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Electrolyte Leakage and Protoplasm Viability of Pummelo Mesocarp Tissue as Influenced by Exogenous GA₃

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Abstract. Studies were conducted to determine whether certain physiological effects of gibberellic acid (GA₃) on the peel of citrus fruits may be attributed to GA₃ interaction with cellular membranes. Excised mesocarp tissue from pummelo [*Citrus maxima* (Burm.) Merrill] fruits was analyzed for electrolyte and K⁺ release over time in varying concentrations of GA₃. Electrolyte leakage and K⁺ efflux was significantly reduced (up to 30%) when tissue was incubated in the presence of GA₃. GA₃ improved the viability of mechanically isolated protoplasts during 72 hr of storage at 7°C, as shown by the use of fluorescein diacetate. These results suggest that some of the GA₃-elicited responses in citrus fruits may be membrane related.

Gibberellic acid (GA₃) has been linked with citriculture since the late 1950s, when it was first used in navel oranges to influence several fruit quality factors (Coggins and Hield, 1958). Since then, this plant growth substance has been shown to modify color development by delaying chlorophyll degradation (Coggins and Hield, 1968) and to reduce or control several physiological blemishes and certain diseases of the peel (Coggins, 1969; Grierson, 1981). GA₃ appears to delay peel senescence for several citrus species.

Although GA₃ has been associated with the alteration of peel

aging for many years, an accepted explanation with regard to the mode of action for the compound has not been delineated. Many investigators have attempted to correlate effects of a plant growth regulator with its binding to a specific cytosolic or membrane-bound receptor. Plant hormones generally exhibit both rapid and slow effects (Ben-Tal and Vainer, 1974; Evans, 1974). It is possible that slow effects are due to transcriptional or translational processes and that rapid effects are due to membrane-mediated responses. Paleg and coworkers found that GA₃ promoted changes in liposomal membrane permeability (Wood and Paleg, 1972) and fluidity (Wood and Paleg, 1974), which may involve complex formation between GA₃ and the charged trimethylamino group of phosphatidylcholine (Wood et al., 1974). In addition, specific interactions between gibberellin and the hydration shell of the choline headgroup of phosphatidylcholine are factors that may affect the conformation of biological mem-

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branes (Janiak et al., 1976). Recent evidence continues to support these early findings. Pauls et al. (1982) has shown that a mixture of GA₄ and GA₇ enhances membrane fluidity in phospholipid liposomes by lowering the gel to liquid-crystalline transition temperature. With regard to the GA₃-induced delay in aging, this may be an important finding because senescence in many systems has been shown to include a dynamic membrane transition from the liquid-crystalline to gel phase (McKersie and Thompson, 1979). This shift has been closely coupled to senescence. (Barber and Thompson, 1980), metabolic dysfunction and cellular damage (Raison and Wright, 1983), and loss of membrane performance and integrity leading to enhanced membrane permeability (Barber and Thompson, 1980). Currently, it is not known if the antisenescence properties of GA₃ are associated with any of these events in citrus fruits.

The objective of our research was to provide experimental evidence as to whether certain physiological effects of GA₃ on the albedo tissue of pummelo peel are mediated through an interaction, either direct or indirect, with biological membranes. Histological characterizations (Coggins, 1969), enhancement of electrical conductivity in senescing whole citrus fruits (Sasson and Monselise, 1977), susceptibility of fruit to fungal decay by sour rot (*Geotrichum candidum*) (Baudoin and Eckert, 1982), and the current model for chlorophyll degradation (Amir-Shapira et al., 1986) in citrus served to motivate this effort.

Materials and Methods

Plant material. Having an extremely thick peel that could provide the amounts of tissue required, pummelo ('Kao Panne' on 'Carrizo' citrange [*C. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] rootstock) fruits were chosen for this work and were obtained from trees grown at the Citrus Research Center, Riverside, Calif. Large (25 cm in diameter), pale-yellow fruits with a naturally soft rind were used for electrolyte leakage measurements and were harvested at a mature stage of development, 9 to 10 months after anthesis. Midseason fruits (6 to 7 months postanthesis), destined for protoplasm isolation, were selected on the basis of their medium size (15 cm in diameter), compact albedo, and light-green surface. Both types of fruits were washed in distilled water and allowed to air dry before use.

