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PHYTOHORMONE BIOREGULATION OF NOMILIN BIOSYNTHESIS IN *CITRUS LIMON* SEEDLINGS

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Key Word Index—*Citrus limon*; bioregulation; inhibition; indoleacetic acid; auxins; abscisic acid; nomilin; biosynthesis.

Abstract—Auxins such as indoleacetic acid, indolebutyric acid, naphthaleneacetic acid and 2,4,5-trichlorophenoxyacetic acid were found to be potent inhibitors of nomilin biosynthesis in young seedlings of *Citrus limon*. Up to 97% of the biosynthesis was inhibited. The auxins used were all effective and inhibited the biosynthesis of nomilin selectively. Abscisic acid was also a potent inhibitor of nomilin biosynthesis and the inhibition was reversed with a cytokinin. Gibberellic acid (GA_3) had no effect on nomilin biosynthesis.

INTRODUCTION

Bitterness due to limonoids such as limonin and nomilin (1) in a variety of citrus juices is a major problem of the worldwide citrus industry and has significant negative economic impact. Limonin is the major cause of limonoid bitterness and is present in all citrus juices. Substantial progress has been made in the study of limonoid biosynthesis in citrus during the past few years [1–4]. Recently, we showed that young seedlings of *Citrus limon* very actively biosynthesize nomilin and that nomilin is the initial precursor of all the limonoids known to be present in *Citrus* and *Citrus* hybrids [1, 3]. We report here that phytohormones such as auxins and abscisic acid inhibit biosynthesis of nomilin in *C. limon*.

RESULTS AND DISCUSSION

Young *Citrus limon* seedlings are excellent tools for studies of the biosynthesis and bioregulation of nomilin (1) since they biosynthesize nomilin very efficiently from acetate [1]. Therefore, studies on effects of phytohormones on nomilin biosynthesis were carried out in young lemon seedlings with ^{14}C-labelled acetate. We found that auxins and abscisic acid were potent inhibitors of nomilin biosynthesis in the seedlings.

Figure 1 shows typical curves of TLC radiochromatograms of the extracts obtained from the lemon seedlings treated with or without 10 ppm of indoleacetic acid (IAA). The major peak shown in the control was identified as nomilin by the procedure described previously [1]. IAA reduced the size of the nomilin peak significantly, showing that IAA is a potent inhibitor of nomilin biosynthesis. This radiochromatographic analysis also suggested that IAA inhibits nomilin biosynthesis selectively because it had no significant effect on nonlimonoid radioactive peaks. The acetone extract obtained from the lemon seedling treated with or without 10 ppm of IAA showed also that there were 10 major peaks on the TLC radiochromatogram, and only the nomilin peak was significantly affected by IAA.

When ^{14}C-labelled acetate was fed simultaneously with IAA to the seedlings, we observed a significant decrease in incorporation of labelled acetate into nomilin. When 20 ppm of IAA was fed and incubated for 2 days, 45, 60 and 70% of nomilin biosynthesis were inhibited.

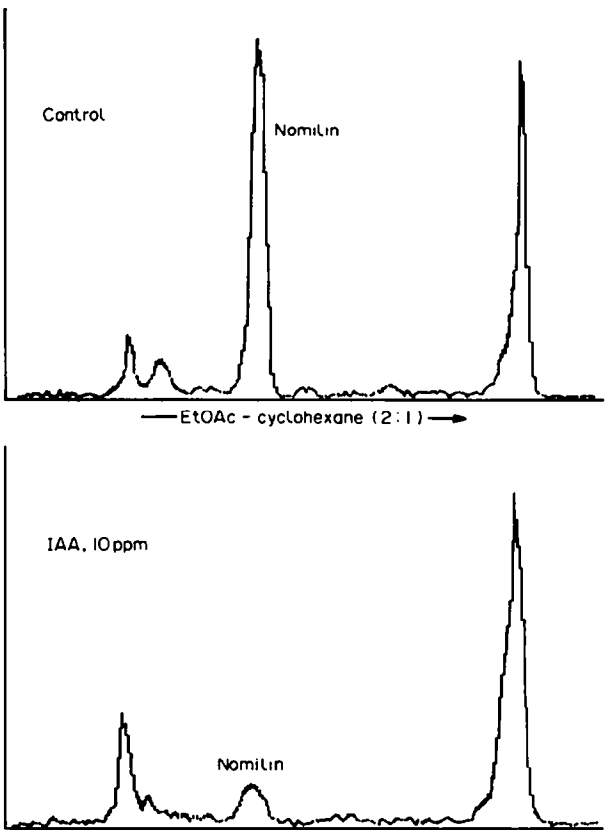
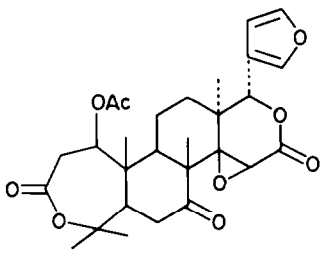


Fig. 1. Radiochromatograms of the extracts obtained from *Citrus limon* treated with or without 10 ppm IAA.



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Increasing the IAA concentration up to 100 ppm did not enhance the inhibition of nomilin biosynthesis. For example, feeding 100 ppm IAA resulted in inhibition of 60, 70 and 72%.

