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CYANOGLUCOSIDE BIOSYNTHESIS IN WHITE CLOVER (*TRIFOLIUM REPENS*)

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Key Word Index—*Trifolium repens*; Leguminosae; white clover; biosynthesis; cyanoglucoside.

Abstract—L-valine-(U-^{14}C), isobutyraldoxime-(U-^{14}C) and 2-hydroxyisobutyraldoxime-(1,3-^{14}C) were fed to white clover shoots which contained or lacked cyanoglucoside. Labelling results indicated that plants which were unable to synthesize linamarin from these precursors lacked the ability to glucosylate 2-hydroxyisobutyronitrile. When L-valine-(U-^{14}C) was fed to cyanoglucoside-producing plants in the presence of cold isobutyraldoxime, radioactivity could be trapped in this compound. No radioactivity was trapped in isobutyraldoxime fed to non-cyanoglucoside-producing shoots demonstrating that this phenotype also lacks the ability to convert valine into the aldoxime. This suggests that the non-cyanoglucoside-producing plants have at least two steps in the biosynthesis of linamarin missing. Differences between the linamarin biosynthetic enzymes of white clover and flax were demonstrated using the inhibitor *O*-methylthreonine and by the failure of cyanoglucoside-producing white clover shoots to synthesize linamarin from fed 2-hydroxyisobutyraldoxime.

INTRODUCTION

Although cyanogenesis (the release of HCN from damaged leaves) has been reported in nearly 1000 species representing 250 genera, only a few species appear to be polymorphic for the character, namely *Lotus corniculatus* and *Trifolium repens* [1]. The principle features of cyanogenesis in white clover were elucidated some thirty years ago by Corkill and his co-workers in New Zealand using polymorphic commercial varieties [2–5]; their conclusions may be summarized as follows. Two related glucosides occur in white clover leaves, linamarin (α-hydroxyisobutyronitrile) and lotaustralin (α-hydroxy α-methyl butyronitrile), and these are hydrolyzed in damaged tissue by an enzyme, linamarase (E.C.3.2.1.21), to produce glucose and an unstable aglycone. The aglycone in turn dissociates to release HCN and either acetone or 2-butanone. The presence or absence of linamarase is determined by alleles of a single gene (*Li*); similarly the presence or absence of both the glucosides appears to be determined by alleles of another independently inherited gene (*Ac*). Only plants which possess at least one dominant allele of both genes are cyanogenic.

In 1960 Butler and Butler [6] showed that L-valine and L-isoleucine are efficient precursors of linamarin and lotaustralin respectively in white clover. Subsequent experiments on the biosynthesis of these cyanoglucosides in linen flax have led to the proposed, multistep pathway shown in Fig. 1 [7] for the formation of linamarin from valine, a primary metabolite. A considerable amount of evidence indicates that isobutyraldoxime, isobutyronitrile and 2-hydroxyisobutyronitrile are intermediates in that pathway. The glucosyltransferase enzyme, which catalyzes the final step (step 4 in Fig. 1), has been purified from linen flax and its specificity studied by Hahlbrock and Conn [8], but the isolation of the other enzymes involved has yet to be achieved. The presence of *N*-hydroxyvaline in this pathway has not been established and

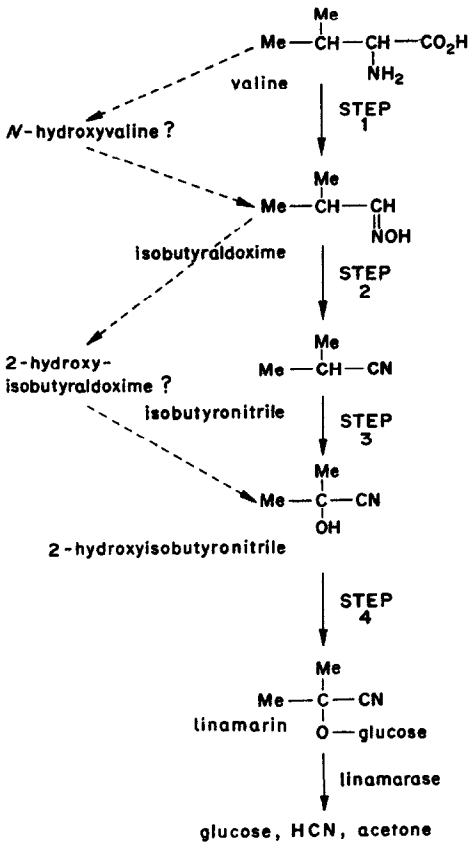

Fig. 1. The proposed pathway for the biosynthesis of the cyanoglucoside, linamarin (after Tapper and Butler [7]).

