Endogenous Growth Substances of Citrus Tissues[1]

Eliezer E. Goldschmidt[2]
Department of Horticulture, The Hebrew University of Jerusalem, Rehovot, Israel

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Studies of growth and development in plants have been imbued with attempts to characterize and identify the endogenous growth substances of plant tissues and to relate changes in their quantities to processes in which they are assumed to participate. Studies of endogenous growth substances can be regarded from the horticultural point of view as complementary to those based upon exogenously supplied growth regulators, leading to better understanding of the developmental processes involved and, eventually, facilitating the practical control of horticultural production.

The unified biochemical basis which is believed to be common to higher plant systems does not, apparently, leave space for distinct hormonal physiology of *Citrus* or any other botanical genus. Nevertheless, the morphological and development differences between plant species might be associated with quantitative or even qualitative changes in endogenous growth substances. Citrus fruits, for instance, with their unique hesperidium structure, might perhaps exhibit special hormonal features which should be elucidated. Hypotheses on hormonal factors specific to *Citrus* tissues have indeed been proposed, as we shall see in the following.

We got involved in characterization and quantitative estimation of growth substances from citrus organs in the course of our studies on growth and development[3]. The present paper tries to summarize and evaluate this work which is scattered and partly still unpublished, along with related contributions from the literature and to critically review our present understanding of the endogenous hormonal balance of citrus tissues in relation to processes of growth and development.

Auxins

The isolation and identification of native plant growth substances has always been fraught with difficulties due to the low concentrations of these compounds in plant material, generally below 1 μg/g fresh weight. These difficulties have led most researchers to avoid the tedious job of purification and to rely largely on bioassays which respond to relatively crude extracts. Qualitative characterization has often been based upon the R_f of the biological activity in one-dimensional paper chromatography and the intensity of the biological response served as a basis for quantitative estimates.

It is generally accepted by plant physiologists that native auxins are indole compounds, particularly β–indolylacetic acid (IAA) which has been identified with certainty in several plant tissues (51). Khalifah, Lewis & Coggins reported nevertheless in 1963 that the biologically active auxin of citrus fruits was neither IAA nor an indole but rather a novel compound which was tentatively designated "citrus auxin" (35). Evidence for the nature of "citrus auxin" consisted primarily of spectrofluorometric spectra determinations and was supported by discrepancies between citrus auxin and IAA in chromatographic behavior and chromogenic sprays (35, 37, 38). The same kind of evidence indicated that native auxins of several plant species unrelated to citrus also had properties of the non-indolic citrus auxin (39). Determinations of the relative fluorescent intensity, assumed to be correlatable with citrus auxin, showed a rapid decrease in auxin concentration during the early stages of fruit development and a further, moderate decrease on a dry weight basis during later stages of fruit growth (38).

The "citrus auxin" hypothesis did not gain further support during the late sixties. The fluorescent material was found to be scopoletin, which is not active in the *Avena* curvature auxin bioassay (c.f. 16) and the biologically active substance has not been identified chemically. The claim for the existence of a specific, non-indolic "citrus auxin" remains therefore unproven.

Physiological and chemical evidence accumulated during the last decade points, on the other hand, to the presence of the ubiquitous indolic auxin IAA in citrus tissues.

Vigorously growing lemon and orange shoots contained a single auxin component which was partitioned and co-chromatographed with labeled IAA and migrated to the same R_f as synthetic IAA in 8 solvent systems in paper chromatography (22). A relatively pure, highly active extract was obtained by employing centrifugal force in the basipetal direction on shoot sections submerged in 20% methanol (20). Extracts obtained by centrifugation which are comparable with diffusates, were almost devoid of growth inhibitors which often mask the biological activity. Difficulties in obtaining the chromogenic reactions typical to IAA were also overcome by using up to 100 g fresh material per sample and employing two dimensional thin layer chromatography (16).

Large amounts of auxin were found also in flower organs (17, 44, Goldschmidt, unpublished data) and particularly in petals which show 2 distinct biologically active R_f zones, appearing, in both extracts and diffusates, one of which corresponds to IAA (9). Excised petals respond to IAA and native auxins by increased curvature of the type evident in opening of flowers and this response has been proposed as an auxin bioassay based on citrus tissues (18).

