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Distribution of ^{14}C Photosynthetic Assimilates in 'Valencia' Orange Seedlings at 10° and 25°C[1,2]

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Abstact. **The distribution of ^{14}C-photosynthate was determined in 8-month-old potted 'Valencia' orange seedlings [*Citrus sinensis* (L.) Osbeck] during 25° and 10°C temperature regimes. Seedlings were pulse-labeled with $^{14}CO_2$ for 3 hr after equilibration with ambient air. Radioactive assimilates were extracted at selected intervals, from leaves, stems, and roots and separated into several biochemical fractions. Low-temperature (10°) exposure resulted in a greater retention of ^{14}C in the sugar fraction of leaves and a lower rate of ^{14}C incorporation into organic and amino acid fractions. Data indicate a lower rate of metabolism of photosynthate and a reduced distribution of ^{14}C in citrus seedlings at 10° than at 25°C.**

Photosynthesis is implicated in the low-temperature cold hardening of many plants (2, 6, 8, 12, 15, 22, 26). Low temperatures altered photosynthate metabolism in *Hedera helix* L., but there was no marked accumulation of ^{14}C-photosynthate in any one metabolic fraction that might be responsible for increased cold hardiness (20). Other studies on carbon photosynthetic metabolism indicate increases in ^{14}C incorporation into glycine and serine during cold acclimation of wheat (18), whereas ^{14}C increases were found in alanine, proline, and C_4-dicarboxylic acids in rape plants (19). The distribution and accumulation of ^{14}C-photosynthates have been studied in cold-sensitive citrus selections but not during low-temperature cold hardening (9, 10, 11, 14). We report data here on the incorporation of ^{14}C during $^{14}CO_2$-uptake studies in continued work on citrus cold hardiness.

Materials and Methods

Plants. Uniform 8-month-old 'Valencia' orange seedlings [*Citrus sinensis* (L.) Osb.] were selected for tests. Seedlings were from open-pollinated seed from the fruit of a selected 12-year-old tree in a citrus planting. Seedlings were grown in a mixture of 1 part sand:2 parts vermiculite:4 parts sphagnum peat moss in 13 × 13-cm plastic pots in a greenhouse under natural daylight; light conditions reached 875 $\mu Em^{-2}sec^{-1}$ [photosynthetically active radiation (PAR)] with 33°C and 40% relative humidity, and no PAR, 20° and 98% relative humidity during nights.

Cold hardening and freeze tests. Seedlings were cold hardened and freeze tested in controlled-environment facilities described previously (23). Separate rooms were used for each of 2 temperature regimes; a constant nonhardening 25°C with 12-hour abrupt light and dark periods, and a constant cold hardening 10° with the same light/dark scheme. Relative humidity was maintained in both instances at 50 ± 5%, and light (86% Cool White fluorescent and 14% incandescent) approximated 350 $\mu Em^{-2}sec^{-1}$ (PAR) at the top of the plants. A total of 90 seedlings were maintained in each temperature regime for 4 continuous weeks.

Cold hardening was tested in a separate freeze room, no PAR, and 50 ± 5% relative humidity. Freeze tests started with 2 hr at 4.4°C, continued with 1.1°/hr decrease to –6.7° for 4 hr, and ended with a return to 4.4° at 1.1°/hr. Seedlings were kept at 25° for 3 hr after freeze tests, and then returned to the greenhouse for 4 weeks for observation of damage. Trees were rated for percentage of leaf and stem survival. Percentage survival data were normalized by arcsin transformation for statistical analyses by the multiple *t* test.

