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FLUOROACETATE METABOLISM OF DICHAPETALUM CYMOSUM

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FLUOROACETATE METABOLISM OF *DICHAPETALUM CYMOSUM*

by

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INTRODUCTION

INTRODUCTION

Background

Dichapetalum cymosum (Hook.) Engl. (poison leaf or gifblaar) is a member of the family Dichapetalaceae and has been recorded in the northern part of the Republic of South Africa as well as in Botswana, Namibia and Zimbabwe (Kellerman *et al.* 1988). Only the apical parts of its branches protrude from the soil and consequently it appears to be pseudo-herbaceous in form. Axillary buds on the primary stem as well as on the adventitious stems can develop into lateral branches (Mogg 1930; Nel 1980). The primary roots can be up to 70 mm in diameter and can reach an underground depth of 3 m (Mogg 1930). The stem which reached a depth of 30 m and which was descibed by Leéman (1939) might in fact have been a root (Nel 1980). Adventitious buds on the roots can give rise to vertically growing stems (Nel 1980). The leaves are alternate and become leathery with age. Only one or two sets of leaves are produced per year. The small flowers are borne in dense clusters and have a white colour. The flowers are bisexual and have a superior ovary and a pentameric calyx, corolla and androecium (Nel 1980). The androecium contains a pentameric staminode whorl (Nel 1980). The fruits are spherical, orange-yellow when ripe and contain one to three seeds per fruit. Seed germination is hypogeal (Vahrmeijer 1970). The flowers, fruits and leaves of *D. cymosum* are parasitized by the caterpillar of a moth (*Sindris albimaculatus* Reg.) This seems to be the reason why the fruits of *D. cymosum* are so rarely found in nature.

D. cymosum causes sudden death to animals and has been recognized as a hazard to livestock in southern Africa since the arrival of the early Voortrekkers in the Transvaal (Steyn 1928; Tannock 1975a). The plant was apparently named as gifblaar (poison leaf) by the Voortrekkers (Steyn 1928). The extensive underground network of roots and stems make it very difficult to eradicate *D. cymosum*. Walsh reported that two or three leaves are sufficient to poison an ox (Steyn 1928). At first it was thought that hydrocyanic acid was responsible for death, but none of this compound was found in the leaves when it was examined at the Imperial Institute in London (Steyn 1928). This was confirmed by Neser and Green at the Veterinary Research Institute, Onderstepoort in Pretoria (Steyn 1928). More attempts to isolate the toxic substance from *D. cymosum* was made by Rimington in 1935. He also did not succeed and concluded amongst other things that the toxic compound did not contain a carboxylic group. Marais subsequently identified the toxic principle of *D. cymosum* as fluoroacetate in 1944 at the Veterinary Research Institute at Onderstepoort (Marais 1944). This was the first report of the natural occurrence of an organic fluorine compound in an organism. Only a few other organic fluorine compounds have since been isolated from organisms.

Fluoroacetate has subsequently been isolated from other *Dichapetalum spp.* in Africa (Vickery & Vickery 1972; Vickery *et al.* 1973), *Acacia georginae* in Australia (Oelrichs & McEwan 1962), *Cyamopsis tetragonolobus* in India (Vartiainen 1984), *Gastrolobium*

spp. in Australia (Aplin 1967), *Glycine max* (Lovelace *et al.* 1968), *Oxylobium spp.* in Australia (Aplin 1967), *Palicourea marcgravii* in South America (De Oliveira 1968), *Streptomyces cattleya* (Sanada *et al.* 1985) and small amounts in tea plants (species not mentioned, Vartiainen & Kauranen 1984). Fluoroacetate is so toxic to animals that the oral lethal dose of fluoroacetate is only 0.06 - 0.20 mg/kg for dogs, 0.15 - 0.62 mg/kg for cattle, 0.25 - 0.50 mg/kg for sheep, 0.30 - 0.70 mg/kg for goats and 10.0 - 30.0 mg/kg for fowls (Kellerman *et al.* 1988). Eland and kudu succumb at relatively high dosage levels of 6.0 - 8.0 mg/kg fluoroacetate and are much less susceptible to *D. cymosum* poisoning than goats, springbok and gemsbok (Basson *et al.* 1982). Fluoroacetate blocks the tricarboxylic acid cycle by its 'suicide conversion' (Figure 1) to the potent aconitase inhibitor, fluorocitrate (Morrison & Peters 1954; Treble *et al.* 1962; Fanshier *et al.* 1964; Villafranca & Platus 1973).

