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Citrus Tissue Culture

STIMULATION OF FRUIT EXPLANT CULTURES WITH ORANGE JUICE

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ABSTRACT

***In vitro* growth of explant (juice vesicle or albedo tissues) cultures from citron (*Citrus medica*), lemon (*C. limon*), grapefruit (*C. paradisi*), sweet orange (*C. sinensis*), and mandarin (*C. reticulata*) fruits was stimulated by addition of orange juice (10% v/v optimum) to a basal medium containing Murashige and Skoog salts, 50 grams per liter sucrose, 100 milligrams per liter *myo*-inositol, 5 milligrams per liter thiamine·HCl, 2 milligrams per liter 2,4-dichlorophenoxyacetic acid and 0.5 milligrams per liter kinetin. In analyzing this effect of orange juice on citron explant cultures, we failed to obtain increased yields by addition of appropriate concentrations of citric acid to the basal medium but obtained growth stimulation when the medium was supplemented with juice from an "acidless" orange variety (cv. Lima). These facts suggest that some component(s) other than citric acid is involved. Addition of the inorganic ash corresponding to 10% (v/v) orange juice to the basal medium had no effect on yields. Similarly, the stimulatory effect of orange juice could not be explained based on its content of sucrose or of organic growth factors already present in the basal medium.**

The use of tissue culture methods for *Citrus* crop species has already had practical benefits. Most notable among these are techniques for obtaining virus-free and mycoplasma-free stocks using *in vitro* grafting of apical meristems from infected plants onto decapitated seedlings (7). As with other woody species which are difficult to culture *in vitro*, future advances with *Citrus* tissue culture will depend on a better understanding of nutritional factors and development of methods for controlling cellular differentiation and organogenesis (4, 9).

To improve techniques for *Citrus* tissue culture, we are investigating conditions that enhance callus culture growth. It has been observed that vigorous growth of tissue cultures derived from fruit of many *Citrus* species requires addition of orange juice to a basal medium containing Murashige and Skoog salts, sucrose, vitamins, and a number of other organic growth factors (6). Erner *et al.* (3), using callus tissues recultured from orange albedo for bioassays, obtained a partial purification of an active component that co-chromatographed with citric acid. On the basis of this evidence and the activity of citric acid in promoting recultured orange albedo, they concluded that the stimulating effect of orange juice is a result of its citric acid content.

In experiments to extend these findings to fruit explants we have observed orange juice-stimulated growth for a number of *Citrus* cultivars. We have failed to observe an effect of citric acid when added as a supplement to the basal medium in the appropriate concentrations. This paper reports results indicating that the stimulation by orange juice of *Citrus* fruit explant cultures is a consequence of some organic factor(s) other than citric acid.

MATERIALS AND METHODS

Chemicals, Composition of Media. Sucrose and the organic growth factors *myo*-inositol, thiamine·HCl, 2,4-D, and kinetin were all obtained from Sigma Chemical Company. Murashige and Skoog salt mixture (5) and Phytagar were obtained from Grand Island Biological Company. The basal medium for *Citrus* explant cultures contained the recommended concentration of Murashige and Skoog salts, 50 g/l sucrose, 100 mg/l *myo*-inositol, 5 mg/l thiamine·HCl, 2 mg/l 2,4-D, and 0.5 mg/l kinetin, and 1% Phytagar (v/v) (pH 5.6). Orange juice, obtained locally and partially clarified by centrifugation at 10,000*g* for 30 min, was added as a supplement prior to autoclaving. Juice from the "acidless" orange variety, Lima, was obtained by hand-squeezing cut fruit. The juice from Lima fruit is pH 6.8 and has titratable acidity of 11 meq/l compared with the juice from Valencia oranges which is pH 3.9 and titratable acidity of 115 meq/l (8).

Plant Materials and Tissue Culture. Young (100 days postpollination) citron, cv. Citron of Commerce, fruit were used for most of the experiments because of the availability of fruit throughout the year. These were obtained from the University of California—South Coast Field Station (Orange County, Santa Ana, Calif.). Lima variety orange fruit was from the University of California—Citrus Experiment Station (Riverside, Calif.). Fruit of other *Citrus* species were purchased locally.