$^{14}CO_2$ exposure. Twelve seedlings per temperature treatment were randomly selected for ^{14}C-labeling 3 hr after temperature regimes were started. Pots were sealed to prevent absorption of $^{14}CO_2$ into the soil, and plants were exposed to $^{14}CO_2$ released from the reaction of 1 ml of 1 M HCl into 50 μCi of premoistened $^{14}CO_2$-barium carbonate (specific activity of 55.7 mCi/mM). Plants were exposed to $^{14}CO_2$ for 3 hr in 104-liter radiation containment chambers constructed of 5-mm-thick clear Plexiglas. Temperatures inside the containment chambers during exposure to $^{14}CO_2$ were within 0.5°C of ambient cold-hardening room temperatures and within 5% of relative humidity. Light conditions were about 275 $\mu Em^{-2}sec^{-1}$ (PAR). Continuous air movement, less than 0.3 m/min, was provided by a small fan inside the chambers. Excess $^{14}CO_2$ after 3 hr was evacuated from chambers into KOH-CO_2 traps with a pressure vacuum system capable of exhausting 20 liters of air/min from the chamber, or a turnover in atmosphere every 5 min. ^{14}C-labeled seedlings, 3 at a time, were randomly selected at 0, 7, 14, and 28 days for tissue analyses. Leaf, stem, and roots were fixed in boiling 76% (v/v) ethanol and stored at −8°C until analyszed. Seedlings not exposed to $^{14}CO_2$ were freeze tested at respective weekly intervals in conjunction with tissued sampling from ^{14}C-plants.

Chemical extraction. Tissue samples were extracted for 6 hr in a soxhlet apparatus after homogenizing with 76% ethanol in a blender. The ethanol extract was evaporated under vacuum at 35°C to dryness, and the ethanol-soluble residue was taken up in glass-distilled H_2O and partitioned 4 times with petroleum ether. Amino acids, organic acids, phosphorylated compounds, and sugars of the aqueous phase were separated by ion exchange resins (3, 21). The purified fractions were evaporated to dryness and then taken up in a known volume of 76% ethanol for storage at −8°C.

The ethanol-insoluble residue was extracted with 20 ml petroleum ether to remove residual lipids and combined with the petro-

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leum ether extract described above to yield a crude lipid fraction. The residues of ethanol and petroleum ether extractions were divided in half for starch and protein extraction. Starch was extracted by boiling the residue in water for 15 min, then hydrolyzing with amyloglucosidase (13). The resultant glucose was then purified by the method of Williams et al. (21).

Proteins were hydrolyzed with 6N HCl at 105°C for 24 hr. The resultant amino acids were purified with Dowex-50 ion-exchange resin after they were neutralized with NaOH and clarified with charcoal. The purified amino acids were evaporated to dryness, taken up in 76% ethanol, and stored at −8°C.

Radioassay. A 200-µl portion of each fraction was burnt in a Packard Tri-Carb sample oxidizer model 306. Absolute radioactivity was determined with a Packard Tri-Carb scintillation counter model 3320.

Autoradiography. In another experiment, incorporation of ^{14}C into specific compounds was examined at 3 hr and at 7, 14, and 28 days of preconditioning. Plants were kept for 1 hr in the presence of 50 µCi of $^{14}CO_2$ inside a 3-liter beaker covered with Saran wrap film. After a 24-hr chase period, leaf tissue was homogenized and extracted with boiling 76% ethanol. The extract was concentrated and chromatographed on Whatman 3-MM paper (1). Radioactive compounds were located by autoradiography using Kodak no-screen X-ray film, and tentative identification was made with cochromatography with known standards.

Results and Discussion

Exposure to 10°C, in contrast to 25°, induced cold acclimation and increased the cold hardiness of 'Valencia' orange seedlings. Seedlings exposed to 10° for 4 weeks averaged less than 10% leaf kill and 0% stem kill, whereas seedlings conditioned at 25° or directly from the greenhouse had 99% leaf and stem kill after freeze tests of –7°.

Uptake of $^{14}CO_2$ at 25°C was not the same as that at 10°(Table 1). Assuming equal access of $^{14}CO_2$ to all trees, 45% of the applied label, or 4.8×10^7 disintegration per min (dpm) of a total 1.11×10^8 dpm was incorporated into seedlings at 25° while 54% of the label, 6×10^7 dpm, was incorporated at 10°. Greater incorporation of $^{14}CO_2$ at 10° than 25° suggests differences in stomatal behavior prior to, or during, labeling and/or differences in respiration and greater ^{14}C losses at 25°. Unequal labeling limited data comparisons to overall trends as percentages of total ^{14}C activity in different tissue fractions during cold-hardening intervals. $^{14}CO_2$ uptake is decreased once citrus leaves are cold hardened (27).