The situation in fruits is apparently far more complex and might be related to the complex structure of citrus fruit. High auxin activity can be found in ovaries and fruitlets during anthesis and fruit set (7, 24, 28, 30, 44), ovaries of seedless cultivars containing more auxin than seeded ones (28), but this activity declines rapidly thereafter, making it difficult to demonstrate auxin activity in crude extracts of fruits, probably due to masking by growth inhibitors (24, 30, 44). Recent studies based on bioassay of purified extracts from *Citrus unshiu* showed a sharp peak in auxin activity 10 days after full bloom followed by a rapid decrease approaching zero levels at 40 days after full bloom (53; Fig. 1). Sol-

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[3]Work on ethylene has not been included in the present article.

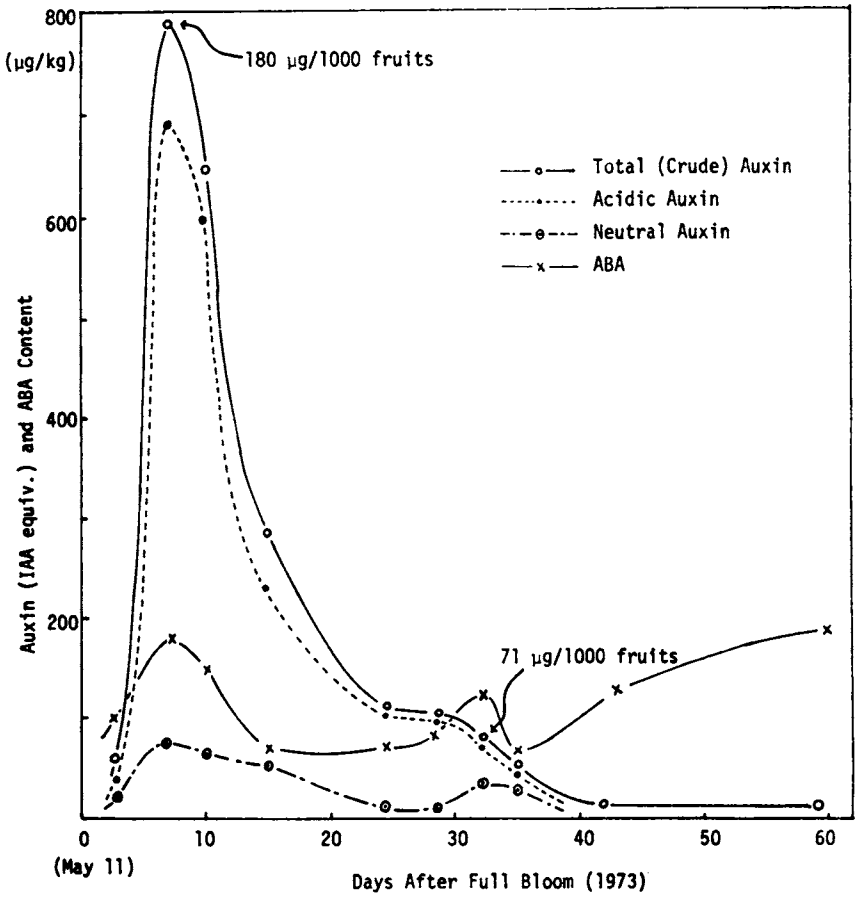

Fig. 1. Kinetics of acidic, neutral and total auxin activities and ABA contents in developing *Citrus unshiu* fruits. Auxins were estimated by the Avena curvature bioassay and ABA determined according to Milborrow (40). Figure kindly provided by Prof. N. Takahashi (c.f. 53).

vent partition and chromatography of extracts from fruit at 3 developmental stages and from various fruit tissues, flavedo, albedo and segments, yielded several prominent zones of growth promotion that did not generally coincide with authentic IAA (24). Nevertheless, Khalifah, who belonged originally to the "citrus auxin" team, showed that citrus fruitlets incorporated ^{14}C-tryptophan into IAA (33).

Auxins of "very young" *C. unshiu* fruitlets have finally been identified by Igoshi et al. (30) using combined gas chromatography – mass spectrometry as IAA (0.65 µg/g) and indoleacetamide (5.3 µg/g). These compounds could not be found in fruits harvested 2 months after full bloom. A neutral auxin component which appeared in young fruitlets has recently been identified by Takahashi et al. (53) as methyl indole-3-acetate.