The fruit for tissue cultures were taken to the laboratory where they were washed with dishware detergent and then rinsed with cold water. Subsequent sterilization steps involved soaking in 0.5% (w/v) sodium hypochlorite for 10 min followed by a brief dip in 95% (v/v) ethanol and flaming. Tissue pieces (20 mg) of juice vesicle or albedo were then cut and planted singly in culture tubes (25 × 150 mm) containing 20 ml of medium. Cultures were incubated in the dark at 27 C for 28 days after which time the tissues were harvested and fresh weights determined.

Preparation of Orange Juice Ash. To hydrolyze pectic substances, a 0.2-ml volume of HNO_3-HCl (1:3) was added to 40 ml of partially clarified orange juice in a quartz crucible. The mixture was evaporated to dryness over a hot plate and then transferred to an ashing oven for 7 hr at 550 C; *i.e.* sufficient time to produce a white ash.

RESULTS

Effects of Orange Juice and Citric Acid on Explant Cultures. Explant cultures of juice vesicles or albedo tissues from *Citrus* fruits proliferate *in vitro* by cell division and expansion for 4 weeks. The final fresh weight yield can be increased substantially by addition of 10% (v/v) orange juice to the basal medium. Increases in yield have been observed with juice vesicle explant cultures of citron (*C. medica*), lemon (*C. limon*), grapefruit (*C. paradisi*), and sweet orange (*C. sinensis*), and with albedo explant cultures of citron, lemon, mandarin (*C. reticulata*) cultivars. Figure 1 shows representative dose/reponse values for three *Citrus* species. The optimum concentration of approximately 10% (v/v) orange juice caused increases in yield as great as 6-fold compared to controls cultured on basal medium.

To determine whether the effect of orange juice was due to its

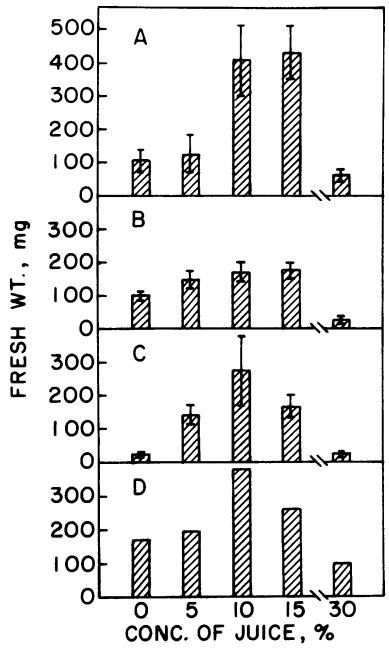

FIG. 1. Effect of concentration of orange juice added as a supplement to basal medium on yields of *Citrus* explant cultures. A: juice of citron; B: albedo tissue of citron; C: albedo tissue of lemon; D: albedo tissue of orange. Growth period was 28 days.

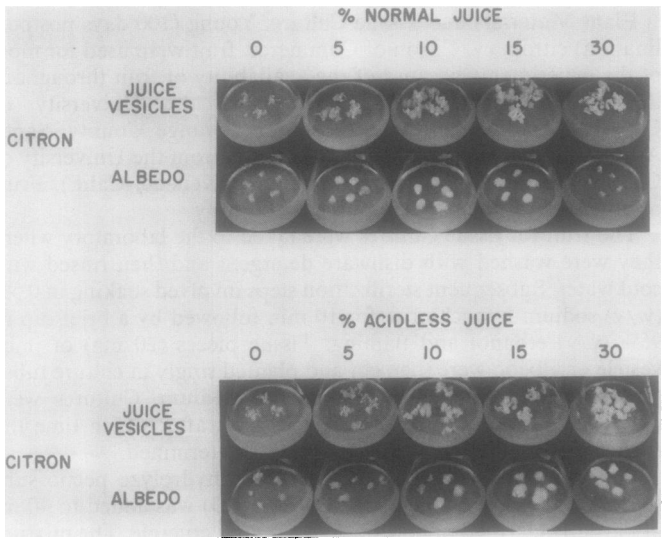

FIG. 2. Comparison of yields of citron juice vesicle and albedo explant cultures grown on media supplemented with Valencia or Lima (*i.e.* "acidless") variety orange juice. Culture flasks each contained 50 ml of medium; growth period was 28 days.

citric acid content (2), we added citric acid in concentrations equivalent to 0.5, 1, 5, and 10% (v/v) orange juice (*i.e.* 0.26, 0.52, 2.5, and 5.2 mM citric acid, respectively). Results of these experiments with citron juice vesicle and albedo explant cultures have been consistently negative. Citric acid was found to be without effect in repeated tests representing over 500 cultures suggesting that some other component(s) of orange juice was responsible for the stimulation of growth.

