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Rapid and Specific Staining for Routes of Liquid Entry into Cucumber Fruit

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Abstract. **A procedure is described for studying routes of liquid entry into and movement within pickling cucumber fruit (*Cucumis sativus* L. cv. Chipper). The internal gases of the fruit are exchanged with oxygen in a closed container. Fruit are then covered with 0.5% aqueous safranin O and allowed to stand for a few hours. Liquid was shown to enter the fruit through epidermal regions of greatest stomatal density, which are near placental tissue. The dye solution then moved through intercellular spaces and cell walls of placental and other mesocarp tissues and vascular elements. The stain facilitated delineation of stomata and visual observation of routes of liquid movement. Liquid entrance apparently is induced due to a partial vacuum created within the O_2-exchanged and liquid-submerged fruit.**

Fleming et al. (3) observed that gas exchange of whole, fresh pickling cucumbers with oxygen greatly influences brining properties of the cucumbers and that the cucumbers rapidly acquired a cured, translucent appearance within a day instead of the usual several weeks required for salt-stock cucumbers. They hypothesized that a partial vacuum was created within O_2-exchanged and brined cucumbers that caused brine to be drawn into the fruit and thereby to fill the intercellular air spaces (which amount to 4% to 6% of the volume of fresh cucumbers) (4), with the resultant cured appearance. They suggested that the O_2 was metabolized to CO_2 when the fruit were brined. The CO_2, being about 80 times more soluble than O_2, was thought to have dissolved in the tissue fluids to a greater extent than the O_2 that it replaced, resulting in a partial vacuum. Occurrence of a vacuum in O_2-exchanged, brined cucumbers has been confirmed (1).

Daeschel and Fleming (2) found that bacteria may enter O_2-exchanged, brined cucumbers and result in gaseous deterioration (bloater formation) of the fruit. Stomata were thought to be points of entry into the fruit. Vascular elements and intercellular spaces of mesocarp tissue were sites of bacterial colonization within the fruit. No conclusive evidence was obtained, however, on routes of bacterial entry into and movement within O_2-exchanged and brined cucumbers. Such information was desired so that means for excluding bacteria from the O_2-exchanged and brined fruit could be developed.

We thought the addition of dye to the aqueous solution in which O_2-exchaged cucumbers are held might provide a unique procedure for determining routes of entry into and movement of liquid, and perhaps bacteria, within brined cucumbers. The purpose of this paper was to describe a procedure that we developed for the rapid and specific staining of routes of liquid entry into and movement within O_2-exchanged and brined, whole cucumbers.

Materials and Methods

Hand-harvested (24–48-hr-old), disease-free, undamaged, 38–44-mm diameter 'Chipper' cucumber fruit were hand-washed and placed in 940-ml glass jars, 2 per jar (Fig. 1). Each jar was fitted with a lid in which 3 rubber serum stoppers (#712300, Bittner Corp.) were mounted. A glass sparger (#12 EC, Kimax) was inserted through one septum and a plastic 10-ml pipette (10 ml in 1/10, disposable Kimble, Owens-Illinois) with 1 cm of the tip removed was inserted through a second stopper. The sparger was connected to a gas flowmeter (Airco, model #A-75U) with a 6.25-mm diameter rubber tubing (Fig. 1). Oxygen was allowed to flow through the jar at a metered rate of 300 cc/min for 1 hr. A 0.5%, w/v, aqueous solution of safranin O (Allied Chemical Co.) was poured via a small funnel through the pipette into the jar. Oxygen flow was continued until the jar was filled with dye solution. A 16-gauge, hypodermic needle was inserted through the third stopper of the jar cap to serve as a gas vent during addition of the dye solution. The needle was removed when the solution rose into the pipette. Thumb pressure was applied intermittently to the third serum stopper to force entrapped gas bubbles out of the jar through the pipette. The pipette was then filled to the 0.0-ml graduation to serve as a dye-solution reservoir. Controls consisted of using the same procedure except that the cucumbers were not gas-exchanged.

Stomatal frequencies were determined from 1–2-mm thick, about 25 mm^2, epidermal strips, viewed at 150× with a light microscope. The strips were taken from the approximate center of the fruit, as viewed longitudinally, and from either "raised" or "recessed" regions, as viewed circumferentially. Raised regions were adjacent to the thickest parts of the mesocarp and the recessed regions were adjacent to the placentae (Fig. 2). Thus, in a 3-carpel fruit, there were 3 raised and 3 recessed regions.

Avenues for gas escape from the fruit were tested by forcing air into cucumbers that were submerged in water. A 6.2-mm diameter glass tube was inserted into the blossom end of a submerged cucumber to its approximate center. Air was introduced into the fruit under sufficient pressure to cause gas bubbles to exit from the fruit (Fig. 5).

