Phytochemistry, Vol. 25, No. 12, pp. 2783–2785, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00

BIOSYNTHESIS OF LIMONOIDS IN *CITRUS*: SITES AND TRANSLOCATION

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(*Received* 11 *March* 1986)

Key Word Index—*Citrus limon*; Rutaceae; lemon; limonoids; biosynthesis; sites; translocation.

Abstract—Sites of limonoid biosynthesis were located in *Citrus limon*. The stem was found to be the major site of nomilin biosynthesis from acetate. Epicotyl, hypocotyl and root tissues were also capable of biosynthesizing nomilin from acetate, but leaves, fruits and seeds did not show this capacity under the conditions used. All the tissues tested were capable of biosynthesizing other limonoids starting from nomilin. *C. limon* was capable of translocating nomilin from the stem to other sites.

INTRODUCTION

Bitterness due to limonoids in a variety of citrus juices is a major problem of the worldwide citrus industry and has a significant negative economic impact. One of our approaches to solve this bitterness problem is to develop a preharvest treatment method for reduction or elimination of bitter limonoids in fruits. For this purpose, it is essential to have a fundamental knowledge of the biosynthesis and metabolism as well as the sites and translocation of limonoids in *Citrus*. During the past few years, substantial progress has been made in the area of limonoid biochemistry in *Citrus* [1–4]. In addition, auxins have been found to be potent inhibitors of the biosynthesis of nomilin, one of the early precursors of all the *Citrus* limonoids known to be present in citrus [5]. In this study, we report sites of limonoid biosynthesis in *Citrus limon* and also report the translocation of limonoids from the sites of biosynthesis to other locations.

RESULTS AND DISCUSSION

The best way and perhaps the only way to find sites of limonoid biosynthesis in *Citrus* is to detach various tissues such as leaves, stems, roots, seeds and fruit tissues, and examine them separately for their ability to biosynthesize limonoids. At first, it was necessary to find whether tissues detached from trees are still capable of biosynthesizing limonoids. Thus, stems of young *C. limon* seedlings were used for this purpose because there was already a strong indication that the intact stem is capable of biosynthesizing limonoids from acetate [1, 2].

Stems were cut to 1 cm lengths and fed with 1 μCi of acetate from the cut end. After 3 days of incubation with a continuous supply of water under dark or light (330 footcandles) at 22°, the stems were analysed for labelled limonoids. The results are shown in Table 1. Detached stems very actively biosynthesized nomilin from acetate. For instance, one experiment showed that 92 200 cpm acetate was incorporated into nomilin under light, which was equivalent to 4.51 % of the original activity. Similarly, two other experiments showed the incorporation of 5.38 and 4.90 % of the original activity into nomilin, respectively. These levels of incorporation were as high as those of intact tissues [1]. Light was not required for the biosynthesis of nomilin from acetate in detached stems of young lemon seedlings as shown by the fact that in the dark 3.60 and 4.90 % of the original acetate activity were incorporated into nomilin. These results indicated that detached stem tissues are capable of biosynthesizing nomilin from acetate, and detached tissues can be used for studies of locating the sites of limonoid biosynthesis.

Table 2 shows the results of limonoid analyses of various sites of *C. limon* fed with labelled acetate or nomilin. The stem tissue was found to be the most active site of nomilin biosynthesis from acetate. Hypocotyl and epicotyl tissues, and roots were also capable of biosynthesizing nomilin from acetate. As suggested previously [6], fruit tissues and seeds, immature or mature, did not biosynthesize limonoids from acetate nor, surprisingly, did leaf tissues under the conditions used. Although these tissues did not biosynthesize limonoids from acetate, they did metabolize the acetate into other labelled compounds. Starting from labelled nomilin, however, all the tissues tested were capable of biosynthesizing other limonoids such as obacunone, obacunoate and limonin

Table 1. Biosynthesis of nomilin from acetate in detached stems of young *Citrus limon* seedlings

	Nomilin	
Conditions	cpm	%
Light	92 200	4.61
	108 000	5.38
	98 000	4.90
Dark	72 000	3.60
	98 000	4.90

One μCi [1-^{14}C]acetate was fed to 1-cm long stems and incubated under dark or light (330 footcandles) for 3 days at 22°.

Table 2. Biosynthesis of limonoids in various sites of *Citrus limon*

Sites	From acetate to nomilin (cpm)*	From nomilin to other limonoids
Stems	92200	+
	(88500)	
Epicotyl and hypocotyl	25400	+
	(2100)	
Leaves	—	
	(—)	+
Roots	8970	+
	(2500)	
Fruit tissues	—	+
	(—)	
Seeds	—	+
	(—)	

*One μCi of labelled acetate was fed to the tissues of young seedlings and incubated for 3 days at 22°.

( ): Duplicate. —: No incorporation of labelled acetate to limonoids. In cases of no incorporation, experiments were repeated at least five times for each tissue. +: Positive incorporation of labelled nomilin to other limonoids such as obacunone, obacunoate and limonin.

