J. Plant Physiol. Vol. 133. pp. 299–303 (1988)

Decomposition of ^{14}C-labelled Cyanamide in *Vitis vinifera* Cuttings

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Received February 25, 1988 · Accepted May 13, 1988

Summary

^{14}C-labelled cyanamide (H_2CN_2) was rapidly metabolized in young shoots of *Vitis vinifera* (experimental cross No. 6234 GM, Geissenheim, FRG) when applied as a pulse (2–2.5 h) through their cut end. Within 20 hours after the beginning of the experiment, about one third of the total activity was found as $^{14}CO_2$, suggesting that a significant amount of the cyanamide was transformed to urea and subsequently hydrolyzed by urease. A significant $^{14}CO_2$-release was observed as early as one hour after cyanamide application. Preliminary results indicate that a part of the decomposition of H_2CN_2 in plant material might be of purely chemical nature.

Key words: ^{14}C-*labelling,* CO_2-*release, cyanamide, cyanamide-decomposition, urease, Vitis vinifera.*

Abbreviations: BuOH = butanol, EtOAc = ethylacetate, MeOH = methanol, SD = Standard deviation, TCA = trichloroacetic acid, TLC = thin layer chromatography.

Introduction

Cyanamide (H_2CN_2, trade name: «Dormex», SKW Trostberg) may be applied to the wood of grapes (and fruit trees) in early spring for an enhanced and uniform bud break. This treatment is especially advantageous in areas with relatively high temperatures during wintertime (Shulman et al., 1983).

Earlier research revealed that H_2CN_2 may be metabolized into urea, arginine, and probably further into guanidinium compounds (Wünsch and Amberger, 1968, 1973; Miller and Hall, 1963). In these studies with species of the *Cruciferae* family and cotton, resp., the rate of decomposition was very high (i.e. most of the H_2CN_2 was metabolized within less than 24 hours after transferring the plants to a H_2CN_2-free medium).

So far, no information about the decomposition rate of H_2CN_2 and its possible metabolites in grapes was available, which might lead to a better understanding of its dormancy breaking effect. Thus, the objective of this study was to quantify the H_2CN_2 metabolization in grapes and to elucidate the consequences of cyanamide applications with respect to plant metabolism.

Material and Methods

Plant material and labelling

Cuttings of *Vitis vinifera* (an experimental cross of «Riesling × Ehrenfelser», No. 6234 GM, staatl. Lehr- und Versuchsanstalt Geisenheim), were rooted and raised hydroponically in an aerated nutrient solution [5 mol · m^{-3} KNO_3, 1.5 mol · m^{-3} $Ca(NO_3)_2$, 1 mol · m^{-3} $MgSO_4$, 1 mol · m^{-3} $NH_4H_2PO_4$, 60 mg/l Fe-EDTA (13 %), 10^{-5} mol · m^{-3} H_3BO_3; other trace elements according to Hoagland and Arnon, 1950]. The plants were kept in a phytotrone (16 h light, 8 h dark, temperature 24 °C/18 °C, 70 % r.h.). The apical 40–50 cm part of the shoot was cut off with a scalpel and kept in a 0.1 N Hoagland solution until starting the labelling period. The cuttings were then placed with the cut end in «Eppendorf reaction vessels», containing 1.75 MBq $H_2{}^{14}CN_2$ (20 h experiment, 1.52 MBq for the 3 d experiment) in 380 µl of a 40 mol · m^{-3} cyanamide solution. The nutrient concentration corresponded to a 0.1 strength Hoagland medium. The tops of the vessels were sealed with «Parafilm» to avoid evaporation of the labelled solution. After about 2 h, the labelled solution had been taken up, and the plants were supplied with a cyanamide-free nutrient solution (as above). Plants were then kept for a further 18 h and 70 h, resp., after $H_2{}^{14}CN_2$-addition («chase»). Throughout the experiment, the individual shoots have

been kept in 3.5 l glass-jars with a water seal, which were flushed continuously with air (about 30–40 changes per hour). The exchanged air was passed through two consecutive traps per plant, each with 10 ml KOH in two experiments. In a separate experiment, where only $^{14}CO_2$ release was monitored, we used 4 traps per plant each with 25 ml of 10% KOH; always far over 90% of the $^{14}CO_2$ were retained by the first trap.