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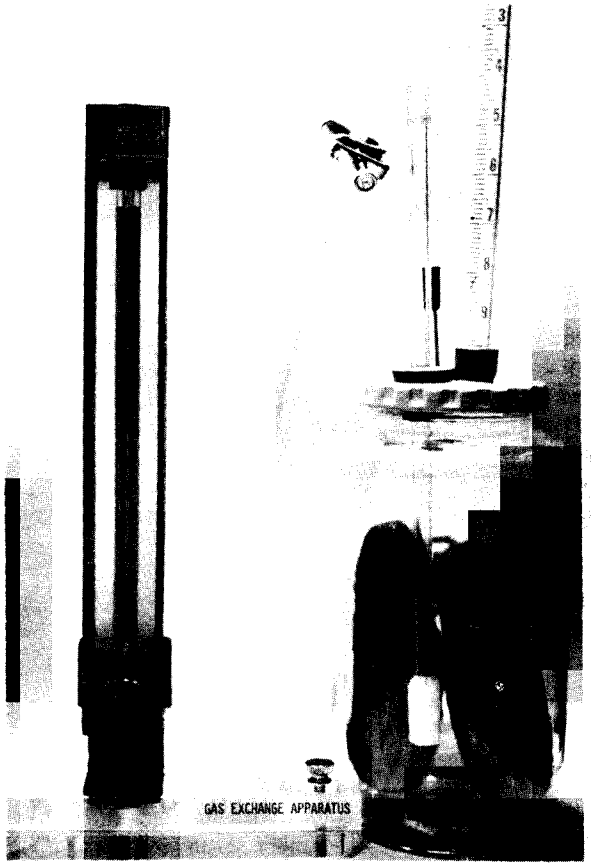

Fig. 1. Gas exchange apparatus.

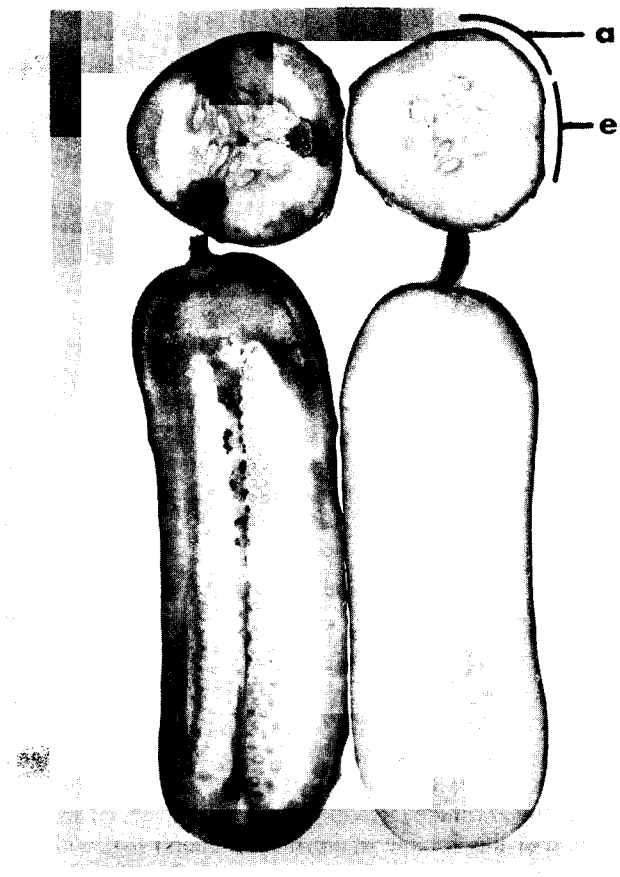

Fig. 2. Transverse and longitudinal sections of O_2-exchanged (left) and nonexchanged (right) cucumbers that were immersed in dye solution for 4 hr. Regions of the fruit epidermis are identified as "e" for recessed and "a" for raised.

Results and Discussion

Dye solution readily penetrated O_2-exchanged, but not nonexchanged, cucumbers during a 4-hr period (Fig. 2). Dye appeared to enter O_2-exchanged cucumbers mostly through the epidermis near placentae (recessed regions). Placentae, and mesocarp cells between placentae and epidermis, were heavily stained (Fig. 2). Dye also entered raised regions of the epidermis, but to a lesser extent than recessed regions. Upon entering through the epidermis, dye penetrated to the interior of the seed area, apparently through vascular elements, cell walls, and intercellular spaces.

In a few of the O_2-exchanged fruit, dye permeated the main vascular bundles that extend longitudinally just beneath the fruit epidermis (Fig. 3). Perhaps in these fruit the attachment scars of the peduncle or perianth afforded additional pathways for dye to enter the cucumbers.

