increases toward an optimum root initiation, the balance of internal growth regulator and possible cofactors may change to bring out the stimulatory effect of auxins on root initiation and overcome the inhibitory influences of cytokinins and gibberellins. Under some conditions exogenous growth regulators can substitute for the effect of temperature on root initiation. The responses of plants to temperature can also be altered by various combinations of exogenous growth regulators.

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Use of Centrifugation to Obtain Auxin Extracts from Cuttings Treated with Terminal Applications of 3-indoleacetic Acid[1]

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Abstract. A method of extracting indole compounds from stem segments of woody cuttings of *Ilex crenata* 'Convexa' by refrigerated centrifugation has been developed. Effective extraction was obtained when segments were immersed in a solution of 40% ethanol (v/v) and exposed to a force of 2750 × *g* for at least 2 hr. Exposure to that force for more than 4 hr did not result in a significant increase in extractable auxin. Quantitative determinations of ^{14}C measured by liquid scintillation counts of the extracts revealed that this method reliably depicted changes in levels of IAA or its metabolized forms in different stem segments as a result of different treatments. Chromatographic separation of extracts revealed that at least 35% of the label was still in the form of IAA after 48 hr. The increased levels of auxin in stem segments was also portrayed by significant rooting increases as determined by rooting index. Centrifugation extracts separated into 2 phases and were removed separately, most of the isotope was contained in the smaller lower phase. Proportionate levels of the 2 phases changed in segments over time and the lower phase disappeared at the time of rooting.

INTRODUCTION

A METHOD of rooting cuttings by terminal application of auxins has been demonstrated (5). Rooting data per se do not indicate how the movement of exogenous auxin or daily changes in total auxin level may be related to observed responses. Such information would give an insight into auxin distribution in the cutting and thereby a possible explanation of the effectiveness of terminal applications of auxin which result in rooting. A direct method was sought, therefore, to measure the amount of auxin and if possible only translocated auxin in cuttings.

Recently Kawase (2, 3, 4) reported that cuttings of *Salix alba* responded to centrifugal force with increased rooting. He determined that the number of roots per cutting increased as force increased. The maximum force was limited by the tenderness of the cuttings since higher forces resulted in loss of foliage or broken stems. Kawase used the method to study only the cofactors associated with rooting, and not auxin. He theorized that the force resulted in accumulations of auxin at the base of cuttings which then acted to stimulate movement of rooting cofactors to that site where increased rooting occurred. When Kawase immersed the basal ends of cuttings in water in centrifuge tubes he found that centrifugation caused substances to be forced from the cuttings which gave a positive bioassay with mung bean seedlings. In later unpublished work Kawase determined that this substance had an Rf value similar to one of Hess's cofactors. It seems quite possible that the application of force coupled with an adequate solvent could result in auxin extraction as well as the extraction of rooting cofactors.

MATERIALS AND METHODS

Unbranched terminal cuttings, 4 to 5 inches long from one clone of *Ilex crenata* Thunb. cv. 'Convexa' were used for experiments. A 1% solution (w/v) of 3-indoleacetic acid (IAA) or 3-indolebutyric acid (IBA) was used for nonradioactive auxin treatments. Solutions were prepared by dissolving crystalline material in hot absolute ethanol and diluting to 40% (v/v) with distilled water.

Terminal or basal dips for all experiments were made by immersing cuttings to a depth of 1 inch in the appropriate solution for 10 seconds. Excess solution was shaken off and cuttings were allowed to air dry on paper towels. Care was taken that the treated terminal or basal ends were not in contact with the paper. Cuttings were then placed in 3 inch plastic pots containing equal parts by volume of sphagnum peat moss and medium grade horticultural perlite. Four cut-

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tings of each treatment were placed in each pot and the pots were placed in full sunlight under intermittent mist in a heated greenhouse with a minimum temperature of 65°F. Mist was regulated by a photocell[3] so that cuttings were not allowed to become dry during daylight hours.