Extraction, cleanup and counting

At the end of the experiment, plants were divided into fully expanded leaves, shoot tip (about the apical 15–20 cm portion of the shoot, including both stem and just expanding leaves) and stem. The plant fractions were weighed, immediately shock-frozen in liquid N_2 and kept frozen until extracted.

Fig. 1: Extraction scheme used to test the metabolization rate of ^{14}C-cyanamide.

Add 50 mmol Na-Phosphat buffer (+ 1% EtOH), pH 6.0 to frozen plant material (2.5 : 1, v/w)
homogenize at ± 0°C in a «Potter-homogenizer» (glass/glass)
centrifuge 10′ at 10,000 × g, → aliquot of supernatant for TLC
reextract pellet twice with 2 ml each of 50 mmol Na-phosphate buffer (pH 6.0)
(sonicate 10 N, 100 W, micro-tip) (under cooling!)
centrifuge (10,000 × g, 10′)
combine supernatants → aliquot for β-counting
→ aliquot for protein precipitation by 10% TCA, heat 1 h to 70°C, centrifuge (10,000 g, 20′)
→ pellet for counting «buffer soluble protein» (+ supernatant)
extract pellet three times with MeOH:EtOAc (1:1, v/v) by sonication, centrifuge (10,000 × g, 10′)
combine supernatants
→ aliquot for β-counting
extract pellet 1 h in 0.05 N NaOH
centrifuge 20′ at 10,000 × g
repeat extraction and centrifuge again
combine supernatants
→ aliquot for β-counting
add TCA to give a 10% solution,
heat 1 h at 70°C,
centrifuge 20′ at 10,000 × g
→ aliquot of supernatant for β-counting
→ pellet for β-counting («NaOH-soluble protein»)
resuspend pellet in water
→ aliquot for β-counting («residual activity»)

For extraction, plant material was re-warmed to about 0°C, 10 mol · m^{-3} phosphate buffer (pH 6.5 + 1% Ethanol) was added (ratio of 1:2.5 w/v), and the mixture ground in a Potter-Elvejhem homogenizer (glass/glass) cooled by an ice bath. If necessary, additional buffer was added to guarantee a uniform homogenate.

An aliquot of about 5 g was used for further separation. The homogenate was centrifuged at 10,000 g, and a part of the supernatant applied directly onto TLC plates (Merck silica gel 0.25 mm, 10 × 20 cm or 20 × 20 cm). The plates were developed in acetonitrile : NH_3 (25%) (95:5, v/v) and butanol : glacial acetic acid : H_2O (4:1:1, v/v) for the separation of cyanamide and some of its metabolites. The distribution of radioactivity over the plate was monitored either with a «Berthold Betascan» or an «IM 3060» β-scanner (Isomess).

The complete extraction procedure is given in Fig. 1. 12 ml of a scintillation cocktail (PCS, Amersham Corp.) were added to the aliquots of the different fractions and the CO_2-traps, and after chemoluminescence had ceased, they were counted in a Phillips-β-counter with automated quench correction.

All experiments were carried out in quadruplicate; CO_2-release was monitored with 5 plants. Figures are given ± standard error.

Throughout the experiments, care had been taken to minimize evaporation of water from the extracts, as reducing to dryness (at least *in vacuo*) may lead to substantial losses of cyanamide (unpublished observations).

Results and Discussion

When applying $H_2{}^{14}CN_2$ via the cut end of *Vitis* cuttings, the most striking result was that almost one third of the total ^{14}C activity was released as $^{14}CO_2$ within 20 h and over 50% during the 72 h (pulse + chase-) period (Table 1). It could be demonstrated by TLC that the activity which was collected in the KOH traps did not derive from eventually evaporated cyanamide (data not shown here). Considering that about

Table 1: Distribution of ^{14}C-activity 18 h and 70 h after applying $H_2{}^{14}CN_2$ as a 2 h pulse (1.75 MBq at 18 h, 1.52 MBq at 70 h, corresponding to about 10 μMol H_2CN_2/plant; values are given ± SD).

Relative distribution of ^{14}C-activity (%)		
chase period:	18 h	70 h
Fraction		
Stem	43.9±6.4	25.1±3.4
Leaves	21.9±3.1	16.8±2.7
Shoot tip	2.1±1.0	1.0±0.9
CO_2	32.0±4.5	57.1±6.7

* Not determined experimentally over the 70 h periode.