We believe that dye solution entered O_2-exchanged and brined cucumbers through regions of least resistance, which appear to be regions of high stomatal density. Microscopic examination of epidermal strips revealed that higher frequencies of stomata occurred in recessed than in raised regions of the epidermis (Table 1). Stomatal guard cells and adjacent tissue were stained selectively, which facilitated locating stomata microscopically (Fig. 4). Most stomata were associated with stained tissue. Some were not, however, indicating that dye solution did not enter these stomata. Whether failure of tissue to be stained was due to stomatal pores being closed or to other factors remains to be determined.

When air was forced into the center of a cucumber submerged in water, gas bubbles exited the epidermis mainly through the

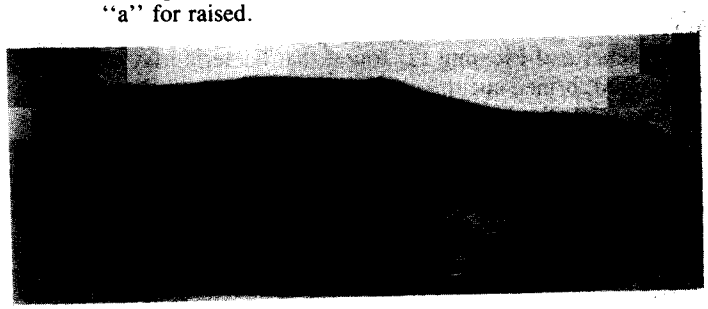

Fig. 3. Stained vascular system of an O_2-exchanged cucumber that was immersed in dye solution for 4 hr. Peel was removed after treatment.

Table 1. Stomatal density in raised and recessed regions of cucumber fruit epidermis.

Surface region	Stomata/field[z]	
	Mean[y]	Standard deviation
Raised	0.37	0.88
Recessed	7.56	6.86

[z]Microscopic field: 1.56 mm^2.
[y]Means determined from five cucumbers. Epidermal strips were obtained from each of the 3 raised and 3 recessed regions of each cucumber. Means are significantly different ($P < 0.01$) by Student's t test.

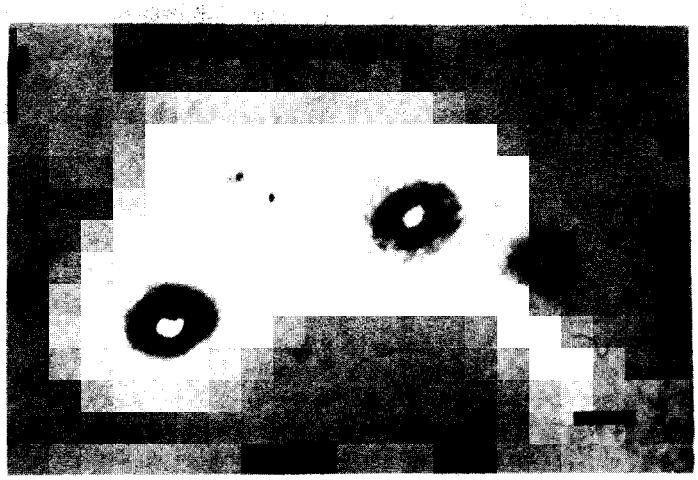

Fig. 4. Stained cells around stomata in the epidermis of an O_2-exchanged cucumber held in dye solution for 4 hr. Bar = 50 μm.

recessed regions (Fig. 5), which correspond to the regions of highest stomatal frequency. Relatively few bubbles exited through the raised regions.

When cucumbers were vacuum-infiltrated with dye solution, staining of tissues was variable and stain localization was poor compared to the O_2-exchange procedure for dye infiltration. In some cases the tissues of the whole cucumbers ruptured under vacuum. Reeve (6) reported a similar problem when vacuum-infiltrating apples with water, and pretreated apples with calcium chloride to prevent cell separation during vacuum infiltration. The cucumber fruit creates its own internal vacuum in the O_2-exchange method of dye infiltration, eliminating problems of tissue rupturing. Thus, the dye solution is more likely to enter and permeate the internal tissues through natural avenues.

Pederson and Albury (5) studied routes of dye movement in brined cucumbers; however, they immersed the fruit in dye solution for 4–24 hr. Our procedure using O_2-exchanged cucumbers is more rapid and provides more discretely stained routes of liquid entrance and movement.

Smith et al. (7) reported difficulty in locating stomata of cucumber epidermis by light microscopy of epidermal strips and replicas of fruit surfaces. Stomata were found to be recessed in the epidermis, which made epidermal replicas for microscopic viewing of limited usefulness. Also, they found a tendency toward clumping of stomata. Discrete staining as reported herein greatly improves stomatal visibility and can aid in locating stomata in epidermal strips for enumeration or other study.

Application of the O_2-exchange method may be useful for dye infiltration of other botanical materials. We have found the method useful with summer squash (*Cucurbita pepo*).

Fig. 5. Bubbles exiting a water-submerged cucumber. Air is being introduced into the center of the fruit through a glass tube. Note that most bubbles are exiting in recessed regions of the epidermis. See Fig. 2 for identity of recessed regions.

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