However, the extent of inhibition was greatly increased when IAA feeding was started two days prior to the acetate feeding (Table 1). For instance, up to 91% inhibition was observed when 10 ppm IAA was fed to the stem of a lemon seedling two days prior to and during the feeding of labelled acetate. Increasing the IAA concentration to 25 and 50 ppm did not enhance the inhibitory action. Other auxins tested include indolebutyric acid, naphthaleneacetic acid and 2,4,5-trichlorophenoxyacetic acid (Table 2). They were also very effective. Up to 97.3% inhibition was achieved with 10 ppm of 2,4,5-trichlorophenoxyacetic acid.

Abscisic acid (ABA) also inhibited nomilin biosynthesis in *Citrus limon* seedlings at a substantially low concentration (Table 3). This inhibition was very potent and required only a few ppm of ABA to achieve up to 71% inhibition. When 3 ppm of ABA were fed to the seedlings and incubated for 2 days under conditions similar to those used for auxin feeding, inhibition was 71, 52 and 46%. When 10 ppm of ABA was fed to the seedlings, young leaves were dehydrated after three days of incubation and the experiment could not be continued.

In addition, it was found that a cytokinin, 6-benzylaminopurine, reversed the inhibitory effect of ABA on nomilin biosynthesis. When 3 ppm of 6-benzylaminopurine was fed to the plants with ABA simultaneously, the above inhibition was reduced to 20, 16 and 14%, respectively. Evidence of antagonism between ABA and cytokinins has been reported. For example, benzyladenine can completely counteract ABA inhibition of growth [5].

Table 1. Inhibition of nomilin biosynthesis by indoleacetic acid in *Citrus limon* seedlings

IAA (ppm)	Biosynthesis	
	Nomilin (dpm)	Inhibition
Control	1 127 900	0
10	100 400	91.1
25	125 000	88.9
50	95 300	91.5

IAA feeding started 48 hr prior to 25 μCi [1-^{14}C]acetate feeding and the IAA feeding continued during 48 hr of subsequent incubation.

Table 2. Inhibition of nomilin biosynthesis by auxins in *Citrus limon* seedlings

Auxins	Biosynthesis	
	Nomilin (dpm)	Inhibition (%)
Control	1 634 700	0
Indolebutyric acid	300 840	81.6
Naphthaleneacetic acid	295 200	81.9
2,4,5-Trichloro-phenoxyacetic acid	44 870	97.3

15 ppm auxin feeding started 48 hr prior to [1-^{14}C]acetate feeding and the auxin feeding continued during 48 hr of subsequent incubation.

Table 3. Influence of abscisic acid, cytokinin* and gibberellic acid on the biosynthesis of nomilin in *Citrus limon* seedlings

Treatments [ppm]	Nomilin biosynthesis		
	Exp. 1 (dpm)	Exp. 2 (dpm)	Exp. 3 (dpm)
Control [0]	1 355 000 (0)†	819 900 (0)	319 000 (0)
ABA [3]	398 800 (71)	396 200 (52)	173 000 (46)
GA_3 [3]	1 300 900 (4)	900 100 (−9)	300 300 (6)
Cytokinin [3]	848 700 (37)	679 200 (17)	294 500 (8)
ABA + Cyto. [3 + 3]	1 089 300 (20)	691 700 (16)	273 500 (14)

*6-Benzylaminopurine.
† % Inhibition is given in parentheses.

Curves of typical TLC radiochromatograms of the extracts obtained from the ABA treated tissues were very similar to those of the controls, but the total radioactivity recovered from the ABA treated tissues was much less than that of the control. ABA appeared to slow down other biosynthetic activities of the seedlings as well as nomilin biosynthesis. Therefore, the action of ABA on nomilin biosynthesis was different from that of the auxins in that ABA was less selective. The cytokinin alone also appeared to inhibit the biosynthesis of nomilin, but inhibition was much less than that of auxins and ABA. Gibberellic acid (GA_3), on the other hand, had no effect on the biosynthesis of nomilin.

Limonoids are biosynthesized in stems, epicotyl tissues and leaves of *Citrus* and translocated to fruit tissues [1, 6, 7]. There is no evidence of the biosynthesis of limonoids from acetate in fruit tissues [7]. Since nomilin is the initial precursor of all the limonoids known to be present in citrus [3], bioregulators such as auxins, ABA or their synthetic analogs might be able to control the accumulation of other limonoids in the tissues. It would

be particularly interesting to determine whether the inhibition of nomilin biosynthesis with auxins in stems and leaves would result in the reduction of intensely bitter limonin in the fruit tissues.

EXPERIMENTAL

Materials. The *Citrus limon* seeds used for germination were from our laboratory, and seedlings (about 10 cm height with 8–10 leaves) were grown in our greenhouse. [1-^{14}C]Sodium acetate (56 μCi/μmol) was purchased from New England Nuclear, Boston, MA. (+)-*cis*-Abscisic acid was obtained from Poling, S. M. and Norman, S. N. of our laboratory and it was 99% pure.

Feeding experiment. Aq. solns of phytohormones were fed with 20 μCi of labelled NaOAc to the seedlings through the stem by the procedure described previously [1]. After two days of incubation in the greenhouse, the young shoot was harvested and used for analysis.

Extraction and analysis of labelled limonoids. Limonoids were extracted from the stem and leaves by the procedure of Hasegawa *et al.* [1]. The extract was then analysed by TLC on silica gel plates with solvent systems: (a) EtOAc–cyclohexane (2:1) and (b) CH_2Cl_2–MeOH (97:3). TLC radiochromatograms were scanned with a Berthold Automatic TLC-linear Analyzer LB 2832.

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