Scientia Horticulturae, 51 (1992) 187–195
Elsevier Science Publishers B.V., Amsterdam

Involvement of rooting factors and free IAA in the rootability of citrus species stem cuttings

O. Sagee[a], M. Raviv[b], Sh. Medina[b], D. Becker[c] and A. Cosse[c]

[a]*Department of Citriculture, Agriculture Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel*

[b]*Department of Ornamental Horticulture, Agricultural Research Organization, Newe Ya'ar 31999, Israel*

[c]*Department of Chemistry, Technion – Israel Institute of Technology, Haifa 32000, Israel*

(Accepted 18 February 1992)

ABSTRACT

Sagee, O., Raviv, M., Medina, Sh., Becker, D. and Cosse, A., 1992. Involvement of rooting factors and free IAA in the rootability of citrus species stem cuttings. *Scientia Hortic.*, 51: 187–195.

Two-year-old trees of cultivar 'Rangpur' lime (*Citrus limonia* Osb.) and of cultivar 'Oroblanco', a triploid pummelo-grapefruit hybrid (*Citrus grandis* Osb. × *Citrus paradisi* Macf.), which had not reached flowering stage, were grown under greenhouse conditions. Cuttings from the last vigorous vegetative flush were taken from each species for rooting experiments. Callus formation and percentage of rooting were determined after 19 and 36 days. Endogenous indole-3-acetic acid (IAA) content and avocado rooting promoter (ARP)-like activity were determined in leaves and bark (cortex) of the lower end of the cuttings on day of excision and 19 days later. Rooting of the easy-to-root 'Rangpur' lime reached 77% after 19 days and 100% after 36 days. At those times the difficult-to-root 'Oroblanco' did not root at all or reached 12% rooting, respectively. At Day 19 the level of free IAA in the bark of 'Rangpur' lime was 3.5 times higher than that at Day 0, and 3.6 times higher than in the 'Oroblanco' bark on the same day. ARP was found in both species at excision day and after 19 days using gas–chromatography analysis. The ARP activity on the day of excision was only slightly higher in the leaves and bark of 'Rangpur' lime than in 'Oroblanco', but after 19 days ARP-like activity rose approximately 45% in the basal bark of 'Rangpur' lime with no such increase in its leaves or in the leaves and bark of 'Oroblanco'. The differences in IAA level and ARP-like activity in the two citrus cultivars appear to be correlated with their ease of rooting, but it is not possible to tell whether the increase in IAA and ARP over the rooting period is the cause or result of root initiation.

Keywords: avocado rooting promoter; cuttage propagation; indole-3-acetic acid; 'Oroblanco'; 'Rangpur'.

Abbreviations: ARF = adventitious root formation; ARP = avocado rooting promotor; BHT = butylated hydroxytoluene; ELISA = enzyme-linked immunosorbent assay; GBq = gigabecquerel; GC = gas-chromatography; IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; PVPP = polyvinylpolypyrrolidone; TBS = tris buffered saline.

Correspondence to: Oded Sagee, Department of Citriculture, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel.

INTRODUCTION

Adventitious root formation (ARF) is regulated by complex interactions between endogenous and exogenous factors which affect the various developmental stages of root formation. In general terms, ARF follows three developmental phases: (1) de-differentiation, in which predetermined cells switch from their normal morphogenetic path to act as mother cells for the root primordia; (2) initiation, in which these cells start to divide and form the distinct structure of a root primordium; (3) elongation, during which the newly formed primordia form vascular connections and later protrude through the surrounding tissues to form roots (Hartmann et al., 1989).

Although many physiological studies have shown that auxin plays a central role in the developmental process of root initiation (Jarvis, 1986), little is known about hormonal factors affecting the de-differentiation stage. The initiation stage includes an auxin-active stage, during which auxin must be supplied continuously for roots to form. This auxin can come from buds or leaves, or may be applied exogenously (Gorter, 1969; Ericksen, 1974; Beck and Sink, 1974; Mohammed and Ericksen, 1974; Pluss et al., 1989). Later on, there is an auxin-inactive stage in which withholding auxin does not adversely affect root formation. At the root elongation and growth stage there is no response to applied auxin (Hartmann et al., 1989). When cuttings are made from easy-to-root plants it is assumed that the endogenous auxin level affects the promotion of adventitious root formation (Hemberg, 1954; Norcini et al., 1975; Weigel et al., 1984). On the other hand, in *Chrysanthemum*, an easy-to-root species, endogenous auxin content does not correlate with root initiation (Stoltz, 1968).

