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LIMONOID BIOSYNTHESIS IN THE STEM OF *CITRUS LIMON*

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

Abstract—Using radioactive tracer techniques, the phloem region of *Citrus limon* stems was shown to be the site of limonoid biosynthesis from acetate. The cortex and inner core regions were found incapable of biosynthesizing limonoids from acetate. However, all the regions were capable of biosynthesizing other limonoids starting from nomilin.

INTRODUCTION

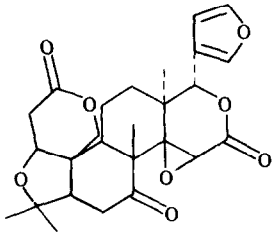
Bitterness due to limonin (**1**) in a variety of citrus juices is a major problem of the citrus industry and has significant negative economic impact. During the past few years, the biosynthetic pathways of the major limonoids have been well established and those of the minor limonoids have been proposed [1–5].

One of our approaches to the bitterness problem has been to develop a preharvest treatment method to reduce the accumulation of limonin (**1**) in the fruit. The significant step toward that goal was our finding that auxins are potent inhibitors of limonoid biosynthesis in citrus seedlings [5]. To determine auxin treatment conditions to reduce limonin accumulation in the fruit, it was necessary to know the exact site(s) of limonoid biosynthesis in citrus. The objective of the present study was to locate the site(s) in question.

RESULTS AND DISCUSSION

Limonoids, mainly nomilin (**2**), have been shown to be biosynthesized from acetate in the stem of citrus and translocated to other locations such as leaves, fruit tissues and seeds [4]. Our previous study showed that detached as well as attached stems of young seedlings are capable of biosynthesizing limonoids from acetate, and suggested that detached stem tissues are ideal tools for studies of limonoid biochemistry [5]. Therefore, in this study, detached stems of young *Citrus limon* seedlings were used to locate the exact site(s) of limonoid biosynthesis from acetate in the stem.

The stem of lemon seedlings was separated into three regions (Fig. 1). Microscopic analyses showed that they are (i) the cortex region consisting of epidermis, collenchyma cells and parenchyma cells (A), (ii) the phloem region consisting of mainly phloem and some paren-



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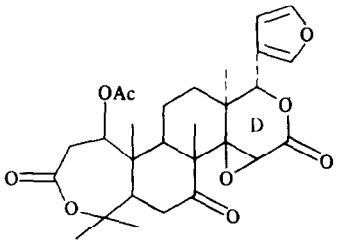

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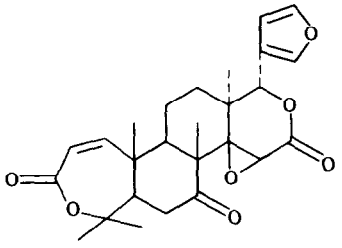

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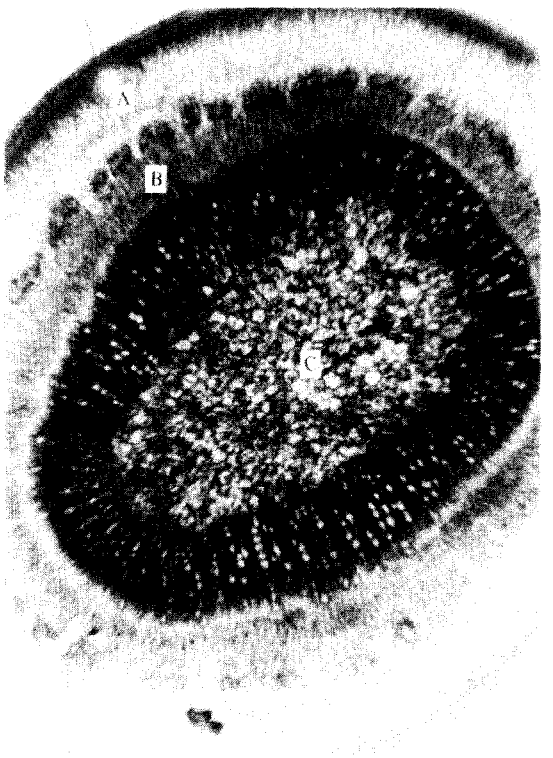

Fig. 1. Cross section of the stem of a *Citrus limon* seedling. (A) cortex region, (B) phloem region and (C) inner core region (× 32 magnification).

chyma cells, fibres and primary xylem (B), and (iii) the inner core region consisting of secondary xylem and pith (C).