The fluoroacetate concentration in the young leaves of *D. cymosum* is much higher than in the older leaves of the plant (Steyn 1928; Melamed 1958; Louw 1968; Von Sydow 1969; Eloff 1968; Eloff 1972b; Tannock 1975b). This makes the plant more toxic with the sprouting of new leaves in spring and also sometimes in late summer. Hall & Cain (1972) found substantial amounts of fluoroacetate in the soil in which *D. cymosum* grows. They speculated that micro-organisms in the soil might synthesize fluoroacetate and that the plant might absorb it from the soil.

Treble, Lamport & Peters (1962) remarked that it is still

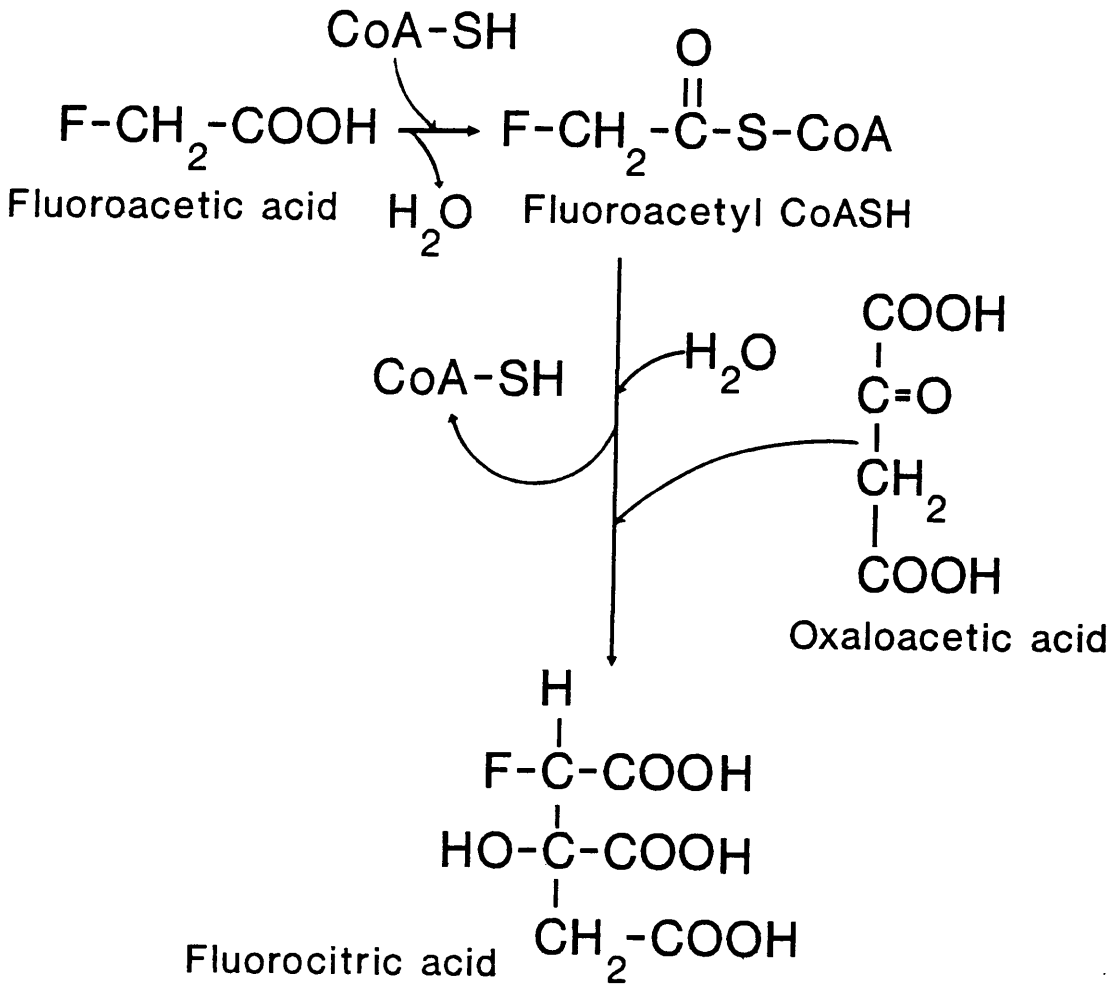

Figure 1 The 'suicide conversion' of fluoroacetic acid to fluorocitric acid.