Assays of electrolyte leakage. One 2.5-cm-wide longitudinal section of rind (epicarp and mesocarp) was carefully excised from each of 10 fruits (replicates) per treatment. Twenty plugs, 4 mm in diameter, 2.5 cm long, uniformly weighing ≈1.5 g, and containing only albedo tissue, were cut from each peel section. The plugs were rinsed for 15 sec with glass-distilled water to eliminate material from cut edges and were then placed in a 100-ml beaker with 30 ml of glass-distilled water containing 0, 3, 29, or 72 μM GA₃ (Abbott Laboratories, North Chicago, Ill.). Each incubation solution had been previously adjusted to a pH of ≈4.2, and a uniform, initial conductivity reading (19 μmhos) had been established by appropriate additions from a 0.5% (w/v) solution of citric acid. The samples were incubated in room light (6 to 8 μmol·s⁻¹·m⁻²) with intermittent and gentle stirring at a constant 24°C throughout the experiment. Electrical conductivity of the incubation solutions was measured at 1-, 3-, 5-, 10-, 15-, 25-, 45-, and 60-min intervals using a conductivity bridge equipped with a dip cell (k = 1.0 cm⁻¹, YSI Model 31, Yellow Springs Instruments, Yellow Springs, Ohio).

An assessment of K⁺ efflux was performed as a separate experiment using 10-fruit replicates and the tissue incubation procedure described in the above paragraph. After a 30-min incubation period, a 5-ml aliquot of the recovery medium was

removed and analyzed to determine the amount of K⁺ diffused from the tissue. Potassium was determined at 766.9-nm wavelength on a Perkin-Elmer Model (460) atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, Conn.) furnished with a 10-cm standard burner head. Total electrolyte content of the mesocarp was evaluated following disruption of the tissue via a freeze-thaw treatment.

Protoplasm isolation. About 30 g of rind plugs (5 mm in diameter) were carefully removed from within 1.5 cm under the peel of each test fruit. The technique allowed the endocarp to remain intact and only the mesocarp and epicarp to be excised. Later, the flavedo portion of the plugs was removed with a single-edged razor blade. The resulting albedo tissue was then plasmolyzed in room light (3 to 5 μmol·s⁻¹·m⁻²) at 24°C by submerging the plugs for 1.5 hr in 400 ml of an incubation solution containing 1.0 M KCl, 0.1 M CaCl₂, 0.01 M 2-(N-morpholino)ethanesulfonic acid (Mes) (pH 6.0), 0.5 mM dithiothreitol, and 0.05% (w/v) bovine serum albumin (BSA). Preliminary experiments suggested that the dithiothreitol and BSA additions improved protoplasm size and yield.

An apparatus (Fig. 1) was designed for the sole purpose of isolating usable numbers of protoplasts by mechanical means that would not require the use of cell wall degradative enzymes. The apparatus was centered around a stainless-steel bar, machined such that tissue plugs could be fed through an off-centered hole to be sliced by a double-edged razor blade. The razor blade, which was fastened to the bottom end of a brass rod using a stainless-steel machine screw, rode against the cutting face in a manner permitting smooth rotation with minimal play. The brass rod itself passed through the center of the device and had its top end connected to a variable-speed electric drill that rotated the rod and attached razor blade. Before use, the brass rod was coated with a small amount of silicon lubrication fluid; excess fluid was removed with a paper tissue. In operation, the lower portion of the device housed the razor blade and was suspended above a 50-ml crystallizing dish containing the incubation solution. With the blade rotating at ≈1900 rpm, tissue plugs were fed into the device with the aid of a fine stream of incubation solution from a wash bottle. Depending on the velocity at which the plugs were fed, slices down to 30 μm thickness could be cut by the apparatus.

