4 days. Our experiments were not carried out at a temperature higher than 29 C since above this temperature wilting of flowers may result from perturbation in the water balance (14). In isolated protoplasts we could use a wider temperature range. After keeping the protoplasts for 10 hr in an aqueous medium at various temperatures (3–51 C) the microviscosity of their plasmalemma was measured. Figure 4A shows that in the temperature range of 3 to 37 C the increase in microviscosity correlates well with the increase in incubation temperature. However, at 51 C substantial decrease in the rate of the change of microviscosity was observed, presumably due to deactivation of enzymes (12). It is therefore plausible that the observed changes in microviscosity with senescence are associated with changes in enzymic activities both in the protoplast and in the whole flower. The dependence of microviscosity (measured at 22 C) on incubation temperature is not uniformly linear in the temperature range from 3 to 37 C (Fig. 4A), yet a plot of log $\bar{\eta}$ (22 C) versus 1/T reveals two distinct linear regions with a discontinuity at around 11 C (Fig. 4B).

Incubation of the protoplasts for 10 hr at various pH values (Fig. 5) gave an optimum curve with a maximal increase in the microviscosity (measured at 22 C) of the plasmalemma at pH 4.4. As shown, at pH > 6, the effect of incubation is reversed, causing an increase in the fluidity of the plasmalemma. Additional 10 hr of incubation of the protoplasts in the same solution caused an increase in the microviscosity of the plasmalemma at all pH values (data not presented), with a profile resembling the one presented in Figure 5. These results indicate that low microviscosity values characterize a younger and therefore more vital state of the plant cell, and can be correlated with the improved viability and longevity of protoplasts (19) and vacuoles (11) upon increasing the pH.

The presence of 5 to 25 mM Ca^{2+} ions in the incubation medium (Fig. 6) increased the rate of the observed change in microviscosity of plasmalemma. In protoplasts prepared in the presence of EDTA, which removes the Ca^{2+} ions (Table I), the microviscosity still increased, though slower. However, the presence of a chelating agent in the incubation medium only had no effect on the microviscosity; this was also the case when the chelating agent was EGTA which is more specific for Ca^{2+} ions. These observations suggest that when the tissue is disintegrated by the enzymes, Ca^{2+} ions are released from the middle lamella and are removed by the chelating agent without getting in contact with the protoplasts. Therefore, the addition of a chelating agent to the incubation medium which was free of Ca^{2+} ions has no effect.

One of the prominent parameters which modulate membrane microviscosity *in vivo* is the mole ratio of free sterol to phospholipids (21, 22). We have determined in different experiments the

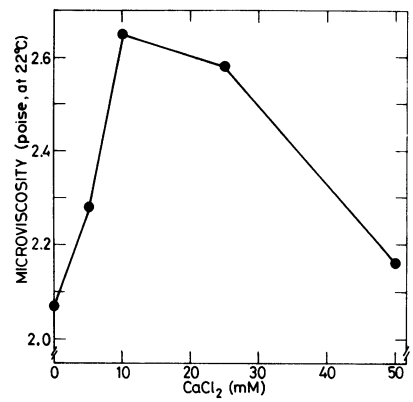

FIG. 6. Changes in microviscosity (22 C) of rose petal protoplast plasmalemma incubated for 10 hr at 22 C with different concentrations of $CaCl_2$ at pH 4.4.

TABLE I. The effect of EDTA (10 mM) in the maceration (16 hr at 22 C) and/or the incubation medium (10 hr at 22 C) on the microviscosity of protoplast plasmalemmas obtained from rose petals.

EDTA in maceration	EDTA in incubation	Microviscosity (poise) at 22 C
-	-	2.72
-	+	2.72
+	-	2.02
+	+	2.07
-	No incubation	1.66
+	"	1.77

TABLE II. The mole ratio of free sterol to phospholipid (± SE) in young and old protoplasts.

	Free sterol : phospholipid (mole ratio)	
	Protoplast obtained from petals of 2 ages[1]	Protoplast aged in vitro[2]
Young	0.28 ± 0.01	0.34 ± 0.01
Old	0.44 ± 0.02	0.68 ± 0.01

[1] Young - stage A, old - stage D [see ref. 2].
[2] Young - fresh protoplast of stage A, old - protoplast of stage A, aged for 24 hr in the incubation medium [37 C, pH 4.4].

TABLE III. Microviscosity and free sterol to phospholipid ratio (± SE) of protoplast plasmalemmas obtained from rose petals at 2 stages (A - young, D - old) as affected by incubation with liposomes. Incubation was for 6 hr at 37 C and pH 6.0

Treatment		Microviscosity (poise)	Free sterol: phospholipid (mole ratio)
Stage	Liposomes		
A	———	2.06	0.22 ± 0.02
A	lecithin + cholesterol (1:1.2, M:M) 0.5 mg/ml	4.05	0.71 ± 0.01
D	———	2.92	0.50 ± 0.03
D	lecithin, 0.5 mg/ml	2.43	0.36 ± 0.01

free sterol and phospholipid contents in either protoplasts isolated from petals of different ages or protoplasts isolated from young petals and aged in aqueous solution (Table II). The results show a marked increase in free sterol to phospholipid ratio with age. This phenomenon was associated with a decrease in phospholipid content while the free sterol level remained essentially constant. Similar observations were recently reported for senescing flowers of *Ipomea tricolor* (1). Supporting evidence to the contribution of sterol to the observed increase in microviscosity of plasmalemma was obtained from the following experiments. Protoplasts were incubated with either lecithin-cholesterol or lecithin liposomes, a treatment which results in either enrichment or depletion of cholesterol from erythrocyte membranes (6), and their microviscosity as well as free sterol to phospholipid mole ratio were measured. When protoplasts from young petals were treated with lecithin-cholesterol liposomes, which increased the sterol to phospholipid ratio, the microviscosity of the plasmalemma also increased. Conversely, treating protoplasts from old petals with lecithin liposomes, and thus decreasing the sterol to phospholipid ratio, resulted in a decrease of the microviscosity of the plasmalemma (Table III).

The *in vivo* increase in the sterol to phospholipid ratio, at least partially results from the activity of phospholipases which cause a decrease in the membrane phospholipid content. Therefore, it seems to us that the change in microviscosity of the plasmalemma is related to the activity of the phospholipases. This possibility is in line with the observed effects of pH and Ca^{2+} on the microviscosity in plasmalemma. The optimal conditions for the activity of plant phospholipases are pH 4 to 5 (9) and the presence of Ca^{2+} (8), which are approximately identical with the requirements for the maximalization of the increase in the microviscosity of the plasmalemma (Figs. 5 and 6).

Our results indicate that senescence of rose petals is accompanied by an increase in the plasmalemma microviscosity, mostly due to the increase of the sterol to phospholipid ratio. This increase is presumably brought about by a decrease of the amount of phospholipids caused by the action of phospholipases.

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