It seems reasonable to believe – and responses to synthetic auxins support this view (2, 43) – that auxins play a role also in the more advanced stages of fruit growth and maturation. However, the fate of endogenous auxins during these later stages of fruit development remains obscure for the time being and awaits further research.

Table 1 contains quantitative estimates of native auxin contents in several citrus tissues.

Gibberellins

The isolation and identification of GA_1 from water sprouts of *C. unshiu* by Kawarada & Sumiki in 1959 (31) was among the first identifications of gibberellins in tissues of higher plants. However, this remained the only orderly chemical identification of gibberellins from citrus and all subsequent studies relied greatly on R_f values and biological activity in various bioassay systems.

Young orange and lemon fruits were reported by Khalifah et al. (36) to contain 3 gibberellin-like substances, 2 of which were tentatively identified as GA_1 and GA_9. The presence of at least 3 gibberellin-like compounds has been described recently also in the flavedo of maturing orange fruits (13). Petals and stamens also show at least 3 biologically active R_f zones (Goldschmidt, unpublished data). Vegetative and generative shoots always contained only a single active R_f zone which behaved chromatographically like GA_1 (21, Goldschmidt, unpublished data).

The existence of polar gibberellin-like activity which did not partition from the aqueous phase into acid ethylacetate but was transferable into butanol has been recognized on several occasions in citrus, mainly in woody twigs, bark and roots (25, 56, Avrech, Goldschmidt & Goren, unpublished data). Both extracts and diffusates from actively growing rootlets were found to contain relatively high gibberellin-like activity which was more or less equally divided between the acid diethylether and butanol fractions (Avrech, Goldschmidt & Goren, unpublished data). These findings might indicate the presence of a translocatable form of gibberellin, perhaps of the bound, glucoside type (52). Further work of isolation and identification is needed at this point.

The concentrations of gibberellin-like substances in stems were correlated with morphological appearance of vegetative (long), mixed-type (medium) and generative (short) shoots (21). Seasonal tests for gibberellin-like activity in bark and woody twigs, whose gibberellin probably originated in root or shoot meristems, showed a large increase towards spring (56). Young shoots and rootlets evidenced abrupt fluctuations which were probably related to alternating growth activities of shoots and roots (56). Ringing resulted in elevated gibberellin-like activity in new lateral shoots, in leaves and in bark above the ring, but ringing, on the other hand, seemed to reduce the levels of gibberellin-like substances in rootlets and in developing fruitlets (25, 56). These apparently conflicting trends have not as yet received an adequate explanation.

Table 1. Estimates of growth substance concentrations in several citrus tissues.

Growth substances	Vegetative shoot tips	Petals	Young fruitlets	Flavedo of mature fruit
Auxins (mg equiv. of IAA/kg fresh wt)	0.25(20)[z]	0.50(9)	0.80(53)	0.05(24)
Gibberellins (mg equiv. of GA_3/kg fresh wt)	4.80(21,42)	0.18(x)	5.00(5)	0.50(5,13)
Cytokinins (mg equiv. of kinetin/kg fresh wt)	–	3.00(x)	4.00(5)	0.30(5)
ABA[y] (mg ABA/kg fresh wt)	–	–	0.10(5,53)	2.50(14)

[z]No. in parentheses indicate references.

[y]Total free and bound ABA.

[x]Goldschmidt, unpublished data.

Gibberellin-like activity has been demonstrated in various phases of fruit development (13, 24, 36, 57). We nevertheless still lack an orderly seasonal follow up. Spectrofluorimetric determinations of GA_1 and GA_3 were performed by Wiltbank & Krezdorn (57) during the initial stages of fruit development. The rate of fruit growth proved to be correlated with concentration of gibberellins during the cell division period. Total gibberellins per fruit increased during the cell enlargement stage concomitantly, or slightly preceded the increase in fruit weight or volume. These findings were interpreted as suggesting a "cause and effect" relationship between certain gibberellins and fruit growth.