The sliced albedo mixture, containing protoplasts and cell wall debris, was first filtered through two layers of cheesecloth to remove the slicings and later through a nylon screen having 64-μm openings to remove the smaller debris. The resulting suspension was transferred into eight 50-ml centrifuge tubes and centrifuged at 180× g for 7 min. Following removal of the supernatants, the pellets were transferred into one 15-mm × 125-mm tube, resuspended in the incubation medium, and centrifuged at 180× g for 7 min. The pellet was resuspended in 5 ml of a suspension medium [0.7 M mannitol, 2 mM Mes (pH 6.5)] containing 18% (w/v) Ficoll (a nonionic synthetic polymer of sucrose; Pharmacia LKB Biotechnology, Piscataway, N.J.). The resulting protoplasm suspension was overlaid with 2 ml of suspension medium containing 12% (w/v) Ficoll and then with 2 ml of suspension medium without Ficoll. This three-layer gradient was centrifuged at 180× g for 30 min. Intact protoplasts were gently collected at the 0%/12% Ficoll interface by using a broad-tipped, air-displacement pipette and were again washed by centrifugation through the suspension medium. The protoplasm pellet collected after the wash was resuspended in the suspension medium, and the number of protoplasts was determined with a hemocytometer.

Fig. 1. Side, top, and bottom views of the device used to mechanically isolate protoplasts from excised mesocarp tissue of pummelo. A stainless-steel bar (A) was machined such that plasmolyzed tissue plugs, fed through an off-centered hole (B) at the base of the device, were sliced by a rotating double-edged razor blade (C). A brass rod (D) passed through the center of the apparatus and was rotated by a variable-speed electric drill (not shown). A stainless-steel machine screw (E) and washer (F) fastened the razor blade to the brass rod and completed the assembly. A stainless-steel rod attached to the back side of the machined bar served as a handle to prevent the bar from turning with the brass rod and razor blade. Dimensions given in millimeters. Side view differs in scale from top and bottom views.

Evaluation of protoplast viability. Two 0.2-ml aliquots of the protoplasm suspension were transferred to 1.5-ml polyethylene conical vials. One vial was treated with 16.7 μ l of water (control), and the other was treated with 16.7 μ l of a 866 μ M solution of GA_3 , giving a final GA_3 concentration in the vial of 67 μ M. A similar technique was performed to generate treatments with GA_3 concentrations of 3 and 28 μ M. To simulate typical citrus fruit storage conditions, protoplasts were held at 7°C in the dark and scored for viability at 0-, 24-, 48-, and 72-hr intervals. The protoplasm viability experiment was conducted six times.

Duplicate samples were withdrawn from each vial and examined for protoplasm viability. Protoplasts were observed with a microscope [American Optical (Buffalo, N.Y.) Model 10 with an AO/Reichert Mercury/Xenon Illuminator (Model 2086, equipped with 490-nm exciter, 510-nm dichroic and 520-nm barrier filters; Reichert-Jung, Buffalo, N.Y.)] by bright-field optics and by epi-illumination fluorescence optics. Protoplasts were stained by adding 0.3 μ l of 5 mg fluorescein diacetate/ml in acetone to 30 μ l of the protoplasm sample and incubating it for 4 to 5 min. The protoplasts were placed on a hemocytometer slide and were first viewed in bright field and then in fluorescence so that both total and viable protoplasm counts could be scored in each sample.

Assessment of bacterial influence. Nonsterile and sterile conditions were compared to evaluate bacterial contamination. Protoplasts were isolated using the procedure described previously

and, in addition, a modified technique was used, where all vials, glassware, and media were sterilized (autoclave at 120°C for 20 min). Mechanically isolated protoplasts were incubated in solutions [0.7 M mannitol, 2 mM Mes (pH 6.5)] containing 0 and 67 μ M GA_3 for 24 hr in darkness at 7°C. Protoplasm viability was then scored, and 100- μ l aliquots of the protoplasm suspensions were plated onto an enriched agar medium [0.7 M mannitol, 1% tryptone, 0.5% peptone, 0.5% yeast extract, 1.5% potato dextrose agar, 2 mM Mes (pH 6.0)] to test for bacterial contamination. Bacterial colonies per petri dish (100 $\times$ 20 mm) were counted after a 25-day incubation period in darkness at 7°C.