In this study, ^{14}C was considered high in the sugar fraction of the leaves up to 7 days after $^{14}CO_2$ exposure. Heavy leabeling of the sugar fraction is common after $^{14}CO_2$ exposure during conditions that favor photosynthesis in plants (4, 10, 18, 19, 20). The disparity in rates of decline in radioactivity in the sugar fraction between 25°C and 10° apparently resulted from different rates of translocation and starch synthesis (Fig. 1, 4). Conversion of labeled sugars into starch helped to cause the rapid decline of ^{14}C activity in the sugar fraction from 0 to 7 days at 10°. The percentage of label in the starch fraction increased 32% of total ^{14}C present in the leaves at 10° from 0 to 7 days. In contrast, the radioactivity declined 44% in the sugar fraction. Low temperatures reduce the translocation of photosynthates in citrus (5), inhibit growth, reduce respiration, and eliminate much of the demand for photosynthetic assimilates and reserves (11, 25). A more rapid rate of starch turnover at 25° than at 10° was indicated in this study, as the ^{14}C activity declined with time at 25° but remained relatively constant at 10°. Other researchers report starch is metabolically inaccessible once formed in lemon trees at low temperatures (11). Our data indicate starch breakdown is slowed, but not stopped, in orange leaves at 10°. Starch depletion in 'Valencia' orange leaves and stems was noted after 3 weeks when young trees were conditioned at 19° and continuous light (24).

Incorporation of ^{14}C into proteins, organic acids, and lipids was generally linear with time in leaves and stems at 25° and 10° (Fig. 1, 2). Noted differences were in rates which were somewhat lower at 10° and incorporation of ^{14}C into amino acids. Incorporation of ^{14}C into organic acids remained relatively low and did not exceed 10% of the total radioactivity found in the leaves. This is less than the incorporation of ^{14}C into organic acids in citrus trees with developing fruit and shoots (10). For plants other than citrus,

Table 1. ^{14}C-labeled fractions extracted from 'Valencia' orange seedlings exposed to $^{14}CO_2$ (50 Ci for 3 hr) at 25° and 10°C and maintained at these temperatures for 28 days in controlled temperature/light rooms.

Days after labeling	^{14}C (dpm)/mg fresh wt)											
	Sugars		Starch		Organic acids		Amino acids		Proteins		Lipids	
	25°	10°	25°	10°	25°	10°	25°	10°	25°	10°	25°	10°
						Leaves						
0	584[z]	1,044*	101	78*	4	3	59	52	8	3	10	8
7	72	180*	11	160*	4	7	38	45	5	9	8	5
14	19	56*	10	56*	5	7	25	29	9	11	9	8
28	2	18*	1	43*	4	6	16	34*	14	16	9	13
						Stems						
0	176	125*	7	9	0	2	8	3	0	0	1	1
7	106	117*	46	102*	12	21*	24	27	3	5	1	4
14	76	59*	40	55*	2	10*	22	35*	1	2	7	2
28	5	22*	24	73*	4	21*	11	22*	5	8	4	9
						Roots						
0	1	4	21	5*	0	7*	1	4	1	0	0	0
7	13	15	43	77*	4	7	10	19*	5	10	4	5
14	12	20*	41	58*	1	2	20	16	4	10*	8	5
28	4	11*	34	74*	3	5	5	17*	5	12*	7	9

[z]Mean of 3 determinations/each of 3 seedlings.
*Mean separation between temperatures, *t* test, significant at 5% level.