In addition to auxin, several other endogenous root-promoting substances were detected in various species (Jarvis, 1986). Endogenous levels of avocado rooting promotor (ARP) were related to clonal (Raviv and Reuveni, 1984) and age (Raviv et al., 1987) differences in rooting ability of avocado (*Persea americana* Mill.) cuttings. When applied to mung bean cuttings as pulses, the root-inducing activity of ARP preceded that of auxin (Raviv et al., 1986b). It was postulated that ARP may serve as an example for a class of compounds that are able to induce or accelerate de-differentation. Recently, ARP was detected in *Laurus nobilis* L. and *Magnifera indica* L. (Becker et al., 1991). Therefore, we were interested in the involvement of ARP in other species with horticultural importance such as citrus. The ARP fraction contains four compounds: 1I (1 acetoxy-2,4-dihydroxy-n-heptadeca-16en); 1II (1 acetoxy-2,4-dihydroxy-n-heptadeca-16yn); 2I (1,2,4-trihydroxy-n-heptadeca-16en); 2II (1,2,4-trihydroxy-n-heptadeca-16yn), of which 2II is

the most active (Raviv et al., 1986a). The development of a gas–chromatography (GC) analytical method for the detection of ARP compounds has enabled us to demonstrate the existence of ARP compounds in species besides avocado (Becker et al., 1991).

The objectives of the present research were to demonstrate the presence of components of ARP in *Citrus* cuttings and to study the involvement of ARP and indole-3-acetic acid (IAA) in the rooting of citrus cuttings. Estimation of ARP by rooting assay and IAA by enzyme-linked immunosorbent assay (ELISA) were carried out in two citrus species, easy-to-root cultivar 'Rangpur' and difficult-to-root cultivar 'Oroblanco', a pummelo-grapefruit hybrid (Sagee et al., 1989).

MATERIALS AND METHODS

Rooting of cuttings. – Two-year-old rooted cuttings of 'Rangpur' (*Citrus limonia* Osb.), sometimes called a mandarin lime (*Citrus reticulata* var. austera Swingle), and 'Oroblanco', a triploid pummelo-grapefruit hybrid (*Citrus grandis* Osb. × *Citrus paradisi* Macf.), were grown in a controlled temperature greenhouse (maximum day temperature 30 °C, minimum night temperature 14 °C). These mother plants trees had not reached flowering stage. Cuttings, 15–27 cm in length, 4–6 mm thick, and with 8–14 fully developed leaves, were taken from the last vegetative flush of ten trees of each species and were placed in a mist-bed with bottom heat of 28 ± 2 °C and a mist regime of a 5 s mist every 10 min. The rooting medium consisted of equal parts of medium-grade Dutch sphagnum peat and shredded polystyrene foam. The cuttings were not treated with rooting hormone. Callus formation and percentage of rooting were determined after 19 and 36 days. Leaf samples and bark (tissue exterior to cambium) from the lower end of the cuttings, 3–4 cm above the cut or the callus were collected for determination of endogenous IAA content and ARP-like activity at Day 0 and Day 19, respectively. The plant tissue was frozen in liquid nitrogen and stored at −80 °C immediately after harvesting and fresh weight determination.

IAA extraction, purification and determination. – Samples of 1 g of frozen plant tissue were placed in 5 ml of 80% methanol containing 100 mmol ammonium acetate, 45 μmol ml^{-1} butylated hydroxytoluene (BHT) and 24 μmol [2-^{14}C]-IAA (2.11 GBq $mmol^{-1}$) as the internal standard. The samples were homogenized (Polytron) for 1 min in an ice bath and then kept in the dark at 4 °C for 30 min, vigorously mixed, centrifuged at 5000 × g for 10 min and the supernatant saved. The pellet was resuspended in 5 ml distilled water and centrifuged as above. Both supernatants were pooled and IAA was subjected to three open-column liquid chromatography steps of polyvinylpolypyrrolidone (PVPP), DEAE-Sephadex and C-18 as described by Sagee et al. (1986).