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in this paper the conversion of valine to isobutyraldoxime is considered as a single step (Step one).

Since Hahlbrock and Conn [9] have shown that the biosynthesis of both linamarin and lotaustralin is catalyzed by the same enzymes, the present study was limited to a comparison of the biosynthesis of linamarin between plants which contain linamarin and lotaustralin (cyanoglucoside-producing plants) and plants which do not (non-cyanoglucoside-producing plants). We have now found that clover plants selected from commercial varieties which lack linamarin and lotaustralin appear to be blocked in at least two points in the biosynthetic pathway.

RESULTS AND DISCUSSION

The end-product inhibition by valine of one of the enzymes involved in its synthesis [10] is one of the few examples of control known in higher plant amino acid biosynthesis. Miflin and Cave [11] have compared the allosteric inhibition of acetolactate synthetase by valine and isoleucine in *AcAc* and *acac* white clover plants. Their results suggest that the control of valine and isoleucine synthesis is equally effective in both genotypes. The possibility that the absence of available valine prevents the production of linamarin in non-cyanoglucoside-producing plants is eliminated by the experiments shown in Table 1, where valine-(U-^{14}C) was fed to plants of both phenotypes.

Table 1 shows that L-valine-(U-^{14}C) is incorporated into linamarin in cyanoglucoside-producing plants but not in non-cyanoglucoside-producing phenotypes. The percentage incorporation varied in cyanoglucoside-producing plants from 4.2 to 6.6%; this is a lower figure than that for flax where about 25% incorporation was seen [12, 13]. The lower incorporation in white clover may reflect the difference in the type of material used; the flax experiments used 5-day-old seedlings where most of the green tissue was cotyledons. Also the total amount of linamarin in the flax tissue was greater than that in the white clover shoots used (about 11 μmol as compared with 3 μmol). The dilution figures given in Table 1 differ from those of flax because no cold valine was added to the clover treatments.

Isobutyronitrile and 2-hydroxyisobutyronitrile are highly volatile compounds and unlikely to accumulate in non-cyanoglucoside-producing leaf tissue. However, isobutyraldoxime is less volatile and measurements were made on the radioactivity present in isobutyraldoxime extracts taken from both types of plants fed L-valine-(U-^{14}C). There was no radioactivity found in the isobutyraldoxime extract from either phenotype and therefore no evidence for the accumulation of this compound in non-cyanoglucoside-producing plants.

In flax the compound *O*-methylthreonine has been shown to inhibit step two [14]; it therefore has two effects on linamarin biosynthesis in this species. It reduces the incorporation of ^{14}C from L-valine-(U-^{14}C) and produces an accumulation of radioactivity in isobutyraldoxime, which leads to the production of labelled isobutyraldoxime glucoside (compound one). L-valine-(U-^{14}C) was fed to white clover shoots in the presence of *O*-methylthreonine in order to test for the presence of step one in non-cyanoglucoside-producing plants. In cyanoglucoside-producing white clover, *O*-methyl-threonine inhibited the incorporation of radioactivity from L-valine-(U-^{14}C) into linamarin by about 70% (Table 1). This is a similar figure to that obtained in flax [14]. However, in contrast to flax, compound 1 (isobutyraldoxime-*O*-β-D-glucoside) was not found in white clover plants of either phenotype and there was no measurable accumulation of isobutyraldoxime in cyanoglucoside-producing plants. No conclusion could therefore be drawn from the lack of isobutyraldoxime accumulation in non-cyanoglucoside-producing plants. It would appear that the inhibition of step 2 by *O*-methylthreonine does not occur in cyanoglucoside-producing white clover and the effect of this compound on linamarin biosynthesis