unknown why *D. cymosum* is not poisoned by fluoroacetate. Eloff is the only author who has shed some light on this aspect. He showed that fluoroacetate can be metabolized by *D. cymosum*. Added fluoroacetate stimulated respiration in the plant, whereas respiration was strongly inhibited by the addition of fluorocitrate (Eloff & Von Sydow 1971). The results of tracer experiments suggest that *D. cymosum* has an active tricarboxylic acid cycle (Eloff 1972a). Because fluorocitrate inhibits respiration and the operation of a tricarboxylic acid cycle in the plant, Eloff & Von

Sydow (1971) speculated that the tolerance of *D. cymosum* to high concentrations of fluoroacetate, may be ascribed to the fact that the 'lethal synthesis' of fluorocitrate does not take place in the plant, most probably because citrate synthetase has different affinities for fluoroacetyl-CoA and acetyl-CoA.

N-methyl-L-alanine (Eloff & Grobbelaar 1967) and N-methyl-L-serine (Eloff & Grobbelaar 1969), two novel amino acids, were isolated from *D. cymosum*. These two amino acids occur in very high concentrations in the plant and together can represent up to 30% of the total nitrogen content of certain parts of the plant (Eloff 1968; Eloff 1972b). N-methyl alanine is synthesized from alanine with methionine as the methyl donor (Eloff 1980a). N-methyl serine is synthesized from serine and N-methyl alanine acts as the methyl donor (Eloff 1980b). These two amino acids could not be traced in another fluoroacetate producing plant, *Acacia georginae*, suggesting that the metabolism of fluoroacetate is not related to that of N-methyl serine or N-methyl alanine (Eloff 1972b).

Scope of the thesis

The fluoroacetate concentration in different parts of *D. cymosum* was determined by Eloff (1972b) with the indirect method described by Louw (1968) and modified by Von Sydow (1969). Tannock determined the fluoroacetate concentration in the leaves of *D. cymosum* with a direct gaschromatographic method (Tannock

1975a; 1975b). One of the aims of the present study was to determine the fluoroacetate concentration in all the different organs of *D. cymosum* by a direct method. Because of the availability of a high-performance liquid chromatograph (HPLC), it was decided to develop a direct, fast and specific method for the determination of the fluoroacetate concentration in *D. cymosum* by using this apparatus.

Hall & Cain's (1972) finding of the presence of high fluoroacetate concentrations in the soils in which *D. cymosum* grows, prompted the study into the possible absorption of ^{14}C-labelled fluoroacetate from the soil by the roots of the plant. The speculation by the above mentioned authors about the possible fluoroacetate synthesis by soil micro-organisms, and the later discovery of *Pseudomonas cepacia* as a regular endophyte of *D. cymosum* (Meyer *et al.* 1990) made it essential to establish unambiguously whether *D. cymosum* is capable of synthesizing fluoroacetate. The production of fluoroacetate by an aseptic *D. cymosum* callus culture as well as by aseptic *D. cymosum* seedlings, were determined with the developed HPLC method.

Another important objective of this study was to establish why *D. cymosum* is not poisoned by the fluoroacetate it contains. The first approach to this question was to establish whether *D. cymosum* is able to degrade fluoroacetate. If the answer to this question is "yes", then the plant's resistance to fluoroacetate can be due to its destruction before it can be converted to fluorocitrate in the mitochondria which can block the tricarbox-

ylic acid cycle. Earlier results showed that the fluoroacetate concentration in young *D. cymosum* leaves is higher than in the older leaves. An attempt was made to ascertain whether this is due to a higher rate of fluoroacetate degradation by the older than by the younger leaves of the plant. It was also established earlier that the plant's endophyte, *P. cepacia*, is capable of degrading fluoroacetate to CO_2 (Meyer *et al.* 1990). It was therefore decided to determine the rate of fluoroacetate degradation by young and old *D. cymosum* leaves, as well as by aseptic *D. cymosum* callus before and after contaminating it with *P. cepacia*.