Erner, Goren and Monselise (5) found that both flavedo and albedo of rough Shamouti orange fruit contained much more gibberellin-like substances than the corresponding tissues of smooth-peeled fruit. Flavedo always showed higher gibberellin-like activity than albedo. Fruits approaching maturation had less gibberellin-like activity than younger fruit according to Erner et et al. (5). Goldschmidt & Galily (13) found that all gibberellin-like components decreased rapidly in flavedo of mature-green fruits upon treatment with ethylene. Gibberellin-like substances were found to build up prior to and during the regreening of Valencia oranges (49). Thus changes in gibberellin-like substances associated with maturation corroborate the well known effects of exogenous gibberellins which delay peel senescence and induce regreening (2, 3, 10, 11, 43).

Cytokinins

The exact nature of citrus cytokinins has never been determined and published data are relatively scarce. Khalifah & Lewis detected cytokinin activity in lemon seed extract in their pioneer work (34). One- and 2-year-old lemon leaves contained higher cytokinin activity than younger leaves (Ilan & Goren, unpublished data), as found recently by Engelbrecht for other plant species (4). Cytokinins were found in all flower organs, but petals were found to be particularly rich in cytokinins (Goldschmidt, unpublished data), although the expansion of petals is generally ascribed to cell enlargement rather than to cell division.

Determinations of cytokinins in rough and smooth fruit showed trends similar to those found with gibberellins, rough containing more than smooth-peeled and flavedo more than albedo (5). Again, fruits approaching maturation had relatively low activity (5). It seems reasonable to assume that cytokinins play an important role in root-canopy relationships, thereby affecting senescence processes and fruit color changes (3, 21). This is another area which calls for further research.

ABA and other growth inhibitors

The abundance of growth inhibitors in citrus tissues and particularly in citrus fruits became evident already during the preliminary stages of our work on endogenous growth substances (44). Large amounts of growth inhibitors might mask the presence of growth promotors or at least interfere with their quantitative estimation, unless careful partitions and chromatographic separations are made. Collection of growth substances by diffusion (or centrifugation) is an alternative method yielding extracts which are practically almost devoid of inhibitors (9, 20).

In citrus, as in other plant material, most attention has been given to the "β-inhibitor" zone whose inhibitory activity has been attributed to phenolic compounds (55). Abscisic acid (ABA) was detected in lemon juice by the optical rotatory dispersion technique soon after its identification as the principal component of the β-inhibitor (40). More recently ABA has been identified in orange flavedo using gas chromatography-mass spectrometry (14). However, it has become gradually evident that citrus tissues, like other plant tissues, also contain large amounts of neutral, non polar inhibitors (14), which resemble the xanthoxin isolated by Taylor and coworkers (8).

It seems now that most of the growth inhibition found in extracts of shoots (19), developing fruit (24, 44) and leaves (Avidan & Goldschmidt, unpublished data) is caused by such neutral inhibitors and not by ABA. ABA accumulates in the course of natural or ethylene-induced maturation while the neutral inhibitor content remains unchanged (14). An upsurge in ABA occurs also in drought-stressed leaves and the neutral inhibitors do not change in this case as well (Fig. 2).

Young fruitlets show a peak in ABA concentration during the first few days after anthesis (53, Button, Addicott and Murphy, unpublished data; Fig. 1). Fruits seem to maintain a stable, relatively low level of ABA subsequently but levels rise slowly when the fruit approach maturation. A decrease in ABA-like growth inhibitors has been found in regreening Valencia fruits (49). Benzyladenine which delays peel color change also reduces the accmulation of ABA (1, 12).

ABA concentrations in mature fruit peel (Table 1) are among the highest reported in the literature. Citrus tissues appear to be unique in the high proportion of "bound ABA," presumably the ABA-glucose ester, which attains 4–10 fold higher levels than free ABA in