Table 1. Incorporation of valine-(^{14}C) into linamarin

Plant	Fr. wt (g)	Compound Administered: *O*-methyl threonine (μmol)	Compound Administered: Valine-(U-^{14}C) (μCi)*	Incorporation from valine-(U-^{14}C): % Converted to linamarin†	Incorporation from valine-(U-^{14}C): Dilution
Cyanoglucoside-producing					
S100/10	0.3	—	1.84	4.2	4200
S100/10	0.6	—	0.92	6.6	14200
S100/1	0.9	—	0.92	6.5	15800
Non-cyanoglucoside-producing					
DWW/1	0.3	—	1.84	0	—
DWW/3	0.5	—	0.92	0	—
DWW/1	0.7	—	0.92	0	—
Cyanoglucoside-producing					
S100/10	0.3	10	1.84	1.8	11700
S100/10	0.6	10	0.92	2.1	53700
S100/10	0.5	20	0.92	2.3	80000
S100/1	0.8	20	0.92	1.2	82000
Non-cyanoglucoside-producing					
DWW/1	0.3	10	1.84	0	—
DWW/3	0.6	10	0.92	0	—
DWW/3	0.7	20	0.92	0	—
DWW/1	0.7	20	0.92	0	—

* Specific activity of 200 μCi/μmol. † Corrected for an assumed loss of carboxyl atom.

Table 2. Incorporation of isobutyraldoxime-(^{14}C) into linamarin

Plant	Fr. wt (g)	Compound administered *O*-methyl threonine (μmol)	Compound administered Isobutyral-doxime-(U-^{14}C)* (μCi)	Incorporation from isobutyraldoxime-(U-^{14}C) % Converted to linamarin	Incorporation from isobutyraldoxime-(U-^{14}C) Dilution
Cyanoglucoside-producing					
S100/1	0.7	—	0.90	1.7	19.0
S100/10	0.7	—	0.90	1.6	19.8
S100/10	0.9	—	0.90	2.0	24.0
S100/1	1.0	—	0.90	2.4	18.2
S100/1	0.7	—	0.90	2.5	18.2
S100/10	0.6	—	0.90	2.6	15.2
Non-cyanoglucoside-producing					
DWW/1	0.6	—	0.90	0	—
DWW/3	0.7	—	0.90	0	—
DWW/3	0.7	—	0.90	0	—
DWW/1	1.0	—	0.90	0	—
Cyanoglucoside-producing					
S100/1	0.6	20	0.90	1.6	16.2
S100/10	0.6	20	0.90	2.0	23.0

* Specific activity of 0.74 μCi/μmol.

must be due to the inhibition of other steps in the pathway.

If the non-cyanoglucoside plants are unable to carry out step 1 but contain the enzymes necessary for steps 2–4, they should convert fed isobutyraldoxime into linamarin. The incorporation of isobutyraldoxime-(U-^{14}C) into linamarin in cyanoglucoside-producing plants is shown in Table 2, but there was no evidence for the synthesis of linamarin from the administered isobutyraldoxime in non-cyanoglucoside-producing plants. These results show that at least one of the steps after aldoxime is missing in non-cyanoglucoside-producing plants. Again some differences between the cyanoglucoside-producing clover and flax material are demonstrated by these experiments. In flax the percentage conversion to linamarin was similar for both valine (25%) and isobutyraldoxime (21%) [12]. In white clover only 2% of the administered isobutyraldoxime was converted to linamarin in comparison with an average figure of nearly 6% for valine. This may reflect the difference in specific activity of the two compounds fed to white clover.

Table 2 also shows that in clover *O*-methylthreonine does not inhibit the incorporation of isobutyraldoxime into linamarin. This explains the lack of isobutyraldoxime accumulation and the absence of compound 1 when *O*-methylthreonine is fed in the presence of L-valine-(U-^{14}C). It is clear that in this species *O*-methylthreonine does not inhibit the conversion of isobutyraldoxime to isobutyronitrile but may inhibit an earlier step of the biosynthetic pathway.