When cuttings were harvested they were washed under running tap water, blotted dry with paper towels and defoliated, one leaf at a time to eliminate tearing of stem tissue. The defoliated stems were then washed, alternately in 95% ethanol and then in water, 3 times. This was done to remove auxin from the outer surfaces of the stem. Cuttings were again blotted dry and allowed to air dry. The 4 cuttings in each treatment group were cut into 1 inch segments. Thus, if cuttings were 4 inches long there were 4 segments. Segments were weighed to the nearest 0.1 g and placed together in a 10 × 75 mm pyrex test tube with 2.0 ml 40% ethanol (v/v) and placed in a refrigerated centrifuge and subjected to a force of 2750 *g* for 4 hr at 75° F ± 1°. Bending of stem segments was eliminated by using special rubber adapters fitted into large centrifuge tubes which provided 2 smaller receptacles which held 2 tubes 10 × 75 mm with a capacity of 3.0 ml each. Only defoliated 1 inch segments were used and with 4 segments in each tube, little space was left for bending of segments due to force.

Extracts were removed from the tube with long tipped 2 ml pipettes calibrated to 0.02 ml and placed in liquid scintillation counting vials. It was observed that the lowest 0.2 ml of extract had a slight pigmentation immediately after centrifugation which eventually disappeared in the solution. This lower portion was removed separately with a 0.2 ml long tipped pipette and called the lower phase (LP), the remaining 1.8 ml was called the upper phase (UP). All samples were divided into these 2 portions. It had been determined colorimetrically that most of the material in the LP had an absorption spectrum like IAA with an absorption peak at 530 nm in Salkowski reagent.

The radiochemical used for all experiments was 3-indoleacetic acid (IAA–2–^{14}C). It was diluted with unlabeled IAA in 40% ethanol to reach a final concentration of 10,000 ppm IAA with 950 ppm in the form of IAA–2–^{14}C and it had a specific activity of 0.09 μC/mM. When solutions were not in use they were stored at 0° C.

Glassware was used only once for all extraction procedures. When extracts were removed from centrifuge tubes the upper and lower phase were placed in separate counting vials. Each phase in its separate vial was placed on a hot plate adjusted to approximately 140 C. When the contents of the vials were evaporated 6 ml of scintillation solution (2, 5 diphenyloxazole) 6.0 g/l (w/v) and 1, 4-bis-2(phenyl oxaolyl)-benzene 9.5 g/l (w/v)[4] were added to each vial, placed in a liquid scintillation counter[5] having a counting efficiency of 74–80%. Experiments were repeated when possible to determine reliability of results. Duplicate sets of cuttings were allowed to root to provide data for comparison with isotopic results. Experiments were designed, to permit statistical analysis using a complete randomized block design.

Ascending chromatographic separations were made on Whatmann 3MM paper in a solvent of isopropanol, ammonia and water (8:1:2 v/v). IAA standards were developed colorimetrically with Salkowski reagent.

To determine if the extract contained IAA after it was translocated in the tissue for 48 hr, terminal applications were made as before and after the exogenous application had dried, cuttings were placed at 75° F in daylight, without mist. Cuttings were inserted through waxed paper into shell vials containing water. After 48 hr defoliation, washing and extraction was done as before. Both phases were brought to a volume of 0.2 ml and a 25 lambda sample was taken from each phase. They were spotted on 3MM Whatmann chromatographic paper with a standard sample of IAA of equal volume. The paper was placed in the chamber and allowed to equilibrate with the solvent of isopropanol, ammonia and water (8:1:2 v/v) for 24 hr. The paper was then inserted into the solvent and removed when the front reached a height of 24 cm. The standard was developed with Salkowski reagent. The sample strips were cut into 12 segments of equal lengths, inserted into counting vials and levels of ^{14}C were determined as before. It was found that 36% of the total count of the UP and 58% of the LP was concentrated at the same Rf as IAA. There were several other concentrations of ^{14}C which indicated presence of other metabolites of IAA.

[3]Solatrol, product of Scientific Instruments Inc.

[4]Dr. K. Simpson, Dept. Agric. Chem., Univ. Rhode Island, Kingston, R. I., personal communication.

[5]Mark 1 model #6860, product of Nuclear Chicago.

