Differential Effects of Sucrose, Abscisic Acid, and Benzyladenine on Shoot Growth and Callus Formation in the Abscission Zone of Excised Citrus Buds

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Israel Giladi, Arie Altman, and Raphael Goren
Department of Horticulture, The Hebrew University of Jerusalem, Rehovot 76-100, Israel

ABSTRACT

The omission of sucrose from the basal medium stimulated callus formation in bud explants of Citrus sinensis (L.) Osbeck. Moreover, it increased the abscisic acid-induced callus proliferation reported earlier in the presence of 5% sucrose (Altman and Goren, Physiol. Plant. 32: 55, 1974). The inhibition of callus formation by the addition of sucrose was not due to the high osmotic potential of the medium. Benzyladenine induced callus formation slightly, in all sucrose concentrations up to 5%. The high level of sucrose was required, however, for the growth of shoots from buds cultured on both basal and benzyladenine-containing media.

The uptake of 14C-sucrose by bud explants was linear for at least 98 hr, and was enhanced significantly by both abscisic acid and benzyladene during the initial 24-hour period. Abscisic acid enhanced the absorption of 14C-sucrose and the accumulation of sugars in buds cultured at high levels of sucrose. More than 50% of the total label accumulated in the callus of abscisic acid-treated explants whereas only 16 and 23% were observed in the growing shoots of control and cytokinin-treated explants, respectively.

Results suggested that while sucrose "starvation" induced initial callus formation, high levels retarded further proliferation of the callus.

The growth of excised cells, tissues, and organs in vitro depends on the supply of various metabolites and growth factors in the nutrient medium. Although many recent studies have dealt with the effects of growth regulators on the development of cultures, very little is known about the control exerted by carbohydrates. It is widely accepted that cultures of explants require an exogenous source of energy and carbon skeleton (16) and that sucrose has been the most effective, although some exceptions to this rule exist (10, 16). Most nutrient media used for tissue cultures contain sucrose, usually at concentrations of 2 to 3.4% (20, 21). For the culture of citrus callus, however, Murishige and Tucker (12) recommended a concentration of 5%, which was applied in our previous studies with Citrus bud explants (1-3).

Up to a certain level, an increase in sucrose concentration in the nutrient medium is followed by increased growth in both callus and organ cultures (7, 16). Recently, it has been shown that carbohydrate deficiency can limit the growth of tobacco callus and that the maximum dry weight obtained depends on the amount of carbohydrate in the medium and not on a particular hormonal regime (18). Welender (19) reported that an increase in sucrose concentration in the medium stimulated root formation in explants of Beta vulgaris only in the presence of relatively high concentration of IAA and kinetin. Navarro et al. (13) studied the effect of sucrose concentration on the shoot growth of cultured lateral buds of Navel orange and found that 3% sucrose induced optimal shoot development, and that 5% already inhibited it.

Previous studies of Citrus bud cultures attributed two distinct morphogenetic phenomena to growth hormones: (a) the induction of callus formation by ABA in the abscission zone (1, 3); and (b) the development of new shoots in the presence of BA (2). Preliminary observations indicated that a low sucrose concentration promoted callus formation and inhibited shoot growth. The results of a detailed study on the effects of sucrose, alone and in combinations with ABA or BA, on callus formation and shoot growth are now reported.

MATERIALS AND METHODS

Plant Material and Culture Conditions. Bud explants were prepared, surface-sterilized, and cultured as described previously (1-3). They were removed from 1- to 3-year-old Shamouti orange (Citrus sinensis [L.] Osbeck) trees grown in containers in a lath-house. Explants were prepared aseptically and planted on a modified Murashige and Skoog medium (12), supplemented with highly purified sucrose (Mallickrodt, Analytical reagent) in order to avoid any side effects by contaminating agents. The concentrations used were specified in each experiment. ABA or BA was added to the medium in a concentration of 10 μM. Bud explants were grown in controlled growth chamber (2), and at the end of the experiment period total fresh weight as well as that of callus and new shoots, was determined; elongation of shoots was recorded by measuring the cumulative length of all of the shoots that developed on the explant.

14C-Sucrose Radioactivity Determination. Bud explants were planted on 1 ml basal medium containing various concentrations of unlabeled sucrose and U-14C-sucrose (600 mCi/mmol, Amer sham, England). Buds were cultured on this medium for various periods up to 96 hr. No significant amount of 14CO2 was released by the buds during the incubation period. At the end of the experiment, explants were lyophilized for 48 hr and oxidized in a Packard sample oxidizer (model 306). Radioactivity was determined by means of Carbo-Sorb II and Permafluor V (8:12, v/v) scintillation liquid in a Packard Tri-Carb liquid scintillation counter (model 3255) equipped with external standardization attachment. Results were corrected for background and quenching and are presented as dpm.