The partially purified extract was methylated with an excess of ethereal diazomethane (Cohen, 1984) and additionally purified by reverse-phase high-performance liquid chromatography (Varian 5500 system equipped with a Supelcosil LC-18-DB fast column, 3.3 cm long). Elution was performed with 50% methanol–water at a flow of 1 ml min^{-1}. The fraction containing methyl IAA was collected, reduced to dryness, redissolved in 50 μl ethanol and 950 μl tris buffered saline (TBS) (50 mmol Tris–HCl, 1 mmol $MgCl_2$, 0.1 mol NaCl, pH 7.8), and assayed within 12 h using an ELISA according to Sagee et al. (1986).

ARP extraction. – ARP was extracted according to a previously described procedure (Raviv et al., 1986b). The crude extract served two purposes: rooting bioassay and GC analysis. For the rooting bioassay the crude extract was paper-chromatographed (Raviv and Reuveni, 1984) and the last section (Rf 0.9–1.0) was used for the bioassay. ARP identification was conducted on leaves only, due to a shortage of basal bark plant material.

GC analysis of ARP. – For the GC analysis an additional purification was conducted. The crude methanolic extracts of the leaves were evaporated to dryness, dissolved in a minimal amount of methanol and filtered using 3 mm Whatman filter paper. The extract was then hydrolyzed at room temperature for 4 h in 20 ml of methanol containing 2% KOH (w/v). The amounts of an individual ARP component in the samples were close to the lower detection limit of the GC, and compounds 1I and 1II were hydrolyzed to 2I and 2II, respectively. Thus, the data presented are of the combined amounts of 1I and 2I (referred to as ARP-A) and 1II and 2II (referred to as ARP-B).

After hydrolysis was completed, 10 ml of distilled water was added to each sample and the methanol was evaporated. ARP was extracted and purified as described earlier (Becker et al., 1991) and samples of 1 μl were injected into a GC equipped with an SE-54 capillary column (25 m long, 0.25 mm i.d.), splitless injector, Chromosorb precolumn inlet, and H_2-flame detector. Detection of compounds ARP-A and ARP-B was performed for all samples using authentic standards and verified by co-chromatography. Retention time under these conditions is approximately 16.9 min for ARP-A and 17.8 min for ARP-B. Currently this method allows only detection of ARP but not quantification (Becker et al., 1991).

Mung bean rooting bioassay. – The mung bean rooting bioassay, originally described by Hess (1965), was conducted using the cultivar 'Berken' mung bean (Vigna Radiata (L.) R. Wilcz.), with several modifications, some of which have been described previously (Raviv and Reuveni, 1984). Additional modifications were: (1) only one cutting was planted in each vial, thus avoiding possible interactions among cuttings. Concentrations of tested compounds can therefore be expressed on a per cutting rather than on a per vial

basis. The solution volume was adjusted daily to 3.5 ml; (2) a selected mung bean line having lower variability and higher responsiveness to root promoters was used. Indole-3-butyric acid (IBA) served as a test compound while selecting this line. Cuttings of this line root faster and root counting was done after 5 days; (3) seedling growth medium was saturated and irrigated with a solution of $Ca(NO_3)_2$ at 140 ppm and H_3BO_3 at 1 ppm. The use of this solution instead of distilled water resulted in healthier and more uniform cuttings.

RESULTS

Roots were visible on 'Rangpur' cuttings at Day 14; after 19 days 77% of the cuttings rooted while the remainder swelled at the base and formed a callus (Table 1). At that time no rooting was observed in the 'Oroblanco' cuttings and only 31% had any basal swelling or had developed a callus (Table 1). After 36 days, 100% of the 'Rangpur' cuttings had rooted, while 12% of the 'Oroblanco' cuttings had rooted and 80% had formed a callus. Root density was slightly higher in 'Rangpur', but the final root length of 'Oroblanco' was almost four times greater than that of 'Rangpur' (Table 1).