Results and Discussion

Extraction, translocation and rooting response. Labeled auxin was used in a series of rooting experiments and counts of ^{14}C were used to determine levels of translocated auxin or auxin products resulting from exogenous applications. Jacobs (1) found that IAA-^{14}C was unchanged after transport through petioles of coleus and bean in 8 hr. A series of preliminary tests with different solvent extracts obtained by centrifugation and developed with Salkowski reagent indicated that indoles could be extracted into solvents by centrifugation. Ethanol was the best solvent tested and extraction times in excess of 4 hr at a force of 2750 × *g* did not result in higher levels of indole in the extracting solution (Table 1). The fourth segment from the tip was used for sample extractions since it was in the region where adventitious roots developed and it had no cut surfaces exposed during auxin application. The results in Table 1 show that there are essentially no differences in extracts obtained at a force of 2750 × *g* after 2, 4, or 24 hr. It is also evident that similar levels of ^{14}C may be obtained with normal gravitation force if segments are soaked in the solvent for 24 hr. Much higher levels of ^{14}C may be obtained for basal segments of cuttings which have been basally dipped in IAA–2–^{14}C for 10 sec but it is not possible to distinguish between translocatable IAA–2–^{14}C and labeled isotope which had been passively carried to that locus. The total amount of extractable material is approximately 1% of that applied.

Table 1. Effect of time and force on extraction of ^{14}C in 40% ethanol from the fourth basal segment of 4 stem cuttings of *Ilex crenata* 'Convexa' 2 days after an application of a 10-sec terminal dip of IAA-2-^{14}C.

Treatment	Force (x *g*)	Time (hr)	Counts per minute[x]
Terminal dip (10 sec)	2750	2	366.3 a
	2750	4	411.3 a
	2750	24	407.0 a
	1	24	373.0 a
Basal dip (10 sec)	1	24	2291.0 b

[x]Treatment means followed by the same letter are not significantly different from each other (P of 0.05).

It is evident from the results in Table 2 that ethanol is superior to water in extraction of ^{14}C from cutting segments. There was no difference between centrifugated extracts obtained after 4 hr at 2750 *g* and those obtained after 24 hr at 1 × *g* in 40%

Table 2. Effect of 2 solvents and 2 extraction times on extraction of ^{14}C from the fourth segment of 5-in cuttings of *Ilex crenata* 'Convexa' 46 hr after terminal applications of IAA–2–^{14}C.

Extraction treatment time (hr)	Force (x *g*)	Solvent	Counts per minute[x]
24	1	40% ethanol	97.6 a
4	2750	40% ethanol	84.0 a
24	1	Water	28.6 b
1	2750	Water	33.6 b

[x]Treatment means followed by the same letter are not significantly different from each other (P of 0.05).

ethanol. Similar extracts obtained in water contained significantly less radioactivity.

An experiment was performed to determine if the centrifugation method could be used to determine levels of ^{14}C in segments from cuttings after exogenous applications of labeled material had been made (Table 3). Definite differences in levels of ^{14}C were found at each locus as a result of treatment. It is interesting that only a small amount of ^{14}C was carried to the apex when the isotope was applied basally. Rooting data (Table 4) were similar to those obtained previously when terminal applications were compared to basal applications of unlabeled IAA (5). Basal applications resulted in highest rooting values followed by terminal treatments and control. Though levels of ^{14}C were 8 times higher in basal segments of cuttings treated with IAA–2–^{14}C than similar segments of terminally treated cuttings, rooting was only 1.3 times higher. The isotope determinations were made several weeks before rooting data were recorded but the relative values probably remained the same throughout the rooting period. This indicates that there is a great deal more auxin or auxin metabolites present in the lowest segment of basally treated cuttings than is needed for root initiation, since adequate rooting took place at the lower concentrations. When the phases were measured separately it was found that the greatest part of the indoles were in the small volume of the LP (Table 5).

Table 3. Total amount of ^{14}C in 4 cuttings of *Ilex crenata* 'Convexa' after treatment with 10-sec dip in 1% IAA–2–^{14}C.