Juice from the Lima cultivar of orange, an "acidless" variety, contains approximately 1/100 the citric acid concentration of the juice from the commercial Valencia cultivar. Figure 2 shows the effects of the two juices in stimulating yields of citron juice vesicle and albedo cultures. It can be seen that juice from the "acidless" orange has definite stimulatory activity in the same concentration range as the normal juice which, again, suggests that some component(s) of the juices other than citric acid is involved.

Inorganic Constituents. The possibility that the enhanced growth of citron explant cultures is a result of inorganic constituents in orange juice was tested by comparing yields obtained on the basal medium with yields on media supplemented with 10% (v/v) orange juice or with the inorganic nutrients in ash corresponding to this same concentration of orange juice. The results of these experiments in Table I show no effect of the ash compared to stimulation by whole juice which indicates that inorganic constituents in orange juice do not cause the increased yields. The recommended single strength concentration of Murashige and Skoog salts is optimal for citron explant cultures (Fig. 3) as it is for recultured *Citrus* callus (6).

Organic Constituents. The effect of orange juice on yields of citron explant cultures in the presence of different sucrose concentrations was investigated to determine whether orange juice furnishes carbohydrate that is present in growth-limiting concentrations in the basal medium. It is known that 10% (v/v) orange juice supplementation would increase the sucrose and total sugar concentrations of the media by approximately 5 g/l and 10 g/l, respectively (1). As expected, orange juice partially restores growth of explant cultures on media lacking sucrose (Fig. 4). The yields of callus are still very low and the magnitude of the increase due to orange juice is much less than that obtained on the optimal sucrose concentrations indicating that sucrose contained in orange juice is not the reason for its activity.

Table II shows the effect of omission of minor organic constituents of the basal medium on yields of citron juice vesicle cultures. The lack of effect resulting from the omission of either *myo*-inositol (treatment c) or kinetin (treatment d) indicates that these growth factors are not essential. Removal of 2,4-D from the basal medium substantially decreases yield (treatment e) but addition of orange juice to these cultures does not significantly improve the yield (treatment f). This shows that orange juice supplies little if any, material substituting for the 2,4-D requirement. Omission of thiamine·HCl has the most dramatic effect on yield (treatment g). This suggests that the stimulation of growth of tissues on medium lacking growth factors but containing orange juice (treat-

Table I. Effects of ash and whole orange juice on yields of explant cultures derived from citron juice vesicle and albedo tissues. Values are means of 5 replicate flasks ± standard error. Growth period was 28 days.

Additions to Basal Medium	Yield (mg fresh wt)	
	Juice Vesicle	Albedo
None	450 ± 27	75 ± 4
Ash of 10% (v/v) Orange Juice	402 ± 35	78 ± 6
10% (v/v) Orange Juice	625 ± 80	142 ± 19

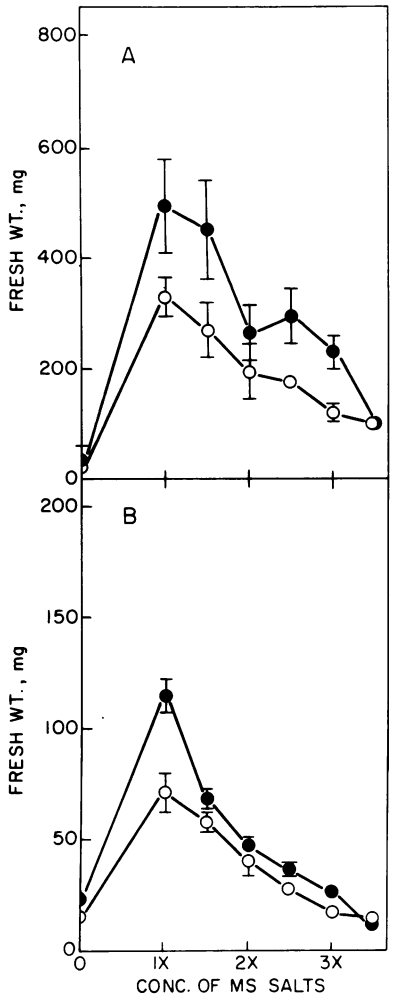

FIG. 3. Effect of increased inorganic nutrient concentrations on response of citron explant cultures to orange juice; 1 × MS salts are the concentrations of inorganics specified by Murashige and Skoog. A: juice vesicles of citron; B: albedo tissue of citron; (○): unsupplemented media; (●): supplemented with 10% (v/v) orange juice. Each point represents five replicate cultures. Growth period was 28 days.