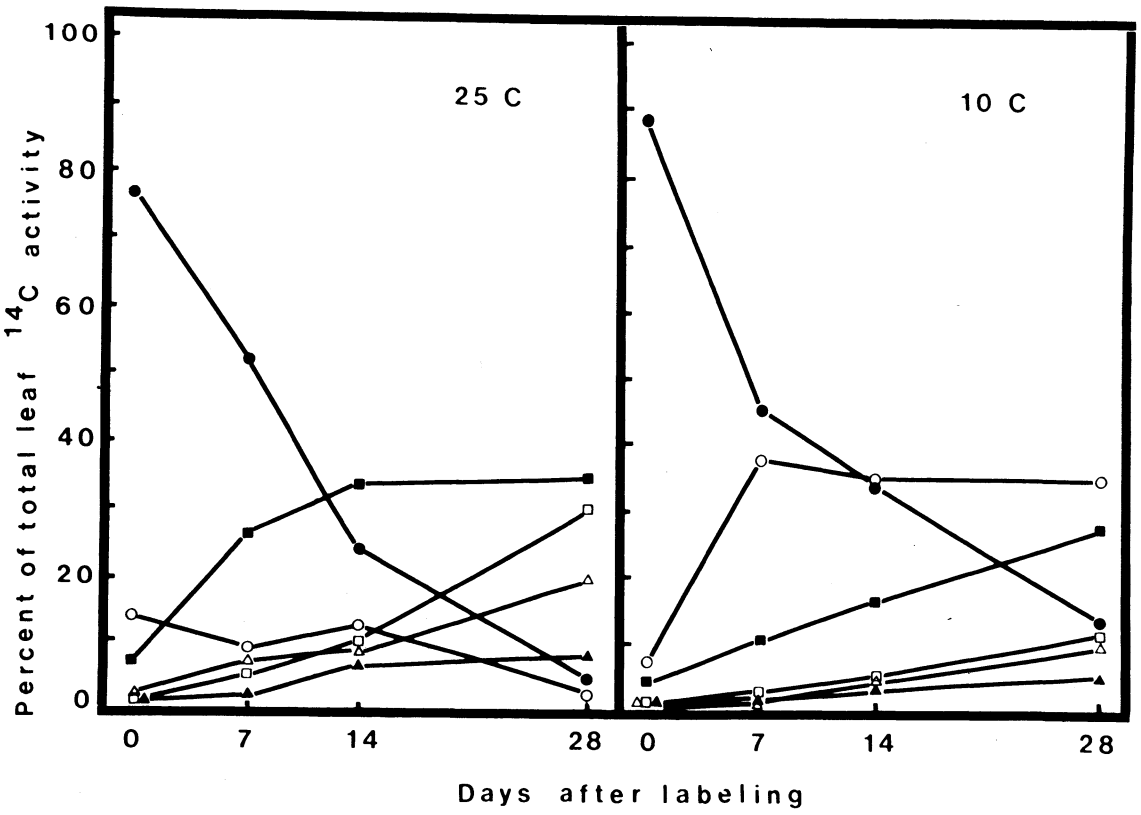

Fig. 1. Distribution of ^{14}C in amino acids (■), lipids (Δ), organic acids (▲), protein (□), starch (○), and sugars (●) in leaves of orange seedlings conditioned at 25° and 10°C.