Results

Membrane permeability to electrolytes. A time-course study of conductivity changes showed a rapid release of solutes during the first 2 min following immersion of the albedo tissue in the incubation media (Fig. 2). However, the efflux concentration from living tissue was low (5 to 50 μ mhos as shown in Fig. 2) in comparison to a concentration of 5154 ± 156 μ mhos from the same quantity of tissue subjected to the freeze-thaw procedure. Leakage rates declined after 20 min, but leakage was still present at the end of the 60-min incubation period. GA_3 (at all concentrations examined) reduced, but did not eliminate, solute efflux. The effect of GA_3 on leakage was observed within the first 5 min of incubation, and, at the end of the experiment,

Fig. 2. GA_3 and electrolyte leakage from mesocarp tissue of pummelo. Plugs were excised from the peel and incubated at 24°C in solutions containing the indicated concentrations of GA_3 . Means for 10 individual fruit replicates $\pm$ SD.

Fig. 3. GA_3 and K^+ efflux from mesocarp tissue of pummelo. Means of 10 individual fruit replicates ($\pm$ SD) after a 30-min incubation period in the indicated concentrations of GA_3 .

net leakage from tissue treated with 72 μM GA_3 was 30% less than from the control tissue.

GA_3 also reduced the efflux of K^+ , a component of total electrolyte leakage, after 30 min of incubation (Fig. 3). The quantity of K^+ released into the external solution from the albedo tissue depended on the concentration of GA_3 , as a 3 μM concentration produced a small reduction in electrolyte loss, while increasing levels further diminished K^+ content of the leachate. This is probably a reflection of the similar trend demonstrated in measurements of total electrolyte conductivity.

GA_3 effect on protoplasm viability. Mechanical isolation of protoplasts from mesocarp tissue of pummelo fruits yielded, on average, 5.0×10^4 protoplast/g of fresh tissue. The protoplasts had a mean diameter of 30 μm . As judged by ability to retain fluorescein generated from fluorescein diacetate, 61% of the protoplasts were viable immediately after isolation. Protoplasts were exposed to three levels of GA_3 ; these had a marked effect on subsequent viability (Fig. 4). The time-course diagram of protoplast survival shows the rapid decline of untreated protoplasts over 72 hr at 7°C. Relative to the protoplasts at the start of the experiment, 34% of the untreated cells survived for 24

Fig. 4. GA_3 and viability of mechanically isolated protoplasts from mesocarp tissue of pummelo. Protoplasts were incubated in the dark at 7°C in solutions [0.7 M mannitol, 2 mM Mes (pH 6.5)] containing indicated concentrations of GA_3 . Means of six experiments $\pm$ SD.

Table 1. Effect of GA_3 on pummelo mesocarp protoplast viability^a under sterile and nonsterile conditions over time.

Incubation time (hr)	Treatment and % viability			
	Sterile		Nonsterile	
	+ GA_3	- GA_3	+ GA_3	- GA_3
0	31.0 $\pm$ 1.7	34.9 $\pm$ 2.8	33.4 $\pm$ 5.9	33.7 $\pm$ 2.5
24	30.6 $\pm$ 2.5	14.5 $\pm$ 3.1	22.2 $\pm$ 2.4	9.8 $\pm$ 2.8

^aViability was determined by the fluorescein diacetate assay; means of three replicates $\pm$ SD.

hr and only 14% endured 48 hr of storage. The GA_3 treatment, however, enhanced protoplast survival, since a 67- μM level had reduced mortality by 25% at 24 hr. At this time interval, there was no significant difference in protoplast viability between the 28- and 67- μM concentrations of GA_3 . Viability of protoplasts declined throughout the 72 hr of evaluation. At all three examination times, survival of protoplasts treated with two higher concentrations of GA_3 was significantly higher than the control. Survival of protoplasts treated with 3 μM GA_3 was significantly higher than the control at 24 and 48 hr, but not at 72 hr.

In an assessment of bacterial and GA_3 influences on protoplast survival, no bacterial colonies were observed in culture of protoplasts isolated under sterile conditions, either with or without addition of GA_3 . For protoplasts isolated under nonsterile conditions, 49 ± 15 bacterial colonies were observed in cultures incubated without added GA_3 , whereas 328 ± 37 bacterial colonies were observed in cultures incubated with 67 μM GA_3 . While sterile conditions favored protoplasm survival, the positive effect of GA_3 on survival occurred in sterile as well as nonsterile conditions (Table 1).

