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NIACIN BIOSYNTHESIS IN LEAF DISCS AND SEEDLINGS OF *CATTLEYA SKINNERI* (ORCHIDACEAE)

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Summary

Seedlings and leaf discs of *Cattleya skinneri* incorporated label from ^{14}C- and ^{3}H-labelled tryptophan into niacin. Label from [^{14}C]glycerol and [^{14}C]aspartate was not significantly incorporated into the vitamin.

Introduction

Numerous attempts have been made to enhance the growth of orchid seedlings or replace the need for fungal symbionts through the addition of vitamins to culture media. In most instances, the effects of vitamins have been inconclusive at best. Niacin is the only exception in that it has enhanced the growth of seedlings of several species (Schaffstein, 1938, 1941; Noggle and Wynd, 1943; Bahme, 1949; Mariat, 1949, 1952; Arditti, 1967b; for reviews see Arditti, 1967a, 1979; Arditti and Harrison, 1977) and may be provided by some mycorrhizas (Hijner and Arditti, 1973). Because of this, orchid seedlings are a good system for the study of niacin biosynthesis in plants.

Animals and some micro-organisms produce niacin via a catabolic pathway starting with tryptophan. Other micro-organisms synthesize the vitamin from aspartic acid and glyceraldehyde-3-phosphate. The evidence regarding the biosynthesis of niacin by flowering plants is inconclusive. Several reports have suggested that the tryptophan pathway is operative, whereas others have maintained that it is not. These differences in opinion are due to the fact that some research was carried out without the use of labelled precursors and other work did not utilize appropriate organisms, techniques or metabolites (for a review see Arditti and Tarr, 1979). The work reported here was carried out to overcome some of these difficulties and obtain information on the pathways of niacin biosynthesis in angiosperms and on the metabolism of orchid seedlings.

Materials and Methods

Seedlings

Seeds of *Cattleya skinneri* were germinated on Knudson C medium (Knudson, 1946) under aseptic conditions in the usual manner (Harrison and Arditti, 1970)

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and cultured on the initial culture medium for 12 months through several transfers. Prior to use the seedlings were pre-incubated for 3 days in autoclaved liquid medium on a shaker.

Leaf discs

Leaf discs, 4 mm in diameter, were taken from greenhouse-grown flowering-size plants of *Cattleya skinneri*. Plants were placed under bright early morning light for 1 h before excision of the leaves. The leaves were surface-sterilized with calcium hypochlorite (Arditti, 1977) before removal of the discs.

Culture and incubation conditions

Cultures (pre-incubation in Knudson C only and incubation with precursors) were maintained on a shaker at room temperature under 18 h photoperiods provided by two 40 W Gro-Lux lamps placed 45 cm above the flasks.

Incubation

Seedlings, 0·5 to 2 g, or leaf discs, 12 to 24 weighing 140 to 400 mg, were incubated in 2 to 5 ml of Knudson C medium containing 50 to 160 μmol tryptophan plus either DL-[benzene ring-U-^{14}C]tryptophan (Amersham FA222) or L-[5-^{3}H]-tryptophan (Amersham TRK 460) at a specific activity of 10 to 20 μCi μmol^{-1}. In experiments with aspartic acid, precursor concentration was 350 μM containing L[U-^{14}C]aspartic acid (Amersham CFB 64) at a specific activity of 50 μCi μmol^{-1}. Glycerol concentration was 100 μM, with a specific activity of 20 μCi μmol^{-1} [U-^{14}C] (Amersham CFB 174).

Seedlings were incubated 4, 7 and 10 days in [^{14}C]tryptophan; 7 days in [^{3}H]tryptophan; 2 to 4 days in [^{14}C]aspartate and 2 days in [^{14}C]glycerol. Leaf discs were incubated for 2 days in each of the precursors ([^{14}C]aspartic acid, glycerol and tryptophan and [^{3}H]tryptophan). All incubations were in filter-sterilized media and replicated twice.

Respiratory CO_2 was collected in ethanolamine:methyl cellosolve (1:3, v/v) or 2 N KOH contained in vials suspended above the incubation mixtures.

Extraction

Tissues were washed with distilled water, homogenized and extracted four times with methanol:chloroform:water (12:5:3, v/v/v; MCW; Bieleski and Turner, 1966). Bound niacin was released by homogenizing and autoclaving for 30 min in 1 N H_2SO_4 (Freed, 1966). Following neutralization with 0·41 N $Ba(OH)_2$ the autoclaved extract was centrifuged and the pellet was extracted four additional times with MCW. The niacin content of the acid extract is identified as total niacin. Aqueous extracts were dried, and the residue was dissolved in the appropriate chromatographic eluant as described below. In this procedure recovery rates of intermediates were 65–100 %.