On the day of excision (Day 0), higher levels of IAA were found in leaves and bark of 'Rangpur' than of 'Oroblanco', but the difference was not statistically significant (Table 2). Although IAA content in the leaves at Day 19 was slightly higher in the 'Rangpur' lime than in 'Oroblanco', it was not statistically different (Table 2). In both species, IAA levels in the bark were higher than in the leaves. At Day 19, when 77% of the 'Rangpur' cuttings rooted, the IAA content in the bark of 'Rangpur' was 3.5 times higher than

TABLE 1

Rooting and callus formation of 'Rangpur' lime and 'Oroblanco' cuttings 19 and 36 days after excision; mean values ± SE

	Species	
	'Rangpur'	'Oroblanco'
Average cutting length (cm)	16.1 ± 1.3	24.4 ± 2.4
Average no. of leaves	8.4 ± 1.6	11.7 ± 1.9
19 days		
Callus (%)	22.5 ± 2.1	30.8 ± 2.2
Rooting (%)	77.5 ± 2.1	0
36 days		
Callus (%)	0	79.3 ± 5.7
Rooting (%)	100	12.2 ± 1.2
Root density[1]	2.6 ± 0.6	2.3 ± 0.4
Average root length (cm)	1.5 ± 0.4	5.5 ± 0.7

[1]Root density was rated on a scale from 1 (tap root only) to 5 (fully covered by root hairs).

TABLE 2

Content of IAA (pmol g^{-1} fresh weight) in leaves and bark (3–4 cm from the base) taken from cuttings of two citrus species 'Rangpur' lime and 'Oroblanco' at excision and after 19 days

Plant species	Leaves		Bark	
	0[1]	19	0	19
'Rangpur'	276±40 a	410±34 a	393±20 a	1333±161 b
'Oroblanco'	206±33 a	338±39 a	308±25 a	372±21 a

[1]Days from excision.
Data represent mean values ± SE. Values within column with the same letter do not differ significantly at $P=0.05$ (Student's t-test).

TABLE 3

ARP-like activity (mung bean bioassay) and type in extracts from leaves and bark of 'Rangpur' and of 'Oroblanco' cuttings at excision and after 19 days

Plant material	Days from excision	Average no. of roots per cutting	ARP type
Leaves			
'Rangpur'	0	10.6±1.47 ab	A, B
	19	10.7±1.48 ab	A, B
'Oroblanco'	0	8.5±1.51 b	A, B
	19	8.3±1.42 b	A, B
Bark			
'Rangpur'	0	8.7±1.35 b	NT
	19	12.6±2.80 a	NT
'Oroblanco'	0	8.1±1.41 b	NT
	19	7.6±1.02 c	NT
Control (water)	-	6.1±0.65 c	-

Mung bean replicates were treated with 50 mg equivalent dry weight of plant extract; rooting data are the mean value ± SE. Values with a common letter do not differ statistically at $P=0.05$ (Duncan's MRT test). NT, not tested.

that at Day 0. At Day 19, IAA levels in the bark of 'Oroblanco' were significantly lower (by 3.6 times) than in the bark of 'Rangpur'. In the 'Rangpur' lime cuttings the bark-to-leaf ratio of IAA was greater than three, while in 'Oroblanco' this ratio was about one.

Using GC analysis, ARP-A and ARP-B were detected in leaves of 'Rangpur' and 'Oroblanco' at excision time and at Day 19 (data not shown). As with IAA, ARP-like rooting activity was slightly higher in leaves and bark of 'Rangpur' lime cuttings as compared with 'Oroblanco' at time of excision, but the difference was not significant (Table 3). At Day 19, the ARP-like rooting activity rose some 45% in the basal bark of the 'Rangpur' lime cuttings, while

no such change was found in 'Rangpur' leaves or in 'Oroblanco' leaves or bark.