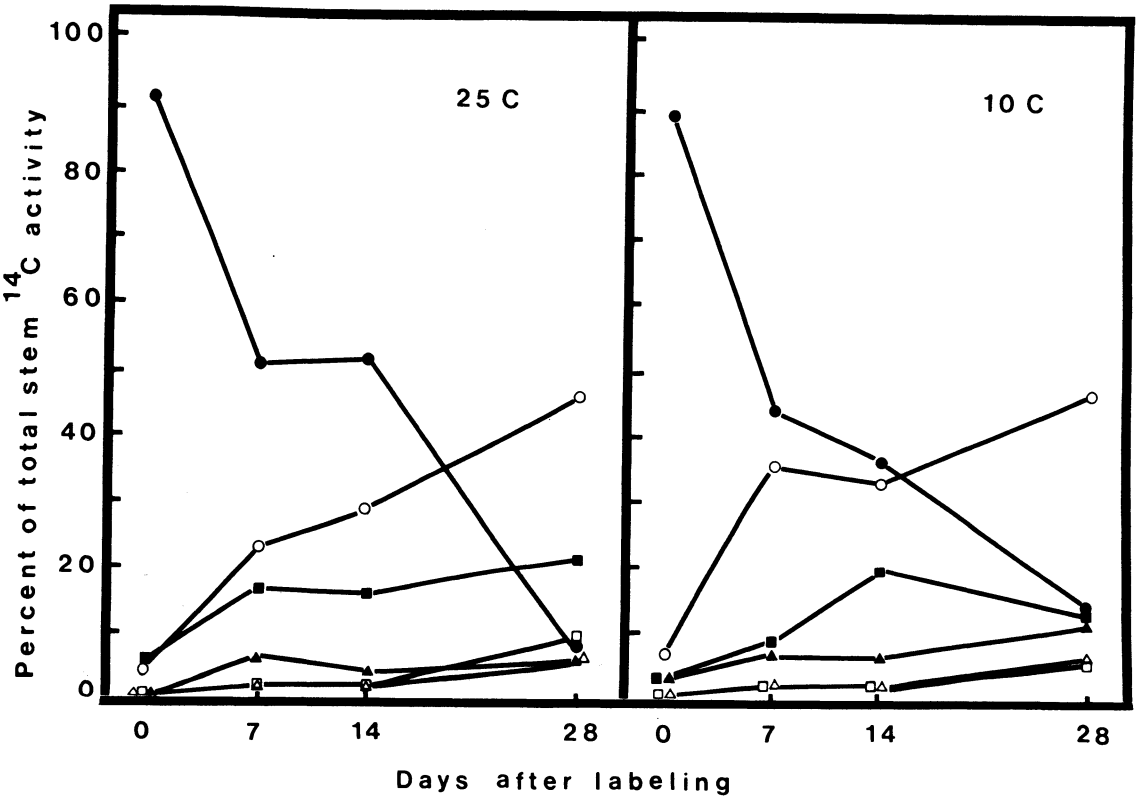

Fig. 2. Distribution of ^{14}C in amino acids (■), lipids (Δ), organic acids (▲), protein (□), starch (○), and sugars (●) in leaves of orange seedlings conditioned at 25° and 10°C.