Assays

Metabolites in the aqueous extracts of plants provided with tryptophan were separated at 20 $\pm$ 2° C by reverse-phase high-performance liquid chromatography (Tarr and Arditti, 1981). In experiments with aspartate and glycerol the flow rate was 1·5 ml min^{-1}, and the eluant was 50 mM sodium tetrabutylammonium phosphate (ion pair reagent) in 10 % (v/v) aqueous methanol, pH 7·0 (Hengen, Seibert and Hengen, 1978) for the first 8 min. After that 75 % aqueous methanol

was introduced for 30 s. At 8·5 min the ion pair reagent was reintroduced. Therefore the column washing step occurred at approximately 14 min. Compounds of interest were quantified by measuring peak areas.

Radioactivity in the pellet, organic portion, CO_2 and 0·5 ml fractions collected from the chromatograph was determined in a Beckman LS7000 liquid scintillation counter using 0·5% PPO and 0·01% POPOP in toluene:methyl cellosolve: ethanolamine (13:11:1, v/v/v) as scintillation fluid for CO_2 samples and 0·4% PPO and 0·05% POPOP in toluene: Triton X-100 (2:1, v/v) for all other fractions.

Results

Incubations with tryptophan

In 4 days of incubation, 0·3% of the total [^{14}C]tryptophan taken up by seedlings was converted to niacin and its metabolites and precursors. The amount increased to 0·4% after a week and 1·4% following 10 days (Table 1). *N*-formylkynurenine was detected after 4 days of incubation (Table 1). Kynurenine and 3-hydroxykynurenine appeared at 7 days. Niacinamide and 3-hydroxyanthranilic acid were detected following a 7-day incubation in ^{3}H-precursor and 10 days in [^{14}C]tryptophan (Table 1).

Respiration of tryptophan by the seedlings, which was relatively low initially (0·6% of the total taken up), dropped even further after 7 days of incubation (0·1%), but increased following 10 days (2·9%, Table 1). Incorporation of [^{14}C]tryptophan in the organic fraction followed a similar pattern, although the percentages were higher (Table 1).

After 7 days, incorporation of [^{3}H]tryptophan into niacin was similar to that of ^{14}C-precursor (0·5 *vs* 0·4%, Table 1). When seedlings were incubated in [^{3}H]tryptophan, 3-hydroxyanthranilic acid and free niacin were detected 3 days earlier than in ^{14}C-incubations (Table 1). The percentage of ^{3}H-label incorporated in the organic fraction at 7 days was nearly 4·5 times greater than that of ^{14}C (Table 1).

Incorporation of label from [^{14}C]tryptophan into indoleacetic acid by seedlings (Table 1) rose from 0·09% of the [^{14}C]tryptophan taken up (4 days) to 45·5% (7 days) and then decreased to 4·9% (10 days).

Incorporation of label into niacin and its metabolites and precursors by leaf discs reached a higher level after 2 days than seedlings showed after even longer incubation periods (Tables 1 and 2). The figures for leaf discs after 2 days were 1·6 and 4·5% for [^{14}C] and [^{3}H] tryptophan respectively. In comparison the seedlings incorporated 0·4 and 0·5% respectively, after 7 days of incubation.

Incorporation of ^{14}C from tryptophan into indoleacetic acid by leaf discs after 2 days occurred at the rate of 2·5% of the total label taken up per day (Table 2).

Glycerol and aspartic acid experiments

Radioactivity (^{14}C) from glycerol and aspartate was not incorporated into niacin during 48 (leaf discs) or 72 (seedlings) of incubation. After 96 h only 0·01% of ^{14}C from aspartate was incorporated into the vitamin by seedlings (Table 3).