DISCUSSION

It is assumed that adventitious rooting depends to a great extent upon auxin levels (Haissig, 1970; Ericksen, 1974; Jarvis, 1986; Hartmann et al., 1989). Studies of auxin metabolism during rooting of mung bean cuttings, using radiolabeled IAA, indicate that IAA is metabolized very quickly (Norcini et al., 1975; Norcini and Heuser, 1988). In the present study we measured the IAA levels in two citrus species which differ in their rooting ability, to see if the difference in rooting is correlated with the endogenous IAA content. The amount of free IAA in the bark of the basal zone of the cuttings taken from the easy-to-root 'Rangpur' lime after 19 days was more than three times that in the difficult-to-root 'Oroblanco' (Table 2). These results support the suggestion that high levels of auxins are associated with promotion of the first stages of adventitious rooting (Haissig, 1970; Ericksen, 1974; Jarvis, 1986; Hartmann et al., 1989). Furthermore, after 19 days, when rooting in the 'Rangpur' lime was 77% (Table 1), it was accompanied by a marked 3.5-fold increase in IAA content, as compared with the level at time of excision (Table 2). As the greatest effect of exogenous auxin on adventitious root formation is during early development (Haissig, 1970; Gaspar and Hofinger, 1988; Hartmann et al., 1989), this IAA increase can have a pronounced effect on the rooting of the cuttings. Since most work which has been done on the role of auxins during adventitious rooting is concerned with exogenously supplied auxins, little is known about the hormone status within the plant. Nineteen days after excision, we found a marked increase of IAA in the basal bark of the cuttings in the easy-to-root 'Rangpur' lime. This might be due to the polar basipetal outflow of auxin through the system that is arrested at the cut end of the cutting (Weigel et al., 1984), owing to decline in IAA oxidase (Mato and Vieitez, 1986), or to the breakdown of bound auxins that might function as a source of IAA during the stage of rooting (Norcini and Heuser, 1988). In this study we show for the first time an increase in endogenous IAA content concomitant with the rooting of citrus cuttings. It should be noted that cuttings of 'Oroblanco' do not root well when their bases are treated with IBA (Sagee et al., 1990). It is possible that in the difficult-to-root 'Oroblanco', IBA and free IAA are metabolized rapidly (Norcini and Heuser, 1988; Wiesman et al., 1989), leaving no free auxin in the tissue. In the easy-to-root 'Rangpur' lime, the higher IAA levels can result from increased synthesis, increased catabolism of conjugated IAA, or inhibition of IAA catabolism.

The existence of ARP compounds in additional species other than avocado was demonstrated recently (Becker et al., 1991). In this report we show evidence, for the first time, of the presence of ARP in citrus. Differences in ARP

levels in the leaves of 'Rangpur' and 'Oroblanco' were detected at the time of excision, but they were not significant and it is not possible to correlate differences in rooting ability of citrus species to ARP level without additional study to confirm the present results. On the other hand, the increase in ARP-like activity in the basal bark of 'Rangpur' cuttings on Day 19 coincides with that of free IAA and may be of significance. A similar trend was found in avocado cuttings (M. Raviv, unpublished observations, 1987).

In conclusion, this work demonstrates that higher levels of IAA and ARP activity appear to correlate with ease of rooting of the citrus cultivars studied, but it is not possible to tell whether the increase in IAA and ARP over the rooting period is the cause or result of root initiation. In the future we intend to clarify whether these compounds play a direct role in the adventitious root formation of citrus cultivars.

ACKNOWLEDGMENTS

We would like to gratefully thank Prof. W P. Hackett and Prof. A. Altman for critical reading of the manuscript, and Dr. O. Reuveni for his advice and help. This research was partially supported by grants from BARD, the US–Israel Binational Agricultural Research and Development Fund, no. I-908-85 to M. Raviv and D. Becker and no. I-1669-89R to O. Sagee.

REFERENCES

Beck, G.R. and Sink, K.C., 1974. The response of poinsettia cultivars to auxins in root promotion of stem cuttings. Scientia Hortic., 2: 231–236.

Becker, D., Raviv, M., Cosse, A., Medina, Sh. and Reuveni, O., 1991. Avocado rooting promoter (ARP) detection in *Laurus nobilis* L. and *Magnifera indica* L. based on gas chromatography. Scientia Hortic., 43: 325–330.

Cohen, J.D., 1984. Convenient apparatus for the generation of small amounts of diazomethane. J. Chromatogr., 303: 193–196.

Ericksen, E.N., 1974. Root formation in pea cuttings. II. The influence of indole-3-acetic acid at different development stages. Physiol. Plant., 30: 158–162.

Gaspar, T. and Hofinger, M., 1988. Auxin metabolism during adventitious rooting. In: D. Davis, B.E. Haissig and N. Sankhla (Editors), Adventitious Root Formation in Cuttings. Dioscorides Press, Portland, OR, pp. 117–131.