The second approach to the intricate question concerning the resistance of *D. cymosum* to high fluoroacetate concentrations, entailed a study of its possible non-conversion to fluorocitrate in the mitochondria of *D. cymosum*. *D. cymosum*'s resistance towards fluoroacetate might be explained by the lack of a functional tricarboxylic acid cycle in the plant. This would have ensured that fluoroacetate would not be converted to the aconitase inhibitor fluorocitrate. Balinsky & Schneiderman (1964) and Eloff (1972a), however provided evidence for the operation of a tricarboxylic acid cycle in *D. cymosum*. It was also shown that fluoroacetate is metabolized by *D. cymosum* (Eloff & Grobbelaar 1972). This suggests that *D. cymosum* does not escape from the lethal effect of fluoroacetate by withdrawing it from the cell's metabolism through storage in the vacuole. A fluorocitrate-insensitive aconitase would also have explained the plant's tolerance to fluoroacetate. This possibility can however, also be

ruled out. The results of Louw *et al.* (1970) showed that the aconitase of *D. cymosum* is inhibited by fluorocitrate. One of the few remaining possibilities is that fluorocitrate, for some reason or another is not synthesized in the mitochondria of *D. cymosum* - possibly because its citrate synthetase has no affinity for fluoroacetyl-CoA. It was therefore decided to determine whether fluoroacetyl-CoA could be converted to fluorocitrate by *D. cymosum*. The condensation of fluoroacetyl-CoA with oxaloacetate by citrate synthetase from porcine heart and a crude enzyme extract from *D. cymosum* mitochondria was monitored by determining the rate of CoASH release.

Another important question that arose after it was discovered that *D. cymosum* normally contains *P. cepacia* as an endophyte, was whether it is *D. cymosum* or its endophyte which is responsible for the synthesis of the two novel amino acids that were discovered in *D. cymosum* by Eloff & Grobbelaar (1967; 1969). To answer the question, a comparative study was made of the occurrence of N-methyl alanine and N-methyl serine in the leaves of *D. cymosum*, an aseptic callus culture of *D. cymosum* and a *P. cepacia* culture isolated from *D. cymosum*.

Structure of the thesis

This thesis consists of contributions in the form of reprints of published papers (Chapters 2, 3 and 4), a manuscript accepted for publication (Chapter 5) and manuscripts under consideration for publication (Chapters 6 and 7).

The fluoroacetate concentration in the different organs of *D. cymosum* as well as the new HPLC method developed for this determination, is described in Chapter 2. This chapter also deals with the possible absorption of fluoroacetate by *D. cymosum* from the soil and the transport of the fluoroacetate to the leaves of the plant. The possible fluoroacetate production by aseptic *D. cymosum* seedlings and callus as well as the establishment of the callus culture, is dealt with in Chapter 3. The isolation of *P. cepacia* from *D. cymosum* and the ability of this bacterium to metabolize fluoroacetate, is described in the fourth chapter. The degradation of fluoroacetate to CO_2 by *D. cymosum* leaves, callus and callus contaminated with *P. cepacia*, is described in Chapter 5. In Chapter 6 more reasons are sought for the plant's resistance to high fluoroacetate concentrations. The metabolism of fluoroacetyl-CoA and acetyl-CoA by a crude *D. cymosum* mitochondrial enzyme extract and citrate synthetase from porcine heart, is dealt with in this chapter. Chapter 7 concerns the occurrence of N-methyl alanine and N-methyl serine in *D. cymosum* and its endophyte *P. cepacia*.

In the General Discussion and Conclusions in Chapter 8 an attempt is made to synthesize a more coherent picture of the results of this study. In it special attention is paid to questions about fluoroacetate production by *D. cymosum* and the plant's tolerance to high concentrations of this poisonous compound is discussed.

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CHAPTER 2

THE DETERMINATION, UPTAKE AND TRANSPORT OF FLUOROACETATE IN *DICHAPETALUM CYMOSUM*

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The Determination, Uptake and Transport of Fluoroacetate in *Dichapetalum cymosum*

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Summary

A fast and sensitive method is described for the determination of fluoroacetate in *Dichapetalum cymosum* using high-performance liquid chromatography. Fluoroacetate concentrations as low as 0.1 μg/g fresh plant material can be determined accurately. The fluoroacetate concentration in the different plant parts was determined. The highest concentrations were found in the immature seeds, flowers and young leaves of the plant. Fluoroacetate can be taken up by the roots and be transported to the leaves of the plant. Foliarly applied fluoroacetate is more readily accumulated by young than old leaves.

Key words: Dichapetalum cymosum, fluoroacetate, high-performance liquid chromatography.