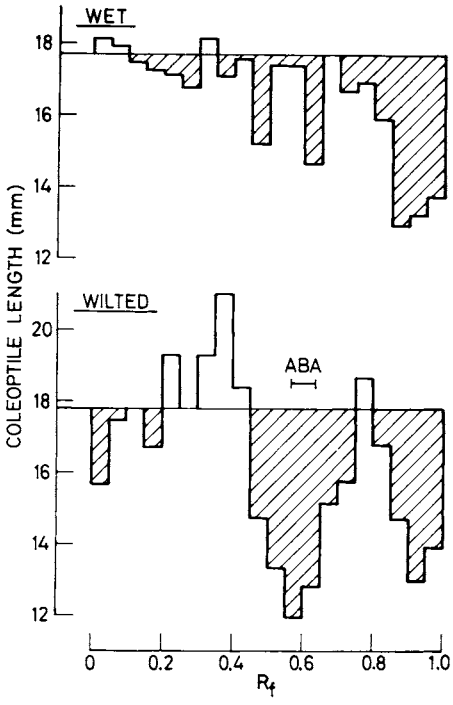

Fig. 2. Growth inhibitor activity of paper chromatographed 80% methanol extracts from wet and wilted Shamouti orange leaves. Note the separation of ABA-like inhibitors (R_f 0.45–0.75) from neutral inhibitors (R_f 0.8–1.0); the latter remaining unaffected by the wilt. (A. Avidan and E. E. Goldschmidt; unpublished).

senescent fruit peel (14). The kinetics of ABA accumulation in ethylene-treated fruit (1, 14) and in water-stressed leaves (Avidan & Goldschmidt, unpublished data) indicates that free ABA starts increasing up to a certain level, beyond which it is transformed into the apparently inactive bound ABA.

Kefeli & Kadyrov have noticed that pigmented plant tissues usually contain more growth inhibitors than pigmentless tissues (32). In accordance with this view are the findings that the pigmented flavedo has more inhibitors than the albedo (12, 24, 50) and that the pigmentless petals have low inhibitor contents (9). However, recent work indicates that the albedo contains about the same amount of ABA as the flavedo and accumulates similar amounts of ABA in response to ethylene (1). The higher content of inhibitors of the flavedo must therefore be explained through the presence of higher levels of neutral inhibitors. Experiments with variegated lemon fruit also showed that white and green flavedo tissue contained comparable levels of ABA and were equally responsive to ethylene (1). Thus, our data do not support the concept of a close relationship between ABA and pigmentation in citrus fruit. Chloroplast and chromoplast pellets were indeed shown not to contain the inhibitor moiety (14). However, ABA

might still be produced inside plastids, as suggested by Milborrow (41), and secreted afterwards into the cytoplasm.

Little can be said about the inhibitors described by Lewis et al. (38) in developing 'Washington' navel orange. Their inhibitor I which accumulates towards maturation might be related to ABA whereas their inhibitor II which decreases slowly throughout the growth period resembles the neutral inhibitors. The inhibitor reported by Rasmussen (49) to accmulate during regreening might also be related to the neutral inhibitors which appear to attain high levels in actively growing tissues.

Other potential growth factors

Citrus tissues contain numerous substances which might play regulatory roles in growth processes and hormonal significance has been assigned to many of them at one time or another. Phenolic compounds have often been mentioned in this context since many of them are active in bioassays, acting either directly or indirectly as cofactors of the IAA oxidase system. An IAA oxidase system with native dialyzable cofactors and inhibitors has been found in citrus seedlings (15). The flavanone glucosides, hesperidin and narigenin, make up to 30% of the dry weight of young 'Shamouti' orange (23) and 'Marsh' grapefruit (29) fruitlets but their relation to fruit growth remains completely unclear at the present time. The pyranocoumarins, seselin and xanthyletin, have been isolated from citrus roots (26, 54) and since seselin is a potent inhibitor of root growth in several plant species it has been proposed to have a similar role in citrus roots (27).

Murashige & Tucker found that subcultures of citrus albedo explants from species other than *C. limon* were dependent for their growth on the supply of orange juice to the medium (46). The requirement for orange juice could not be replaced by any of the known classes of growth regulators and it has been suggested that orange juice contains a special unknown growth factor (46). Nitsch assumed nevertheless that some native cytokinin was involved (48). A recent study has shown, however, that most of the growth promotive activity of orange juice can be attributed to citric acid which is, of course, a well known component of citrus juice (6). Synthetic citric acid will replace most of the requirement for juice (6; Fig. 3).

Perspective

Have the studies of endogenous growth substances in citrus lived up to their expectations? The same question should, of course, be raised also with regard to studies of endogenous plant growth substances in general.

On one hand, it is clear that our knowledge of endogenous growth substances in citrus tissues is still fragmentary and inaccurate, being based mainly on bioassays, in the absence of physical and chemical data. On the other hand, we are nowadays aware that even a perfect knowledge of a tissue's hormonal contents at a certain moment, cannot in itself tell us much about the role of the hormones in the system, unless a complete picture of the hormone's turnover can be obtained. High concentrations of a growth substance in a tissue is often taken as evidence that the substance plays an important regulatory role in that tissue, but low concentrations can still be associated with high rates of both synthesis and consumption, as is known, in fact, for many important metabolites which never pile up in the tissue. Hence, even curves of seasonal changes in growth substances are sometimes open to more than a single interpretation.