Table 3. Translocation of [^{14}C]nomilin from the stem to other locations in *Citrus limon* seedlings

Locations	Total activity (cpm)	Nomilin activity (cpm)
Stem	30500	7200
	(39800)	(20600)
Leaves	48000	23100
	(55700)	(38900)
Hypocotyl and epicotyl	5200	3700
	(14300)	(11800)
Roots	4800	3200
	(13100)	(10100)

100000 cpm (150000 cpm) of [^{14}C]nomilin was fed to the stem of a seedling at 2 cm above the ground and incubated in a greenhouse. After 24 hr (18 hr) of incubation, each location was analysed separately for total radioactivity and labelled nomilin.

from nomilin. We also observed previously the conversion of obacunone to obacunoate and limonin, and obacunonate to limonin in all the sites tested [2–4].

Previous work showed that nomilin is the major, if not only, limonoid biosynthesized and accumulated in the stems of young citrus seedlings such as *C. limon*, *C. paradisi* and *C. sinensis* [1]. ^{14}C-Labelled nomilin isolated from the stem consistently had a specific activity several times greater than that isolated from the leaves [1]. This evidence and the results obtained in this study show that nomilin is biosynthesized in stem tissues, and suggest strongly that nomilin is the major limonoid translocated from the stem to other locations.

In order to provide additional evidence to support the above, we designed experiments to find whether *C. limon* is capable of translocating nomilin from the stem tissues to other locations. When 120000 cpm of nomilin was fed to the stem of a young *C. limon* seedling at a location 2 cm above the ground, radioactivity was distributed to the whole seedling after 24 hr of incubation (Table 3). TLC radiochromatographic analyses of each tissue extract showed that labelled nomilin was positively detected in all the locations tested. This experiment clearly shows that *C. limon* is capable of translocating nomilin from stem tissues to other locations.

When labelled acetate was fed to stems of young citrus seedlings such as *C. limon*, *C. paradisi* and *C. sinensis*, up to 5% of the original activity was incorporated into nomilin [1]. When labelled acetate was fed to a detached fruit, either mature or immature, activity was not incorporated into any limonoids. However, when a 0.5 g size fruit attached to a 3-cm stem was fed with 10 μCi of [^{14}C]acetate through the cut end of the stem, activity was incorporated into nomilin in the stem (183000 cpm) and in the fruit (23800 cpm) after 24 hr of incubation. After 48 hr, in addition to labelled nomilin, labelled limonin (5500 cpm) was also observed in the fruit, suggesting that nomilin was first biosynthesized in the stem and translocated to the fruit, where nomilin was further metabolized to limonin.

When [2-^{14}C]mevalonic acid was fed by injection or absorption to orange fruits, Datta and Nicholas [7] also observed no incorporation of radioactivity into limonin. However, they obtained radioactive limonin from germinated Valencia orange seeds. We believe that, most likely, the labelled limonin found by them had been biosynthesized in the root and stem portions of germinated seeds. We could find no labelled limonoids biosynthesized from mevalonate [6] or acetate in dormant Valencia orange or lemon seeds.

Leaves were once thought to be the major site of limonoid biosynthesis [6] because when labelled acetate was fed to lemon fruits, the activity was not incorporated into limonoids. However, when labelled acetate was fed to leaves of lemon trees, the activity was incorporated into limonin in the fruit adjacent to the fed leaves [6]. In this case, the labelled acetate most likely migrated to the stem where it was converted to nomilin. The nomilin was then further translocated to the fruit tissues and there it was converted to limonin. It is very difficult to prove negative findings. Leaves could be a site of limonoid biosynthesis starting from acetate, but the data show that they are apparently not the major site. The results obtained thus far indicate that the stems are the major site of nomilin biosynthesis from acetate, and suggest also that the stem nomilin is then translocated to other locations such as leaves, fruits and seeds. There, other limonoids are further biosynthesized from nomilin.

EXPERIMENTAL

Materials. Citrus limon seedlings (10 cm high with five or six leaves) and trees bearing fruits used in this study were grown at the Pasadena Laboratory. [1-^{14}C]Acetate (56 mCi/mmol) was purchased from Dupont New Products, MA. [^{14}C]Nomilin (1 mCi/mmol) was prepared as described in ref. [1].

Feeding of labelled materials. Labelled materials were fed to the stem of a seedling at 2 cm above the ground through a wet string by the procedure described in ref. [1]. They were then incubated in our greenhouse. For detached tissues, they were fed through the cut area and incubated at 22° under dark or light (330 footcandles).

Extraction and analysis. Tissues fed with radioactive material were extracted by the procedures described previously [1]. The extracts were spotted onto silica gel plates which were developed with solvent systems (a) EtOA–cyclohexane (3:2), (b) CH_2Cl_2–MeOH (97:3) and (c) CH_2Cl_2–EtOAc (3:2). TLC radiochromatograms were scanned with a Berthold automatic TLC-linear analyser LB 2832. Total radioactivity was counted with a Beckman liquid scintillation system, LS-3133P.

Identification of labelled metabolities. Nomilin was identified as described in ref. [1], and other limonoids such as obacunone, obacunoate, deacetylnomilin and limonin were also identified by the procedures published previously [2, 3].

Acknowledgements—The authors thank Dr. V. P. Maier for helpful discussions in the preparation of this manuscript and also Mr. H. Quach for the preparation of *Citrus* seedlings.

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