Table 2: Relative concentration of ^{14}C-cyanamide in the 50 mmol Na-phosphate-buffer extractable ^{14}C-activity after feeding $H_2{}^{14}CN_2$ to *Vitis*-shoots; data from TLC scans [plates: silica gel, 250 μm, developed in acetonitrile: 25% NH_3 (95:5, v/v), values ±SD].

^{14}C activity in the cyanamide fraction (%)		
chase period:	18 h	70 h
Fraction		
Stem	30.9±10.7	2.4±1.1
Leaves	32.3± 6.5	2.6±1.3
Shoot tip	25.1± 4.9	6.2±1.9

* Hardly detectable over the background level.

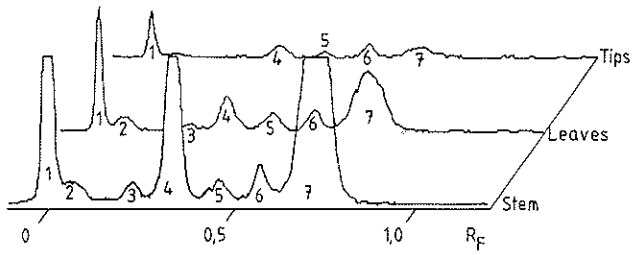

Fig. 2 a: 3 D – plot of TLC-scans (silica gel, solvent system: acetonitrile/25 % NH_3, 95 : 5, v/v), watersoluble ^{14}C-activity, 18 h chase after application of $H_2{}^{14}CN_2$ (pulse: 2 h); peak identification: 1 = proteins and various metabolites (unidentified), 2 = unknown, 3, 4 = unknowns (may contain urea), 5 = dicyandiamide, 6 = thiourea (??), 7 = cyanamide.

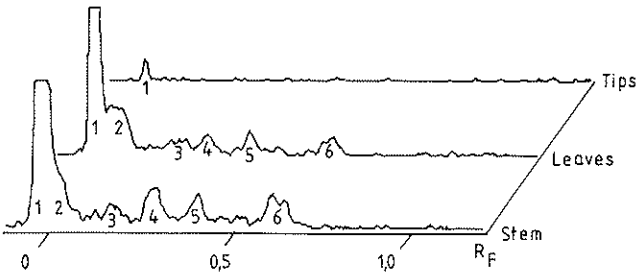

Fig. 2 b: same as Fig. 2 a, but 70 h chase period.

30 % of the buffer extractable activity in stem and leaves, and only 25 % in the shoot tip, co-chromatographed with authentic cyanamide (Table 2), about 70 % of the applied cyanamide were thus metabolized within the chase period of 18 h. The rate of cyanamide decomposition by *Vitis* shoots (the average fresh weight was 12.3 g) may thus be estimated to be around 1.1–1.2 μmol/g FW · h. Consequently, only a minor part of $H_2{}^{14}CN_2$ remained unmetabolized after 72 h. These results confirm earlier observations of a rapid decomposition of cyanamide (Wünsch and Amberger, 1968; Hofmann et al., 1954).

Several ^{14}C-metabolites were found in the TLC-scans, but most of them could not yet be determined unequivocally. Traces of dicyandiamide found in the extracts are probably derived from impurities in the cyanamide preparation (about 2.2 %). Fig. 2 a, b shows the distribution of ^{14}C activity in scans of TLC plates loaded with the buffer extracts and developed in acetonitrile/25 % NH_3 (95 : 5, v/v). Those metabolites which were identified earlier (Hofmann et al., 1954; Wünsch and Amberger, 1968) run mostly near the origin. Separation of the extracts with other solvent systems lead to the positive identification of the following metabolites: urea, guanylurea, and probably arginine. It was found that most of the ^{14}C-activity disappeared from the chromatographically mobile compounds within 3 d (Fig. 2 b), and about 32 % of the buffer extractable activity became insoluble in 10 %

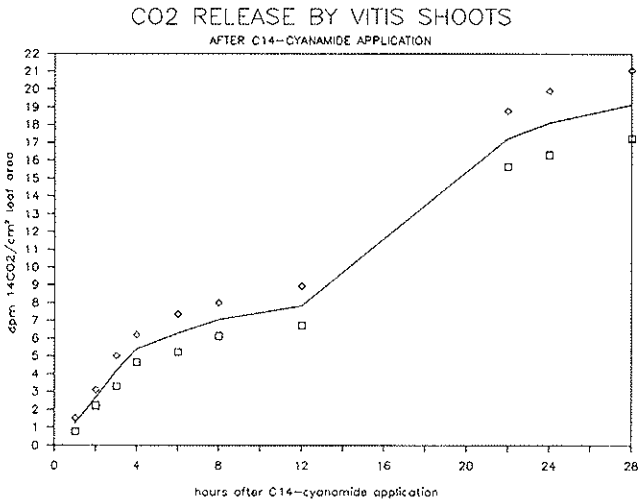

Fig. 3: $^{14}CO_2$ release of *Vitis vinifera* – shoots after feeding with $H_2{}^{14}CN_2$, (values are given as dpm/cm² leaf area); data are shown ± SD (□, ◇).