Convincing evidence for the physiological importance of an endogenous growth substance has, in fact, been obtained in some cases through sophisticated use of exogenous growth regulators, even without measurements of endogenous hormonal contents. For example, the role of gibberellins as native inhibitors of flowering in citrus has been demonstrated through careful treatments with growth retardants and gibberellins (21, 42, 45, 57).

And yet, data for endogenous growth substances of citrus in various areas corroborate the general physiological views and are in good agreement with morphological observations and growth regulator experiments. Such a comprehensive view has been achieved for the development of vegetative and generative shoots whose gibberellin-like substances and auxin contents correlate with their elongation (21). A meaningful picture emerges also for maturing citrus fruit peel. Natural as well as ethylene-induced maturation involve an upsurge in ABA concomitant with a decrease in gibberellin-like substances (1, 10, 12-14). Data for changes in auxins and cytokinins in this system are still missing, however.

The hormonal control of fruit set and development continues to be a central, greatly unresolved problem. The young fruitlet is an extremely active meristem containing large amounts of auxins, gibberellins, cytokinins, ABA and other growth inhibitors all of which act in concert to control fruit development. What happens after the first few weeks, when the concentrations of auxin in the tissue diminish? Is the growth of flavedo, albedo and juice sacs, which vary in proportion from one cultivar to another, dependent upon the same kind of hormonal balance? These are some of the questions which await further investigation. Another area which invites further study is the hormonal interrelationship of roots and tops which appears to be of uttermost importance in trees (3, 42). The problems in obtaining sap from citrus trees cause extra difficulties which do not exist in grapes and apples. The studies conducted so far on the hormonal balance of ringed orange trees (25, 56) emphasize the need for a more sophisticated approach to the investigation of the hormonal interactions between citrus tree organs.

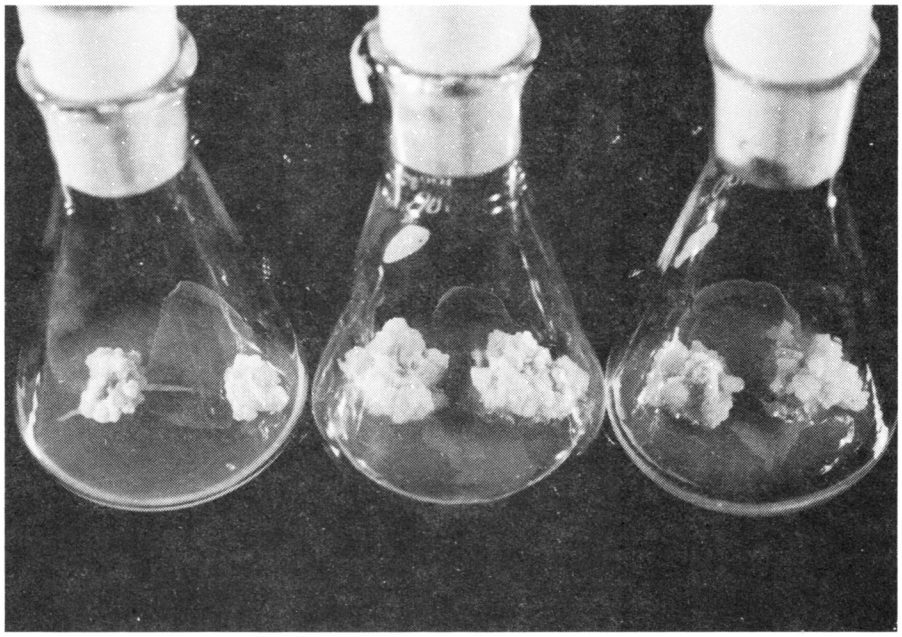

Fig. 3. Effects of citric acid and orange juice on growth of orange albedo explant tissue culture. From left to right: Control, citric acid (2,000 mg/liter) and orange juice (100 ml/liter (Y. Erner, O. Reuveni and E. E. Goldschmidt; unpublished).

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