Each region and whole stem were incubated with [^{14}C] acetate to find which region is the site of limonoid biosynthesis from acetate. Radiochromatographic analyses of the extracts showed that labelled acetate was incorporated into nomilin (**2**) in the whole stem (Fig. 2), which confirmed the previous results [4]. The combined region of cortex and phloem had incorporation similar to the whole stem (Table 1). Figure 2 also shows that the phloem region is the site of limonoid biosynthesis from acetate in the stem. Limonoid peaks, mainly nomilin (**2**), were found only in the phloem region of the stem. There was no incorporation of the radioactivity into limonoids in the cortex and inner regions, except for one of the six replicate experiments with the cortex in which 0.043% of the original radioactivity was incorporated into nomilin (**2**), while the other five gave zero incorporation (Table 1). This small but positive incorporation was most likely due to a small amount of phloem tissue that was not completely separated and remained attached to the cortex. Microscopic examination revealed that the separated cortex region occasionally included a small portion of the phloem tissues. Further careful investigation with longer incubation times, up to five days, and larger sample sizes confirmed that both the cortex and inner regions were incapable of biosynthesizing limonoids from acetate.

In two replicate experiments with the whole stem, 1.09 and 0.77% of the original radioactivity was incorporated into nomilin (**2**); in the phloem region, 3.21, 2.89 and 2.80% of the original radioactivity was incorporated into

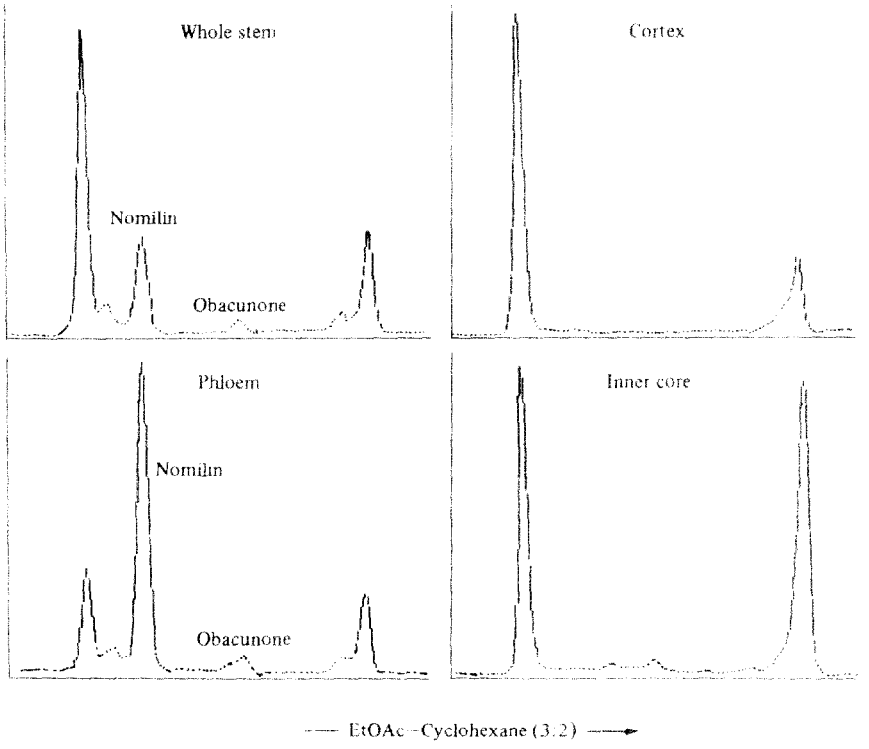

Fig. 2. Radiochromatograms of extracts of various *Citrus limon* stem tissues fed with sodium [1-^{14}C]acetate.

Table 1. Biosynthesis of nomilin from sodium [1-^{14}C] acetate in various stem tissues of *Citrus limon* seedlings

Tissues	Acetate fed (μCi)	Nomilin cpm	Nomilin Incorporation (%)
Whole stem	5	109200	1.09
	5	76800	0.77
Cortex plus phloem region	10	153700	0.77
Cortex region	5	0	0
	10	0	0
	5	4200	0.043
	5	0	0
	5	0	0
	5	0	0
Phloem region	5	320900	3.21
	10	575500	2.89
	5	279700	2.89
Inner core region	5	0	0
	5	0	0
	5	0	0
	5	0	0
	5	0	0

nomilin (Table 1). It is quite understandable that the extent of the incorporation of labeled acetate into nomilin in the phloem region was higher than that in the whole stem.