2-Hydroxyisobutyraldoxime-(1, 3-^{14}C) has been shown to be incorporated into linamarin when fed to linen flax [15]. In addition to a 12% conversion of 2-hydroxyisobutyraldoxime to linamarin, about 36% of the aldoxime fed to flax was directly glucosylated to 2-(glucosyloxy)-isobutyraldoxime. Table 3 gives the results of feeding 2-hydroxyisobutyraldoxime-(1,3-^{14}C) to white clover. There was no incorporation of radioactivity from the oxime into linamarin in either phenotype. Paper chromatography in 2-butanone–acetone–water of the ethanol extract from cyanoglucoside-producing plants did, however, show two major labelled compounds. One compound had an R_f of 0.87, which corresponded to that of "unmetabolized" 2-hydroxyisobutyraldoxime; the other compound had an R_f of 0.38. Linamarin had an R_f of 0.47–0.56 in this solvent system, depending on the temperature of the laboratory, but the unknown compound (R_f 0.38) did not co-chromatograph with an authentic sample of linamarin when rechromatographed in butanol–water. Treatment of the unknown compound with 2,4-dinitrophenylhydrazine in dilute HCl gave a mixture of methacrolein-2,4-dinitrophenylhydrazone and 2-hydroxyisobutyraldehyde-2, 4-dinitrophenylhydrazone.

These results are consistent with the proposal that the

Table 3. Incorporation of 2-hydroxyisobutyraldoxime-(1,3-^{14}C) into linamarin

Plant	Fr. wt (g)	Compound administered 2-Hydroxyisobutyraldoxime -(1,3-^{14}C) (μCi)*	Incorporation from 2-hydroxyisobutyraldoxime % Converted to linamarin	Incorporation from 2-hydroxyisobutyraldoxime % Glucosylated
Cyanoglucoside-producing				
S100/1	0.6	0.23	0	17.2
S100/10	0.6	0.23	0	19.6
Non-cyanoglucoside-producing				
DWW/1	0.7	0.23	0	0
DWW/3	0.5	0.23	0	0

* Specific activity of 0.28 μCi/μmol.

Table 4. Incorporation of valine-(^{14}C) into linamarin in shoots treated with isobutyraldoxime

Plant	Fr. wt (g)	Compound administered L-valine* (U-^{14}C) (μCi)	Isobutyraldoxime (μmol)	Incorporation from Valine-(^{14}C) % Converted to linamarin†	% Trapped as isobutyraldoxime
Cyanoglucoside-producing					
S100/10	1.5	3.68	43.2	2.9	0.3
S100/1	0.6	1.84	21.6	3.5	0.7
Non-cyanoglucoside-producing					
DWW/1	1.3	3.68	43.2	0	0
DWW/3	0.7	1.84	21.6	0	0

* Specific activity of 200 μCi/μmol. † Corrected for an assumed loss of carboxyl atom.

unknown compound is 2-(glucosyloxy)isobutyraldoxime [15]. It appears therefore that about 18% of the 2-hydroxyisobutyraldoxime was converted to 2-(glucosyloxy)-isobutyraldoxime in cyanoglucoside-producing plants. Although the hydroxyaldoxime was not converted to linamarin in this species, it was readily glucosylated. In non-cyanoglucoside-producing plants only one major labelled compound was found in the ethanol extract. It had an R_f of 0.87 and was assumed to be the unreacted 2-hydroxyisobutyraldoxime; there was no evidence for the presence of 2-(glucosyloxy)-isobutyraldoxime in these plants. Further the amount of unreacted 2-hydroxyisobutyraldoxime recovered from noncyanoglucoside-producing plants was greater than that recovered from cyanoglucoside-producing plants, where there had been some metabolism of the compound. The fact that 2-hydroxyisobutyraldosime is readily glucosylated in cyanoglucoside-producing plants but not in noncyanoglucoside-producing phenotypes suggests that a glucosyltransferase enzyme is missing from plants of the latter phenotype and this may be the enzyme catalyzing step 4 (Fig. 1) in the cyanoglucoside biosynthetic pathway.

Table 4 gives the results of an experiment in which L-valine-(U-^{14}C) was fed to clover shoots in the presence of cold isobutyraldoxime, in order to further test for the presence of isobutyraldoxime as an intermediate in cyanoglucoside biosynthesis in the non-cyanoglucoside-producing phenotype of white clover. When 21.6 μmol of isobutyraldoxime were fed to the cyanoglucoside-producing clover shoots a measurable quantity of radioactivity, given as L-valine, was trapped as the aldoxime. In contrast there was no accumulation of radioactivity in the isobutyraldoxime extracted from non-cyanoglucoside-producing plants, although (as determined by colour of the isobutyraldehyde-2,4-dephenyl-hydrazine band on TLC plates) the cold isobutyraldoxime was extracted from shoots of this phenotype. This experiment shows that step one is also missing from the non-cyanoglucoside-producing white clover plants.