Table 3: Extractability of ^{14}C-activity after application of $H_2{}^{14}CN_2$ to *Vitis vinifera* cuttings (2–2.5 h pulse, chase periods as indicated («protein» = precipitated by 10 % TCA at 70 °C; values are given as % of the total activity in the respective plant parts).

† chase period (n)	Extractant plant part	H_2O/buffer* → «protein»		MeOH/EtoAc (1 : 1 v/v)	0.05 N NaOH → «protein»		insoluble in 0.05 N NaOH
	stem	84.6	0.8	7.6	3.3	0.9	6.5
	±Sd	(±3.9)	(±0.5)	(±2.9)	(±0.5)	(±0.1)	(±2.1)
18	leaves	84.2	0.3	8.3	2.2	1.0	5.4
	±Sd	(±5.1)	(±0.1)	(±4.6)	(±0.4)	(±0.4)	(±0.5)
	shoot tip	85.7	0.3	5.2	3.2	0.8	6.0
	±Sd	(±1.3)	(±0.2)	(±1.7)	(±0.3)	(±3.0)	(±1.3)
	stem	68.1	32.0	12.0	5.0	0.4	15.0
	±Sd	(±6.5)	(±8.6)	(±8.9)	(±1.5)	(±0.2)	(±4.2)
70	leaves	76.0	22.6	9.0	4.3	0.1	10.8
	±Sd	(±4.2)	(±3.5)	(±2.7)	(±1.6)	(±0.05)	(±1.7)
	shoot tip	86.2	31.9	6.7	2.9	0.2	5.3
	±Sd	(±6.5)	(±8.3)	(±4.0)	(±2.5)	(±0.7)	(±3.7)

* buffer = extractant: 50 nmol NaH_2PO_4 + 1 % ethanol

TCA, i.e. was bound in the protein fraction (Table 3). This coincides with observations for other plants of the *Cruciferae*-family (Wünsch and Amberger, 1968).

In a separate experiment, the time course of $^{14}CO_2$-development was followed when feeding $H_2{}^{14}CN_2$. Significant amounts of activity were found one hour after the beginning of the experiment (Fig. 3). A cyanamide-hydratase has been isolated by Stransky and Amberger (1973) from *Myrothecium verrucaria* Alb. u. Schw., which catalyzes the reaction of $H_2N-C\equiv N + HOH \rightarrow H_2N-\overset{O}{\overset{\|}{C}}-NH_2$. A comparable enzymatic activity in higher plants has not been observed by these authors. Nevertheless, the results presented here suggest that higher plants may as well incorporate a high percentage of the applied cyanamide via urea or a similar substance, which in turn might be further decomposed (by urease). Although the formation of urea from cyanamide may be under enzymatic control in higher plants, to a certain extent this might also occur spontaneously. We happened to observe a decomposition of ^{14}C-cyanamide in frozen leaf extracts stored at −20 °C over 4 months and a concomitant increase of ^{14}C-activity in a fraction which co-chromatographed with urea in butanol : glacial acetic acid : water (4 : 1 : 1) (Fig. 4 a). It may thus tentatively be assumed that H_2CN_2 decomposition in higher plants is (in part) of purely chemical nature, although this was questioned by Latzko (1955). But as this author used heat to inactivate enzymes, other possibly reactive compounds might have been altered as well. It cannot be ruled out, though, that the observed substance is not urea, as the main peak (No. 4) in Fig. 4 a co-chromatographed with guanylthiourea in BuOH/$CHCl_3$/CH_3COOH/MeOH/H_2O (40 : 24 : 10 : 8 : 10), and the respective rF in this system is slightly different from urea (peak No. 3 in Fig. 4 b). Further experiments are needed to assess the formula of the (transitory) metabolites. As can be seen from Table 3, most of the ^{14}C-activity remains in a water-/buffer soluble form over the entire 3 d period. About one fourth to one third of the ^{14}C was incorporated into a TCA-precipitable fraction («protein»). Recently, Vilsmeier and Amberger (1988) with grape cuttings found a significant incorporation of ^{15}N into the protein fraction and a sixfold increase of arginine derived from ^{15}N-labelled cyanamide.