Treatments	Segment location (in)	Number of days after application	
		2	9
		Counts per minute	
Terminal dip			
Cold IAA	Apex	0	1.4
	2nd	0	1.0
	3rd	0	0.0
	4th	0	0.0
	5th	0	0.0
	Total	0	2.4
Terminal dip			
IAA-2-^{14}C	Apex	1163	490
	2nd	198	207
	3rd	211	171
	4th	75	192
	5th	217	214
	Total	1864	1274
Basal dip			
IAA-2-^{14}C	Apex	53	16
	2nd	116	101
	3rd	337	251
	4th	1091	1085
	5th	3704	1791
	Total	5301	3244

Table 4. Effect of terminal or basal applications of IAA–2–^{14}C and IAA in 10 sec dips at 1% concentration in 40% ethanol (v/v) on rooting of stem cuttings of *Ilex crenata* 'Convexa.'

Treatment	Rooting index[x] Mean[y]
Control	28.8 a
Terminal dip	47.2 b
Basal dip	61.2 c

[x]Scores are based on total cumulative length of roots of four cuttings in each replication.
Key = 0–¼″=1, ¼–½″=2, ½–1″=3, 1–2″=4, over 2″=5.
[y]Treatment means followed by the same letter are not significantly different from each other (P of 0.05).

Table 5. Determination of ^{14}C extracted by centrifugal force (2750 × *g*) from groups of 4 one inch segments of *Ilex crenata* 'Convexa' 9 days after applications of IAA–2–^{14}C.

Treatment 10-sec dip	Segment (in)	Lower phase (0.2 ml)	Upper phase (1.8 ml)
		Counts per minute	
Terminal	Apex	341	148
	2nd	131	76
	3rd	150	20
	4th	164	27
	5th	197	17
	Total	985	189
Basal	Apex	16	0
	2nd	101	0
	3rd	244	6
	4th	1043	41
	5th	1666	124
	Total	3071	172

When the 2 phases were followed separately for extended periods (Table 6) it was found that the amount of ^{14}C in the lower phase remained relatively constant at each locus until the fourth week when callus formation took place on the base of the cutting and root primordia were formed. At this time there was no ^{14}C in the LP of the lowest segments, but there were higher counts in ^{14}C in the UP of the segment of basally treated cuttings but no noticeable increase in ^{14}C in the basal segment of terminally treated cuttings.

All data obtained by this centrifugation method were consistent in that there were no reversals in trends and results consistently depicted changes in auxin levels in cuttings which had been treated with terminal applications of IAA–2–^{14}C. This method does appear to be useful when applications were made to more mature specimens in measuring changes in translocatable auxin as a result of terminal treatment. It may not be as reliable in determining levels of basally applied auxin, particularly in the basal segment, since it is not possible to separate translocatable auxin from labeled isotope bound in or passively carried to that segment any more than the apical segment can be reliably measured for translocatable auxin when terminal treatments are used.

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Table 6. Amount of ^{14}C in the lower phase and upper phase of centrifugated extracts from stem segments of cuttings of *Ilex crenata* 'Convexa.'

Treatment in 10 sec dips	Locus	Number of days after application									
		1		8		15		22		29[x]	
		Counts per minute									
		LP	UP	LP	UP	LP	UP	LP	UP	LP	UP
Terminal dip IAA-2-^{14}C + Cold IAA	Apex	1107	4238	1174	2186	1129	2068	1037	1413	1242	1589
	2nd	282	1293	280	411	367	566	281	323	529	701
	3rd	163	746	258	300	330	322	259	220	395	287
	4th	83	163	120	129	116	120	0	113	0	293
	Total	1635	6439	1832	3026	1942	3076	1577	2079	2166	2870
Basal dip IAA-2-^{14}C + Cold IAA	Apex	30	22	60	50	66	48	61	45	82	45
	2nd	114	76	291	131	248	178	162	130	214	117
	3rd	699	542	1187	502	1058	597	1030	640	969	580
	4th	8958	7196	8305	4854	4770	2642	0	5901	0	6341
	Total	9801	8836	9843	5539	6142	3465	1253	6716	1265	7083

[x]Cuttings had rooted by the 29th day, roots were not included in samples.