A small but positive radioactive obacunone (**3**) peak was also identified in both the whole stem and the phloem regions. No other limonoid peaks were detected under the conditions used. Nomilin (**2**) has been shown to be a direct precursor of obacunone (**3**) in the biosynthetic pathways of limonoids in citrus [2]. Therefore, obacunone (**3**) shown in the phloem region and whole stem was apparently a metabolite of nomilin (**2**). These results clearly show that the biosynthesis of limonoids from acetate occurred only in the phloem region of the stem.

Data obtained thus far showed that the phloem region of stems was the only site of limonoid biosynthesis from acetate in citrus. Other tissues such as leaves, fruit tissues and seeds were either incapable of biosynthesizing limonoids from acetate or had a very low capacity. However, they have been found capable of biosynthesizing limonoids starting from nomilin and obacunone [4]. In this study, we found that the phloem, inner core and cortex regions were also capable of biosynthesizing obacunone (**3**) from nomilin (**2**), and limonin (**1**) from obacunone (**3**). When 38 200 cpm of [^{14}C] nomilin was fed to the phloem, inner core and cortex regions by the procedures similar to the labelled acetate feeding method, a small but positive amount of radioactivity was incorporated into obacunone in all three regions. The extent of the incorporation was approximately 1%. When 47 000 cpm [^{14}C] obacunone was fed to each of the three regions, 4, 8 and 24% of the radioactivity was converted to limonin in the phloem, cortex and inner core regions, respectively.

The conversion of nomilin (**2**) to obacunone (**3**) is catalyzed by nomilin acetyl-lyase which has been isolated from cell-free extracts of *Corynebacterium fascians* [6], but it has not yet been isolated from citrus. Thus far, we have observed a large accumulation of nomilin in young citrus seedlings. This is most likely due to the lack of nomilin acetyl-lyase activity in the seedlings. In support of this, we have shown on many occasions through feeding of labelled substrates that other enzymes leading from obacunone to limonin are present in the seedlings. Therefore, this enzyme is under a different regulatory control from the other enzymes and is definitely related to the age of the tissue. As the fruit grows, the activity increases. Consequently, the bioregulation of this enzyme is attractive as an approach to the control of limonin bitterness in citrus.

Auxins such as 1-naphthaleneacetic acid, indoleacetic acid, indolebutyric acid and 2,4,5-trichlorophenoxyacetic acid have been shown to be potent inhibitors of nomilin biosynthesis in the stem and cause less accumulation of limonin in the fruit [5, 7]. This inhibition occurred when auxins were fed to the seedling through a wet string penetrating the stem. Both ends of the string were placed in a reservoir bottle where the feeding solution was supplied. This feeding method, however, is not practical in field operation. To be practical the method must allow introduction of auxins into the phloem region of the stem.

EXPERIMENTAL

Materials. Citrus limon seedlings (10–20 cm in height) were grown in our greenhouse. Na[1-^{14}C]acetate (54 mCi/mmol) was purchased from Dupont New Products, Billerica, Massachusetts. [^{14}C]Nomilin was biosynthesized from labelled acetate with lemon seedlings by the procedures of ref. [1]. [^{14}C]Obacunone was enzymatically prepared from [^{14}C]nomilin with nomilin acetyl-lyase [6].

Procedures. Stems (4 mm in diameter) were cut into pieces 1 cm long and separated into 3 sections using a scalpel and forceps (Fig. 1). Each section was placed in a *V*-shaped small vial and 5 or 10 μCi labelled acetate in aq. soln was added to each vial. After two days of incubation at 22°, the tissue was washed thoroughly with H_2O and radioactive compounds were extracted by procedures described previously [1]. [^{14}C]Nomilin and

[^{14}C]obacunone feedings were carried out by the procedures similar to the acetate feeding.

The extracts were spotted on silica gel TLC plates and developed with the following solvent systems: (a) EtOAc–cyclohexane (3:2); b) CH_2Cl_2–MeOH (97:3); (c) EtOAc–CH_2Cl_2 (2:3) and (d) toluene–EtOH–H_2O–HOAc (200:47:15:1, upper layer). TLC radiochromatograms were obtained with a Berthold Automatic TLC-Linear Analyzer LB 2832. Total radioactivity was counted with a Beckman Liquid Scintillation System, LS-3133P.

Nomilin was identified by the procedures described previously [1], and other limonoids such as limonin and obacunone were also identified by the procedures described previously [2, 8].

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