Sugar Analysis. Total sugars were analyzed according to Dubis et al. (6). Samples were ground by mortar and pestle in a solution composed of 4 ml double-distilled H2O, 2 ml of 5%
phenol, and 10 ml concentrated H$_2$SO$_4$. The color which developed during the incubation period was determined by means of a Zeiss PPQ II spectrophotometer at 490 nm. Data were calculated with a calibration curve for sucrose.

Experimental Design. Five individual bud explants were used for each treatment intended to determine $^{14}$C-sucrose uptake and 10 additional buds for the determination of total sugar content. Data on callus formation and shoot growth represented at least 10 separate cultures. Experiments were repeated two to three times.

RESULTS

Callus Formation and Shoot Growth. Previous investigations (1, 3) established that ABA specifically induced callus formation in buds grown on Murashige and Skoog medium containing 5% sucrose. There were, however, some indications that lower sucrose concentrations also promoted callus formation. The induction of callus by ABA in 5% sucrose was therefore compared with that by media containing various concentrations of sucrose, without ABA (Table I). The highest total fresh weight was obtained in the medium containing ABA; in medium containing neither sucrose nor ABA, a small increase over basal medium (5% sucrose only) was recorded (Table I).

Since the increase in fresh weight was due mainly to callus formation, the fresh weight of callus was determined separately. A clear-cut negative correlation between sucrose concentration and callus formation was observed in the absence of ABA. The site of callus formation—the abscission zone between the petiole and the branch (1)—remained unchanged and shoot growth was observed only in the intermediate sucrose concentrations.

The possibility of osmotic effects was examined by comparing callus growth in several concentrations of sucrose and manniitol, separately and in combinations. The addition of manniitol to a concentration equal to 10% sucrose (in terms of osmotic potential) did not prevent callus formation when sucrose was omitted from the medium; in the presence of sucrose, callus formation was markedly inhibited, regardless of manniitol (Table II). It therefore seemed that the suppression of callus induction and growth in the presence of sucrose was due not to a rise in osmotic potential of the medium, but to an inhibitory metabolic effect of sucrose.

In view of the above and keeping in mind that both ABA and BA have specific morphogenetic effects on bud development in culture (2), attention was focused on the effects of different levels of sucrose, separately and in combinations with ABA or BA. Results indicated (Table III) that in the presence of ABA, all of the sucrose concentrations tested caused callus formation in the abscission zone. The incidence of callus formation in the absence of ABA was 100% only when sucrose was omitted. About half of the buds formed callus in the presence of BA, regardless of sucrose supply. The effect of sucrose concentration on callus formation was more evident when fresh weight of the callus, i.e. its over-all growth in 21 days, was considered. Sucrose definitely inhibited ABA-induced callus formation, but the inhibition was not evident when bud explants were grown on a medium supplemented with BA. Again, callus formation in the absence of ABA was highest when no sucrose was added, although vigorous growth resulted in few of the callus-forming buds of 5% sucrose cultures (compare with Table I). Development of new shoots on bud explants was greatest in a medium of BA and 5% sucrose, but not BA alone. The intermediate value of sucrose (2.5%) yielded a lower incidence of bud emergence and slight retardation of shoot elongation. Growth of shoots was evident in some of the buds, while 10 $\mu$M ABA totally inhibited shoot growth (Table III).

Sucrose Uptake. Although it is assumed that sucrose content of tissues reflects sucrose concentration in the medium, the best of our knowledge documenting data are not yet available. It was therefore of interest to know whether the specific effects of sucrose and of ABA or BA on callus formation and shoot growth were related to differences in absorbed sucrose. $^{14}$C-Sucrose

<table>
<thead>
<tr>
<th>Hormones</th>
<th>None</th>
<th>ABA, 10 $\mu$M</th>
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<tbody>
<tr>
<td>Sucrose conc.</td>
<td>0.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Total fr wt of explant, mg</td>
<td>39.3±3.4</td>
<td>33.6±3.4</td>
</tr>
<tr>
<td>Callus fr wt, mg/bud</td>
<td>7.1±1.2</td>
<td>3.1±1.3</td>
</tr>
<tr>
<td>Callus fr wt, relative to ABA</td>
<td>47.7±2.4</td>
<td>20.8±2.4</td>
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<tr>
<td>Total elongation of shoots, mm/bud</td>
<td>0</td>
<td>1.9±1.3</td>
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Table II. Comparison of Sucrose and Mannitol on Callus Formation in Bud Explants

Data recorded after 25 days in culture. Sucrose and/or manniitol were included in the basal medium. 2.63% w/v mannitol + 5% w/v sucrose in terms of osmotic potential. Values within a column are not significantly different when followed by the same letter (at the level of p=0.01)