Gorter, C.J., 1969. Auxin-synergists in the rooting of cuttings. Physiol. Plant., 22: 497–502.

Haissig, B.E., 1970. Influence of indole-3-acetic acid on adventitious root primordia of brittle willow. Planta, 95: 27–35.

Hartmann, H.T., Kester, D.E. and Davies, F.T., 1989. Plants propagation. Principles and Practices. 5th edn., Prentice-Hall, Englewood Cliffs, NJ, pp. 199–255

Hemberg, T., 1954. The relation between the occurrence of auxin and rooting of hypocotyls in *Phaseolus vulgaris* L. Physiol. Plant., 7: 323–331.

Hess, C.E., 1965. Rooting co-factors, identification and functions. Proc. Int. Plant Propag. Soc., 15: 181–186.

Jarvis, B.C., 1986. Endogenous control of adventitious rooting in non-woody cuttings. In: M.B. Jackson (Editor), New Root Formation in Plants and Cuttings. Martinus Nijhoff, Dordrecht, Netherlands, pp. 191–222.

Mato, M.C. and Vieitez, A.M., 1986. Changes in auxin protectors and IAA oxidases during rooting of chestnut shoots in vitro. Physiol. Plant., 66: 491–494.

Mohammed, S. and Ericksen, E.N., 1974. Root formation in pea cuttings. IV. Further studies on the influence of indole-3-acetic acid at different development stages. Physiol. Plant., 32: 94–96.

Norcini, J.G. and Heuser, C.W., 1988. Changes in the level of [^{14}C]indole-3-acetic acid and [^{14}C]indole acetyl aspartic acid during root formation in mung bean cuttings. Plant Physiol., 86: 1236–1239.

Norcini, J.G., Heuser, C.W. and Hamilton, R.H., 1975. Changes in free and conjugated indole-3-acetic acid during initiation and early development of adventitious roots in mung bean. J. Am. Soc. Hortic. Sci., 110: 528–533.

Pluss, R., Jenny, T. and Meier, H., 1989. IAA-induced adventitious root formation in green wood cuttings of *Populus tremula* and formation of 2-indolone-3-acetyl aspartic acid, a new metabolite of exogenously applied indole-3-acetic acid. Physiol. Plant., 75: 89–96.

Raviv, M. and Reuveni, O., 1984. Endogenous content of leaf substance(s) associated with rooting ability of avocado cuttings. J. Am. Soc. Hortic. Sci., 109: 284–287.

Raviv, M., Becker, D. and Sahali, Y., 1986a. The chemical identification of root promoters extracted from avocado. Plant Growth Regul., 4: 371–374.

Raviv, M., Reuveni, O. and Goldschmidt, E.E., 1986b. Evidence for the presence of a native, non-auxinic rooting promoter in avocado. Plant Growth Regul., 4: 95–102.

Raviv, M., Reuveni, O. and Goldschmidt, E.E., 1987. The physiological basis for loss of rootability with age of avocado seedlings. Tree Physiol., 3: 115–122.

Sagee, O., Maoz, A., Mertens, R., Goren, R. and Riov, J., 1986. Comparison of different enzyme immunoassays for measuring indole-3-acetic acid in vegetative citrus tissues. Physiol. Plant., 68: 265–270.

Sagee, O., Shaked, A., Hemo, M. and Hasdai, D., 1989. Rooting of citrus cuttings. Alon hannotea, 43: 1365–1374 (in Hebrew with English summary).

Sagee, O., Shaked, A. and Hasdai, D., 1990. Rooting of cuttings from gibberellin and benzyladenine-treated citrus trees. J. Hortic. Sci., 65: 473–478.

Stoltz, L.P., 1968. Factors influencing root initiation in an easy- and a difficult-to-root *Chrysanthemum*. Proc. Am. Soc. Hortic. Sci. 92: 622–626.

Weigel, U., Horn, W. and Hock, B., 1984. Endogenous auxin levels in terminal stem cuttings of *Chrysanthemum morifolium* during adventitious rooting. Physiol. Plant., 61: 422–428.

Wiesman, Z., Riov, J. and Epstein, E., 1989. Characterization and rooting ability of indole-3-butyric acid conjugates formed during rooting of mung bean cuttings. Plant Physiol., 91: 1080–1084.