Discussion

Orchid seed germination and seedling development are enhanced by 3-hydroxyanthranilic acid, kynurenine, quinolinic acid and niacin (Noggle and

Table 1. *Fate of* [^{14}C] *and* [^{3}H]*tryptophan in* Cattleya skinneri *seedlings*

	Values are for 100 mg seedling tissue							
	4 days		7 days				10 days	
	DL-[benzene ring-U-^{14}C] Tryptophan		DL-[benzene ring-U-^{14}C] Tryptophan		L-[5-^{3}H]Tryptophan		DL-[benzene ring-U-^{14}C] Tryptophan	
Substance	Amount, tryptophan equivalent (nmol)	Percentage of total tryptophan taken up	Amount, tryptophan equivalent (nmol)	Percentage of total tryptophan taken up	Amount, tryptophan equivalent (nmol)	Percentage of total tryptophan taken up	Amount, tryptophan equivalent (nmol)	Percentage of total tryptophan taken up
Tryptophan	209	30·3	63·8	11·7	2·2	1·5	213·0	27·1
N-Formylkynurenine	1·0	0·1	0·7	0·1	0·02	0·01	5·2	0·7
Kynurenine	—	—	0·8	0·1	0·2	0·1	1·6	0·2
3-Hydroxykynurenine	—	—	0·3	0·1	—	—	—	—
3-Hydroxyanthranilic acid	—	—	—	—	0·3	0·2	1·0	0·1
Niacin unhydrolyzed	—	—	—	—	0·02	0·01	0·8	0·1
Total niacin	0·9	0·1	0·4	0·1	0·1	0·1	0·8	0·1
Niacinamide	—	—	—	—	0·1	0·1	1·7	0·2
Niacin plus its intermediates and metabolites	1·9	0·3	2·2	0·4	0·7	0·5	11·1	1·4
Indoleacetic acid	0·6	0·09	247·3	45·5	0·8	0·5	38·6	4·9
Miscellaneous	364·4	52·8	78·7	14·5	3·6	2·4	230·8	29·4
CO_2	4·2	0·6	0·4	0·1	—	—	22·5	2·9
Pellet	67·9	9·8	133·6	24·6	116·5	77·2	180·7	23·0
H_2O	—	—	—	—	5·4	3·6	—	—
Organic fraction	41·6	6·0	17·7	3·3	21·7	14·4	88·2	11·2
Total taken up	689·6	99·8	543·7	100·1	150·9	100·1	784·9	99·9

Table 2. *Fate of* [^{14}C] *and* [^{3}H]*tryptophan in* Cattleya skinneri *leaf discs*

Substance	Leaf discs (per 100 mg, 2-day incubation)			
	DL-[benzene ring-U-^{14}C] Tryptophan		L-[5-^{3}H]Tryptophan	
	Amount, tryptophan equivalent (nmol)	Percentage of total tryptophan taken up	Amount, tryptophan equivalent (nmol)	Percentage of total tryptophan taken up
Tryptophan	63·3	39·5	105·8	60·6
N-Formylkynurenine	1·3	0·8	5·8	3·3
Kynurenine	—	—	0·5	0·3
3-Hydroxykynurenine	0·6	0·4	—	—
3-Hydroxyanthranilic acid	—	—	0·1	0·1
Niacin unhydrolysed	—	—	0·5	0·3
Total niacin	0·4	0·2	0·8	0·5
Niacinamide	0·4	0·2	—	—
Niacin plus its intermediates and metabolites	2·7	1·6	7·7	4·5
Indoleacetic acid	7·9	4·9	2·3	1·3
Miscellaneous	25·7	16·0	20·0	11·5
CO_2	0·1	0·1	—	—
Pellet	42·8	26·7	21·8	12·5
H_2O	—	—	—	—
Organic fraction	17·9	11·2	17·0	9·7
Total taken up	160·4	100·0	174·6	100·1

Wynd, 1943; Bahme, 1949; Mariat, 1949, 1952; Arditti, 1967a; for reviews see Arditti, 1967b, 1979; Arditti and Harrison, 1977). Tryptophan inhibits the growth of seedlings at the protocorm stage, but promotes that of older, leaf-bearing ones (Arditti, 1967a). For this reason leaf discs and 1-year-old seedlings with well developed (albeit small) leaves were used in these experiments. Both the seedlings and leaf discs converted tryptophan into niacin (Table 1). Leaf discs did so more efficiently, probably because growth and metabolic rates of seedlings are low (Arditti, 1967b, 1979; Rao, 1980). Bacterial conversion can not be a factor in these experiments because the leaf discs were surface-sterilized by an agent known not to damage or inhibit growth of orchid tissues (Arditti, 1977). The seedlings were cultured under sterile conditions and all incubations were made in sterilized media.

Cattleya orchids fix carbon via the Crassulacean acid metabolism (CAM) pathway. In a hybrid of *C. bowringiana* and *C. forbesii*, highest acidity levels occur between midnight and 07.00 h under full sunlight in Singapore (Goh *et al.*, 1977). In the shade deacidification is slower but becomes accelerated if the plants are moved to full sunlight. Since no information is available regarding the effects of acidification on orchid leaf metabolism, the plants were placed under high light intensity to allow for deacidification, before removal of leaf discs.