Thus the non-cyanoglucoside-producing white clover plants lack the enzyme activities necessary to carry out both step one and step four of Fig. 1. This must be either the result of mutation in two genes, each of which caused the loss of one function, or the result of a mutation in a single gene resulting in the loss of both activities. In a similar study of the genetic control of *o*-hydroxycinnamic acid in *Melilotus alba*, Haskins and Kosuge [16] concluded that the *Cu* locus controls the *o*-hydroxylation of *trans*-cinnamic acid. In this system, which has many similarities to the cyanogenic polymorphism in white clover, the *Cu* locus appears to control the activity of a single enzyme, and this is consistent with the genetic evidence that the *Cu* locus is a single gene. The genetic background of the white clover plants used in this study is not known. However, it is clear that although the cyanoglucoside polymorphism in white clover has commonly been used as an example of a single gene controlled biochemical difference, the loss of two enzyme activities in the non-cyanoglucoside-producing plants tested in this work means that the single gene nature of this cyanoglucoside polymorphism must be questioned.

EXPERIMENTAL

Materials. Isobutyraldoxime-(U-^{14}C) [7] was a gift from Dr. B. A. Tapper, Applied Biochemistry Division, DSIR, Palmerston North, New Zealand. 2-Hydroxyisobutyraldoxime-(1,3-^{14}C) [15] was genersouly provided by Dr. H. Zilg.

Selection of plant material. A number of white clover seedlings which lacked linamarase activity were selected from polymorphic commercial varieties using the picrate test [2] and a purified ethanol extract of linamarin. The picrate test and a partially purified preparation of flax linamarase was then used to classify these seedlings into plants which contained linamarin and lotaustralin (phenotype, cyanoglucoside-producing, linamarase negative) and plants which had the phenotype, non-cyanoglucoside-producing linamarase negative.

Methods. Labelled compounds were fed to white clover shoots cut from mature plants; these shoots consisted of the meristem, stem and developing leaves back to and including the first expanded leaf of the shoot. Compounds were administered in the light for 4 or 7 hr in 200 μl of dist. H_2O. Additional H_2O was given as required. When isobutyraldoxime was fed no special precautions were taken to limit the loss of this volatile compound. In an open vessel containing 200 μl H_2O and 11 μmol of isobutyraldoxime, only 50% was lost after 1 hr. Under exactly similar conditions the white clover tissue took up 200 μl H_2O in 1–2 hr and the surface of the liquid was covered by the tightly packed stems and leaf tissue. In the inhibition and trapping experiments *O*-methylthreonine or isobutyraldoxime were dissolved in 200 μl H_2O and fed at the same time as the labelled valine. After treatment shoots were washed, weighed and ground in liquid N_2. The frozen powder was first extracted in cold (4°) Et_2O with 0.5 g anhydrous Na_2SO_4 [14]. Extraction was carried out using two 1-ml vols Et_2O. The Et_2O extract was quickly drawn off with a Pasteur pipette and the plant residue then further extracted with two 25-ml vols boiling 80% EtOH for 5 min. Isobutyraldoxime in the ether extract was identified by converting it to the 2,4-dinitrophenylhydrazone of isobutyraldehyde with warm dil acid soln [14]. After 2 hr, the vol was reduced and the mixture chromatographed in toluene on thin layers of Si gel against authentic marked compounds. Using this method

80% of the isobutyraldoxime in the ether extract could be recovered as isobutyraldehyde-2,4-dinitrophenylhydrazone from the TLC plate. Linamarin in the EtOH extract was identified by PC on Whatman 3MM paper in (*n*-butanol–H_2O (50:9) and 2-butanone–Me_2CO–H_2O (15:5:3). The amount of linamarin present was estimated by enzymatic hydrolysis of the chromatographed compound using a partially purified preparation of linamarase from flax [7]. The HCN released was trapped in 1 M NaOH and estimated using C_5H_5N–benzidine reagent [17]. The production of HCN from single leaves was detected by suspending small strips of filter paper soaked in sodium picrate over a leaf which had been treated with toluene [2]. The picrate test was carried out in small sealed tubes incubated at 30° for 12 hr. Radioactivity was estimated by scintillation counting using a triton/toluene solvent mixture.

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