Discussion

This paper represents an initial attempt to evaluate a potential relationship between cellular membranes and some gibberellin-mediated responses in citrus fruits. Leakage of electrolytes, as judged by conductivity increase, has been shown to be a simple and useful experimental method for assessing damage to cellular membranes. Enhanced electrolyte leakage during particular stages of plant development has recently been characterized in several organs, including germinating seeds (Hill et al., 1988), cotyledons (Keppler and Novacky, 1986), bacterially stressed roots

(Cahill et al., 1985), and senescing leaves (McKersie et al., 1982) and fruits (Nur et al., 1986). This leakage generally implies modifications in tonoplast or plasmalemma permeability. Assuming that the results acquired with excised albedo cores are indicative of intact peel physiology, the time-course study of solute efflux from the mesocarp of pummelo rind (Fig. 2) provides further support for the practicality of the conductivity method in assessing membrane integrity in these aged tissues, as influenced by GA₃. The observed alteration in tissue permeability was detectable at a GA₃ concentration of only 3 μ M, which is substantially less than the previously recorded concentration of 13,000 μ M required to elicit a response in egg phosphatidylcholine liposomes (Hester and Stillwell, 1984).

The changes observed in the conductivity of leachates over time is considered to be a nonspecific indication of total ionic leakage through membranes and has been adopted as a suitable estimate of membrane integrity and permeability (Simon, 1977). In this light, K⁺, a more specific component of the electrolyte leakage, was analyzed as a means to verify the previous conductivity surveys. Previous reports have shown K⁺ efflux to be a valuable diagnostic and sensitive tool in evaluating membrane damage and subsequent metabolic upset in aged apple slices (Nur et al., 1986) and senescing leaves (McKersie et al., 1982). The response pattern for K⁺ leakage from albedo tissue resembled the leakage kinetics and treatment effects demonstrated in the more-general conductivity procedure. Thus, GA₃ may have a beneficial impact on membrane integrity.

While the influence of GA₃ on membrane properties has been inferred from conductivity and specific ion leakage studies, it must be kept in mind that these conclusions are based on data obtained from an intricate and intact tissue system. Ionic contributions from mechanically injured cells and noncytoplasmic extracellular spaces, the buffering effects of the cell wall, and interactions of adjacent cells may impose a complex source of error affecting a meaningful interpretation of the data. Protoplast systems, on the other hand, remove "these cell wall hindrances, thus providing direct access to the plasmalemma. Enzymatically isolated protoplasts have an inherent disadvantage, however. Damage to the plasmalemma resulting from the very digestive enzymes used to prepare the protoplasts themselves (Galun, 1981) and various stresses that occur during the enzymatic isolation process have resulted in reduced protoplasm viability (Hartman and Hock, 1985). The mechanically isolated protoplasm system used in this study provides a means of studying some plant physiological responses without the added complications of enzymatic digestion.

Protoplast survival has been assumed for several years to be an indication of an intact biological membrane, clearly demonstrated via retention of fluorescein by the nonleaky membranes of living protoplasts and its release through the modified membranes of dead protoplasts (Widholm, 1972). This technique was used by Briggs et al. (1984) and other investigators to aid in the evaluation of plasmalemma transformations in response to a selective fungal toxin. In this study, however, the viability of mechanically derived protoplasts was examined as an indirect assessment of plasmalemma functionality and integrity in the presence of varying concentrations of GA₃. Various reports have documented certain factors governing protoplast survival, which are, perhaps, unrelated to any GA₃ influence (Cassells and Cocker, 1982; Cassells and Tamma, 1986).

The overall emphasis of this investigation involved an evaluation of GA₃ behavior in albedo protoplasts and intact albedo tissue. The effects of GA₃ on protoplast viability and tissue

electrolyte leakage may have implications with regard to the site of action of GA₃. Since a GA₃ concentration of 3 μ M produced preservative effects on both the solute efflux capacity from intact tissue and the survival of protoplasts, it is apparent that the site of GA₃ action is in the symplast, possibly related to cellular membranes.

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