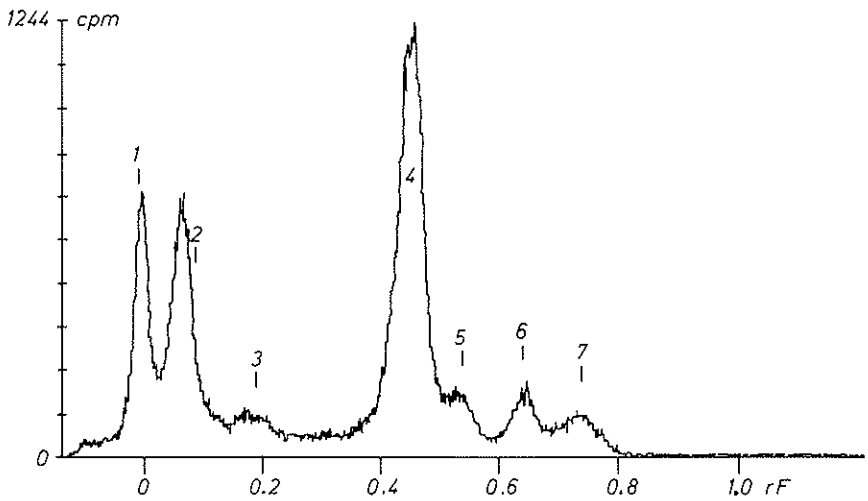

Fig. 4 a: TLC-scans of extracts from $H_2{}^{14}CN_2$-fed *Vitis*-leaves (extracts were kept frozen at −20 °C for over 4 months); silica gel-plates (0.25 mm) were developed in BuOH/CH_3COOH/H_2O (4 : 1 : 1, v/v); peak identification: 1 = start, immobile metabolites, 2 = unidentified metabolite, 3 = guanidinium (?), 4, 5 = urea/guanylthiourea (??), 6 = dicyandiamide, 7 = cyanamide.

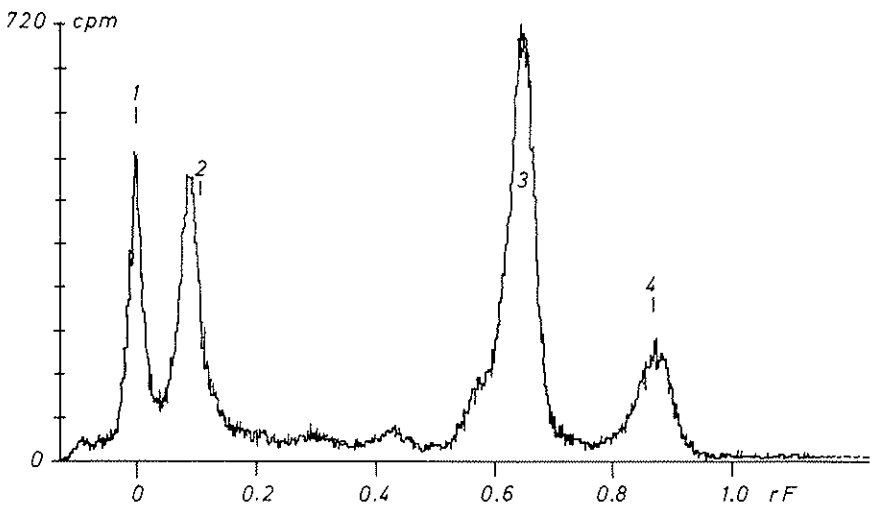

Fig. 4 b: The same as Fig. 4 a, but the solvent system was BuOH/$CHCl_3$/CH_3COOH/MeOH/H_2O (40 : 24 : 10 : 8 : 10, v/v), peak identification: 1, 2 = as above, 3 = guanylthiourea (?), 4 = cyanamide.

The possibility that part of the $^{14}CO_2$, developing during the experiment might have been assimilated again, makes it difficult to identify metabolites occurring only in trace amounts. It is therefore required to work in complete darkness or with dark-grown cell cultures in order to give a full account of all early occurring metabolites of cyanamide in higher plants.