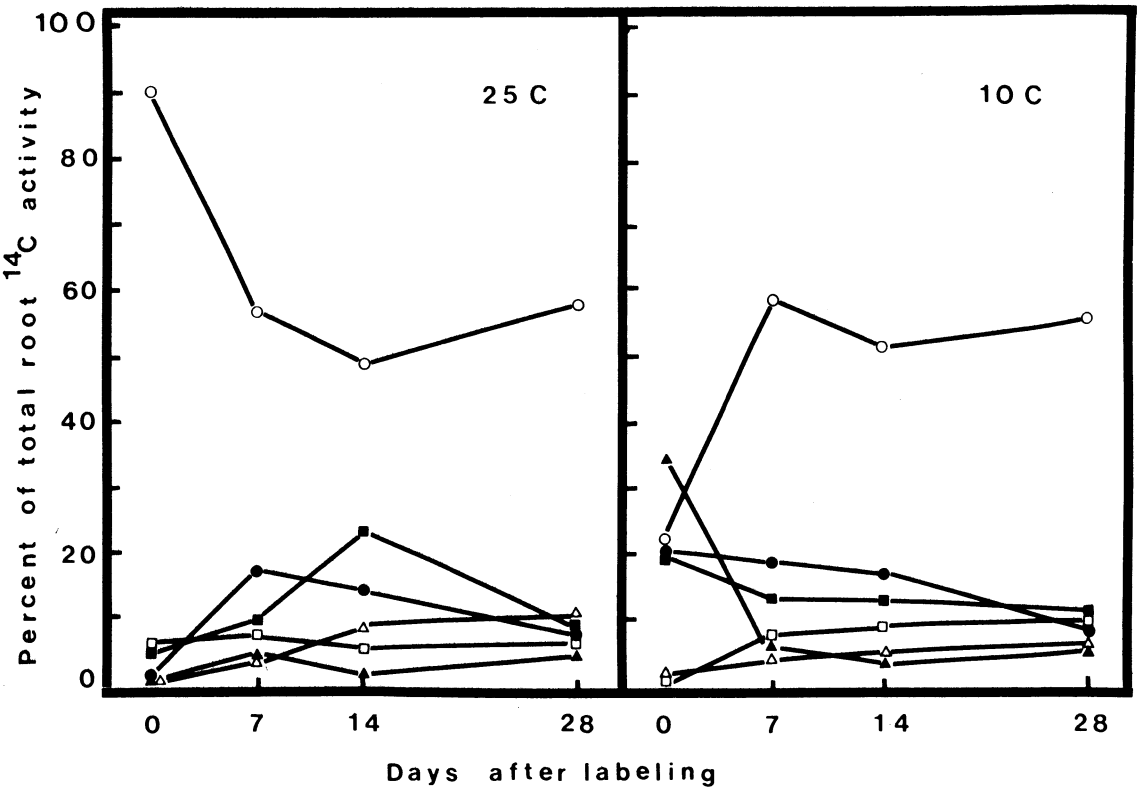

Fig. 3. Distribution of ^{14}C in amino acids (■), lipids (Δ), organic acids (▲), protein (□), starch (○), and sugars (●) in leaves of orange seedlings conditioned at 25° and 10°C.