Tryptophan labelled with ^{3}H was used to confirm results obtained with [^{14}C]tryptophan and to determine whether the nature of the label may present problems. The levels of [^{3}H]kynyrenine and [^{3}H]niacin as well as percentage conversions were similar for both labels.

Tryptophan is a precursor of IAA and a number of additional compounds in plants. The amounts of IAA produced were determined, since it has been

Table 3. *Fate of ^{14}C from aspartate and glycerol in* Cattleya skinneri *seedlings and leaf discs*

	Seedlings (per 100 mg)				Leaf discs (per 100 mg)			
	L-[U-^{14}C]Aspartate				L-[U-^{14}C]Aspartate		[U-^{14}C]Glycerol	
	72 h		96 h		48 h		48 h	
Substance	Amount, aspartate equivalent (nmol)	Percentage of total taken up	Amount, aspartate equivalent (nmol)	Percentage of total taken up	Amount, aspartate equivalent (nmol)	Percentage of total taken up	Amount, glycerol equivalent (nmol)	Percentage of total taken up
Niacin	—	—	1·0	0·01	—	—	—	—
Other	845·4	59·6	9298·0	83·9	56·8	13·1	267·8	88·1
CO_2	0·7	0·05	1·0	0·01	106·4	24·6	4·3	1·4
Pellet	493·3	34·8	1598·0	14·4	230·0	53·1	8·8	2·9
Organic fraction	79·3	5·6	184·4	1·7	40·0	9·2	23·0	7·6
Total taken up	1418·7	100·0	11082·4	100·0	433·2	100·0	303·9	100·0

suggested that there may be competition for tryptophan as precursor of the auxin and the vitamin (Galston, 1949). Our results suggest that such competition does not exist in orchids since the percentage of tryptophan converted to niacin increased with time regardless of IAA production (Table 1). Other products of tryptophan were not assayed because they have no bearing on the problem of niacin biosynthesis.

Levels of IAA increase in orchid seedlings as development proceeds (Arditti, 1967b). On the other hand, the activity of IAA peroxidase in *Vanda* (Orchidaceae) shows reverse behaviour: it is highest during early stages of development and decreases during periods of maximal growth and differentiation (Alvarez and King, 1969). In addition, exogenous IAA brings about an increase in peroxidase activity. These reports support the idea that IAA is capable of enhancing the activity of the enzyme that destroys it (Alvarez and King, 1969). If a similar relationship occurs in seedlings of *Cattleya skinneri*, higher IAA levels would lead to increased peroxidase activity and subsequently reduced amounts of auxin. This is indeed so: the amount of ^{14}C-labelled IAA increased from 0·09 % (4 days) of the total tryptophan taken up to 45·5 % (7 days) and then dropped to 4·9 % (10 days). This suggests that the seedlings use some of the exogenous tryptophan to produce auxin which, on reaching a certain level, elicits peroxidase activity. If so, our observations are in agreement with previous reports on orchid seedlings (Alvarez and King, 1969) and the suggestion that peroxidase controls the levels of IAA in plant tissues (Galston, 1967).

The conversion rate of [^{14}C]tryptophan to IAA at 7 days (45·5 % of tryptophan taken up) is very high. At the moment the only explanation for this may be the fact that some parts of orchid plants (pollinia for example and possibly also ovules) contain unusually high levels of auxin (for a review see Arditti, 1979). It is possible, therefore, that seedlings, too, may be capable of accumulating similar concentrations. Another puzzling aspect of IAA production by orchid seedlings is the difference between incorporation of [^{14}C]tryptophan (45·5 %) and [^{3}H]tryptophan (0·5 %) after 7 days.

Label from [^{14}C]aspartate was not significantly incorporated into niacin and only an inconsequential amount of ^{14}C from glycerol could be detected in the vitamin (Table 3). The incorporation of ^{14}C from aspartate was too low (0·01 %) to be of any importance. If real, this conversion was probably the result of recycling of label through tryptophan. Both aspartate and glycerol may have alternative uses in orchid seedlings. However, even with such uses higher incorporation into niacin would be expected if these compounds were its precursors.

Altogether our findings show that orchid seedlings synthesize niacin via the tryptophan degradation pathway rather than one involving aspartic acid and glyceraldehyde-3-P. Thus the suggestion that the tryptophan pathway has been 'conclusively excluded' from the flowering plants (Henderson *et al.*, 1959) must be re-examined.

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