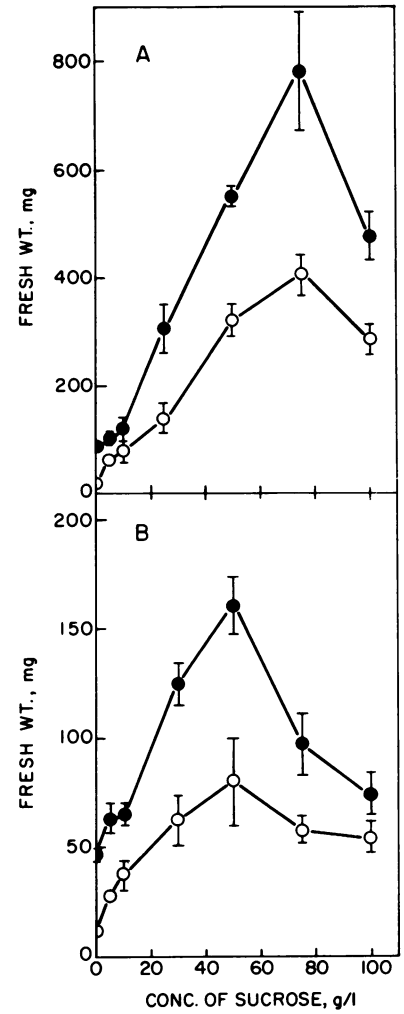

FIG. 4. Effect of sucrose concentration on response of citron explant cultures to orange juice. A: juice vesicles of citron; B: albedo tissue of citron; (○): unsupplemented media; (●): supplemented with 10% (v/v) orange juice. Each point represents five replicate cultures. Growth period was 28 days.

Table II. Yields of juice vesicle explant cultures in the absence of growth factors. The + indicates presence of a growth factor, - absence. Concentrations of growth factors are as indicated in the Materials and Methods. Orange juice was added to 10% (v/v). Values are means of 5 replicate flasks ± standard error. Growth period was 28 days.

Treatment	Growth Factors					Yield (mg)
	i-Inositol	Kinetin	2,4-D	Thiamine·HCl	Orange Juice	
a	+	+	+	+	+	721 ± 53
b	+	+	+	+	-	518 ± 34
c	-	+	+	+	-	532 ± 93
d	+	-	+	+	-	509 ± 73
e	+	+	-	+	-	300 ± 55
f	+	+	-	+	+	390 ± 61
g	+	+	+	-	-	147 ± 19
h	-	-	-	-	-	90 ± 6
i	-	-	-	-	+	348 ± 48

ment i *versus* treatment h) may be at least partially a result of the thiamine content of the juice (1). Enhanced growth of cultures on the basal medium supplemented with orange juice (treatment a *versus* treatment b) is not a consequence of thiamine, however, because the basal medium contains the optimum thiamine·HCl concentration (6).

DISCUSSION

The stimulation of *Citrus* tissue cultures by orange juice supplements has now been extended to explant cultures derived from a number of fruit types. Unlike the stimulation of recultured callus of orange albedo, the effect of orange juice on citron explant cultures cannot be attributed to citric acid based on the lack of effect of citric acid added to the basal medium and the demonstrated stimulation of growth caused by juice from the "acidless," variety of orange.

Explant cultures derived from juice vesicle tissues of citron fruit have shown the highest degree of growth stimulation in response to orange juice supplements. Our evidence with these cultures suggests that neither inorganic constituents nor the carbon source sucrose is the component of orange juice causing increased yields. It seems unlikely, also, that the orange juice furnishes stimulatory quantities of those organic growth factors already present in the basal medium.

Whether the active organic component(s) in orange juice is a known plant growth substance remains to be determined. Unanswered also is the question of the relationship, if any, of the *in vitro* response to potential factors regulating fruit growth and development in *Citrus*.

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