<table>
<thead>
<tr>
<th>Sucrose</th>
<th>Mannitol</th>
<th>Buds forming callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu$M</td>
<td>$\mu$M</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
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<td>100 a</td>
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<tr>
<td>5</td>
<td>0</td>
<td>100 a</td>
</tr>
<tr>
<td>5</td>
<td>2.63</td>
<td>10-20 b</td>
</tr>
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</table>

Table III. Combined Effects of Hormones and Sucrose Concentration on Callus Formation and Growth in Bud Explants

Data recorded after 21 days in culture; ± = standard error. Values within a line are not significantly different when followed by the same letter (at the level of p = 0.05)
uptake was continuous and linear for at least 98 hr, and at essentially a uniform rate (Fig. 1). No significant changes in the uptake, whether expressed on a unit bud basis or dry weight basis, were evident during the culture period. The uptake of 14C-sucrose during a 24-hr incubation period in media of various specific radioactivities was altered by the addition of increasing amounts of 14C-sucrose at constant concentration (5%) of unlabeled sucrose, expressed in the positive linear regression $y = 1.303x$ (y = dpm/dry matter; x = dpm/tube). Uptake of 14C-sucrose in the initial 24-hr culture period of buds was enhanced significantly by both ABA and BA (Fig. 2). Nevertheless, whereas ABA enhanced sucrose uptake only in sucrose concentrations above 2.5%, BA stimulated the absorption in all sucrose levels. ABA also altered the endogenous sugar content of buds grown at various sucrose concentrations (Fig. 3). A decrease of 50 to 60% in endogenous sucrose was evident in buds of sucrose-less medium, with or without ABA (10.14 ± 0.53 mg/g fresh weight was detected at zero time). In buds cultured at higher levels of sucrose (5 and 15%), the amounts of accumulated sugar were considerably higher. The content of sugar in buds cultured on 2.5% sucrose, however, was greater for basal medium than for medium supplemented with ABA.

The data indicated that ABA and BA may affect both types of growth (callus or shoots) and sucrose uptake. A study of the effect of both hormonal substances on the distribution of 14C-sucrose in various zones of bud explants (Table IV) revealed that total radioactivity was highest in buds cultured with ABA and lowest in those of BA. However, more than 50% of total label was accumulated in the callus of buds cultured with ABA, while the shoots grown on basal and BA media accumulated only 16% and 23% of total label, respectively. The greater accumulation of radioactivity in shoots of buds cultured with BA was related to the more vigorous growth of these shoots. The data indicated the existence of a preferential accumulation of 14C-sucrose in actively growing zones of bud explants.

**DISCUSSION**

This present study dealt with two distinct effects of sucrose concentration in the medium on excised citrus buds: the stimulation of shoot growth by increasing concentrations of sucrose, and the induction of callus by omission of sucrose from the medium (Tables I and III).

The promotive effects of higher sucrose levels on lateral bud growth are well documented, especially when conditions *in vitro* are inadequate for significant photosynthesis (13). The observations that these effects on shoot growth are more evident in the presence of BA (Table III) were expected, since cytokinins are known to enhance the growth of lateral buds, both in intact plants (14) and *in vitro* (2, 13). The mode of action of cytokinins in such systems may be due both to the induction of numerous adventitious shoots in bud (2) and leaf explants (17), and to the release of existing lateral buds from apical dominance by pro-
producing a greater accumulation of nutrients in the developing bud (14, 15). The latter suggestion is supported by the findings that BA enhances both the uptake of H\textsuperscript{14}C-sucrose by explants (Fig. 2) and its accumulation in the growing shoots (Table IV).

The induction of callus in cultured explants by omitting sucrose from the basal medium has not been reported previously. It is noteworthy that another morphogenetic phenomenon, i.e., asexual embryo formation in habituated callus of Citrus sinensis (L.), is also stimulated by sucrose “starvation” (8). Callus induction and proliferation have usually been attributed to wounding and to auxin and cytokinins (20, 21). In Citrus bud explants, callus formation is specifically induced by ABA, and it has been proposed that this occurs in two stages: (a) activation of certain cells in the abscission zone by ABA; and (b) enhancement of subsequent proliferation of callus by GA\textsubscript{3} (3).

It is suggested that sucrose “starvation” may enhance the accumulation of endogenous ABA to levels that are critical for the induction of callus. Stress conditions have been shown to produce a rise in endogenous ABA (11) and ethylene (5) which may also be involved in callus formation in the present system (ref. 9, and Goren and Altman, unpublished data).

The proliferation of already formed callus may be inhibited by high levels of sucrose in the medium. Indeed, sucrose concentrations above 2.5% repress proliferation of callus of various plants (7). Low concentrations of sucrose favor the initiation of numerous shoots in tobacco callus and depress the growth of callus (4). The combined effect of ABA and sucrose “starvation” in producing the greatest proliferation of callus (Table III) supports this hypothesis. The role of endogenous ABA in this system is now under study.

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