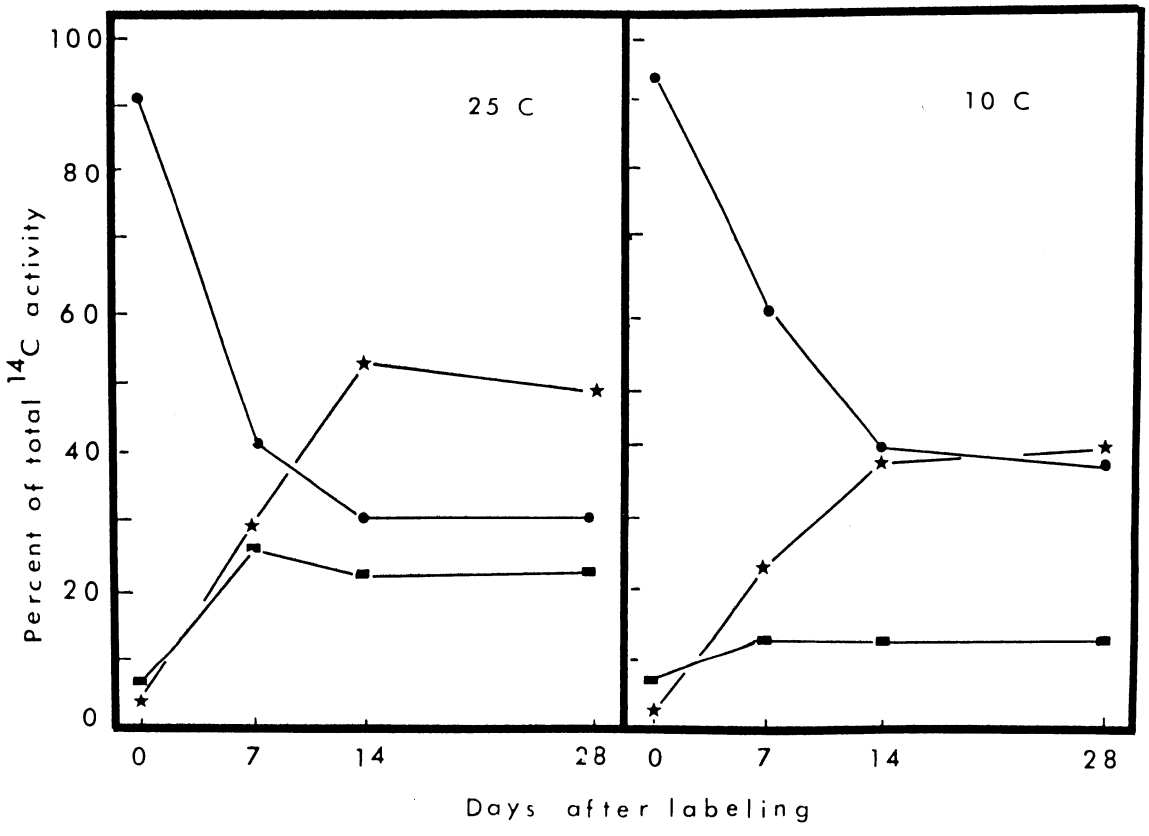

Fig. 4. Distribution of ^{14}C in amino acids (■), lipids (Δ), organic acids (▲), protein (□), starch (○), and sugars (●) in leaves of orange seedlings conditioned at 25° and 10°C.

organic acids accounted for at least 50% of the ^{14}C activity in the leaves during low temperatures(20).

^{14}C-photosynthate translocated to the roots apparently was rapidly transformed into starch and largely remained in that form throughout the 28 days regardless of temperature (Fig. 3). Other metabolites were labeled to a lesser extent, comprising less than 50% of the total radioactivity up to 28 days. The reason for the initially high ^{14}C activitity in organic acids of the roots is not known. Exposure to 10°C may temporarily slow root metabolism, which resulted in an initital increase becoming less evident with continued acclimation.

Differences were found in the allocation of ^{14}C-photosynthates to the roots of citrus seedlings at the different temperatures. The largest portion of ^{14}C activity was found in the roots of seedlings at 25°C after 14 days and at 10° after 28 days (Fig. 4). In field studies (7), ^{14}C accumulated in the roots of apple trees during the autumn at rates greater than we observed for citrus. Such observations suggest differences in partitioning of photosynthates between subtropical, evergreen citrus, and temperate, deciduous fruit trees.

In our study, a higher percentage of the label would be expected at 10°C than at 25° in one of the fractions isolated if low temperatures promoted the synthesis of a compound(s) responsible for cold hardiness that is dependent on photosynthesis. Amino acids, proteins, lipids, and organic acids are ruled out on a quantitative basis, since the incorporation of ^{14}C on a percentage basis of total ^{14}C was less at 10° than 25°. Low temperatures are known to stimulate protein synthesis in red pine (17), but in our study, incorporation of ^{14}C into proteins was equal to or less at 10° than at 25°. Starch had a higher ^{14}C content at 10° than at 25°, but starch is largely ruled out as a protective substance since its insoluble properties minimize the potential to protect against freeze stresses.

Changes in cells that increase cold hardiness may be more qualitative than quantitative. A shift in the metabolism of 1 or 2 compounds may occur within a metabolic fraction (16). Our study would not detect such changes if such a shift in metabolism of ^{14}C-phytosynthate caused increased cold hardiness in the seedlings. Autoradiography did not indicate major differences in labeling patterns of ethanol-soluble compounds extracted from leaves conditioned for 28 days at 10° and 25°C. Following a 24-hr incorporation period, ^{14}C activity was found in the amino acids: proline, alanine, asparagine, serine, glycine, and gamma amino butyric; organic acids: citrate and malate; and sugars: sucrose, glucose,and fructose. Several compounds were not identified and merit consideration in continued work. We did not find increases in ^{14}C in the organic and amino acid fractions that were noted in other studies on wheat (18) and rape plants (19).

The greater retention of ^{14}C in the sugar fraction of citrus leaves exposed to 10°C suggests low temperatures reduced the metabolism of ^{14}C-photosynthates in citrus, but there was no accumulation in a specific biochemical fraction that would account for increased cold hardiness. This has been found with plants other than citrus (20). Probably of more importance is the distribution of ^{14}C-photosynthates into specific cellular sites, which was not investigated in cold hardening of our citrus seedlings.

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