The standard deviations were relatively high in our experiments (see Tables and Fig. 2), but this reflects the normal variability between individual shoots and gives more realistic figures.

Presently, experiments are being undertaken to further identify the transitory metabolites after H_2CN_2-application. The results presented here clearly show that cyanamide is rapidly decomposed by *Vitis vinifera* plants, similar to earlier observations with other species (Wünsch and Amberger, 1973; Hofmann et al., 1954), and that its metabolites are mainly incorporated into the protein fraction. To which reactions of cyanamide its dormancy-breaking properties can be attributed, is still a matter of speculation. As cyanamide is a very reactive compound, it may exert multiple effects on plant metabolism.

Acknowledgements

The authors wish to thank Dr. Haisch, (Bayer. Landesanstalt f. Bodenkultur, Munich), Isomess Inc. and Berthold K. G. for generously providing the facilities for TLC-scanning, Prof. Dr. Hock (chair of Botany, Freising-Weihenstephan) for the permission to use his scintillation counter, and Prof. Dr. Schaller (Staatliche Lehr- und Versuchsanstalt Geissenheim) for the plant material, as well as the «SKW Trostberg AG» for financial support.

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J. Plant Physiol. Vol. 133. pp. 304–309 (1988)

Carbon Gain, Water Conservation and, Expression of CAM during Leaf Development of *Senecio Medley-Woodii*

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Received April 27, 1988 · Accepted May 19, 1988

Summary

CO_2 gas exchange and transpiration of each of the eleven youngest leaves of *Senecio medley-woodii* were monitored separately. The youngest leaves exhibited a marked net CO_2 release associated with a low transpiration. With increasing leaf age a typical C_3 photosynthesis pattern was measured and the transpiration was on a high level. In older leaves a shift to the crassulacean acid metabolism (CAM) occurred and the transpirational water loss for the 24 h period decreased. As a consequence of the shift to CAM a substantial improvement of the water use efficiency resulted. A correlation could be established between leaf area on the one hand and nocturnal accumulation of malate and increase in osmolality on the other. The importance of minimizing water loss at early stages of leaf expansion is discussed in connection with the shift to CAM and the water economy of the whole plant.

Key words: Senecio medley-woodii; Asteraceae; crassulacean acid metabolism; gas exchange (CO_2); leaf age; transpiration; water use efficiency.

Abbreviations: CAM = Crassulacean acid metabolism; VPD = Water vapour pressure deficit.

Introduction

The Crassulacean acid metabolism (CAM) plants generally grow in arid habitats and are characterized by an efficient water economy (Winter, 1985; Ting, 1985; Lüttge, 1987). On one hand, the inverted stomatal rhythm reduces transpirational water loss in general and on the other hand, the diurnal transpiration decreases rapidly with the onset of drought (Kluge and Ting, 1978; Osmond et al., 1979; Ruess and Eller, 1985). CAM plants are highly versatile in their response to environmental conditions (Lüttge, 1987) particularly to water vapour pressure deficit (VPD) of the air and to the water availability in the soil (Ting, 1985; von Willert et al., 1985). The expression of CAM is also modulated by developmental changes (Winter and Lüttge, 1976; von Willert et al., 1976). In many CAM plants, young leaves perform C_3 photosynthesis and with maturation a shift towards CAM occurs (Jones, 1975; Nishida, 1978; von Willert, 1979; Acevedo et al., 1983; Holthe et al., 1987).

In *Brophyllum fedtshenkoi* leaf resistance to water vapour during daytime was found to be lower in the younger leaves compared with the older ones (Jones, 1975). This may indicate that the transpiration rates of younger leaves are higher than those of the older leaves. This has been verified in the cases of *Opuntia basilaris* (Hanscom and Ting, 1977) and *Senecio medley-woodii* (Eller and Ruess, 1986) but the information about the relationship between expression of CAM and water economy of maturing leaves is still scarce.

The objective of this study was to investigate the daily carbon gain and the expression of CAM in connection with water conservation and water use efficiency. For this purpose, CO_2 exchange and transpiration of the eleven youngest leaves of *Senecio medley-woodii* were monitored separately to quantify the progressive accentuation of CAM and its effectiveness in water conservation.

Material and Methods

The CAM plant used in this study is a member of the Asteraceae, *Senecio medley-woodii* Hutchins. from the province of Natal (Republic of South Africa). Cuttings were propagated in the