Introduction

Dichapetalum cymosum (Hook) Engl. (Gifblaar) was the first plant in which an organic fluorine compound was found when Marais (1944) discovered that its toxic component is fluoroacetate. Fluoroacetate has since been discovered in several other plant species (Baron et al. 1987). The natural fluoroacetate concentration in plants is usually very low. The earliest analytical methods proved inadequate and more sensitive procedures had to be developed (Vartiainen and Kauranen 1984). Several chromatographic methods (Tannock 1975 a, Vartiainen and Kauranen 1984, Vickery et al. 1973, Yu and Miller 1970) and a ^{19}F NMR spectroscopic method (Baron et al. 1987) for the determination of fluoroacetate in plant extracts have been published. The limit of detection with the latter method is estimated to be 4 μg/g fresh material. Fluoroacetate concentrations of 0.1 μg/g fresh material can be detected with gas chromatographic methods. However with these methods derivatization of fluoroacetate is necessary. Ray et al. (1981) described a high-performance liquid chromatographic (HPLC) method for the determination of fluoroacetate in canine gastric content. This method, although involving a lengthy extraction procedure, is specific for fluoroacetate and has a sensitivity of 1–50 μg/g fresh material. Derivatization of fluoroacetate is necessary for detection at 254 and 280 nm. The derivatization reaction is difficult to drive to completion and requires a large excess of the derivatization agent (Ray et al. 1981).

Eloff (1972) determined the fluoroacetate concentration in different parts of *D. cymosum* with the indirect method described by von Sydow (1969). They measured the total fluorine and inorganic fluorine content of the samples and assumed the difference to be due exclusively to fluoroacetate. Eloff (1972) found that the fluoroacetate concentration differs a great deal in the different plant parts which he analyzed.

Eloff (1972), Tannock (1975 b) and von Sydow (1969) reported much higher fluoroacetate concentrations in the young leaves than in the old leaves of *D. cymosum*. The question now arises if fluoroacetate is synthesized at a higher rate in the young leaves than in the old leaves and/or if young leaves can mobilize fluoroacetate from the other organs including old leaves. It is possible that fluoroacetate is formed elsewhere in the plant and is transported to the leaves of the plant. Hall and Cain (1972) found high fluoroacetate concentrations in the soils in which *D. cymosum* grows. It is possible that the plant may be able to absorb this fluoroacetate and transport it to it's leaves.

The aim of this study was to develop a direct, fast, sensitive and specific method for the determination of fluoroacetate by means of HPLC, to use the method in assaying the fluoroacetate concentration in the different organs of *D. cymosum* and also to determine if fluoroacetate can be taken up

through the roots and be transported to the leaves of the plant.

Materials and Methods

Materials

Samples of *D. cymosum* were collected in December 1987 in the field near Pretoria (Republic of South Africa) for fluoroacetate determination. The transportation studies were done with approximately one-year-old *D. cymosum* seedlings which were grown from seed (collected at the same site as described above) and cultivated in a glasshouse (27 °C day and 21 °C night temperature). [1,2-^{14}C]Fluoroacetate (22 Ci/mol) was obtained from Amersham, England.

Methods

Extraction procedure

Composite leaf, stem, root and fruit samples of field-grown *D. cymosum* plants were prepared by cutting up and thoroughly mixing the material from several plants. The fruit were dissected into pericarp and seed. A known amount of authentic fluoroacetate was added to one of two identical samples of each of the organs whereafter all the samples were processed in the same way. The fresh material was blended with an ultra turrax in 0.1 M NaOH and heated in a water bath at 80 °C for two hours. The suspension was filtered through Whatman no. 1 filter paper with suction and the filtrate concentrated in a rotary evaporator at a temperature not exceeding 40 °C. After the volume of the concentrate was determined, 1 ml was acidified with 1 ml 9 M H_2SO_4 in a test tube. Diethyl ether (5 ml) was added for the extraction of organic acids. The mixture was vortexed and then centrifuged. The ether layer was removed and the aqueous phase similarly extracted three more times with diethyl ether. The combined ether extracts were evaporated to dryness in a beaker placed in a fume cabinet. The residue was dissolved in 1 ml 0.0035 M H_2SO_4, filtered through a syringe-fitted Millex-Ha filter (porosity 0.45 µm) and analyzed by means of HPLC.

In one experiment 3 replicates of the same composite leaf sample were separately processed in order to determine the variability of the results. In another experiment several subsamples from one composite leaf sample were enriched with different amounts of fluoroacetate before being processed. In this way an attempt was made to ascertain the sensitivity of the method.

Chromatography (HPLC)

Solvent, 0.0035 M H_2SO_4; flow rate, 0.6 ml/min; oven temperature, 55 °C; chart speed, 0.25 cm/min; sensitivity, 0.005–0.5 absorbance unit full scale; wavelength monitoring, 206 nm; injection volume, 20 µl; column, Aminex Ion Exclusion HPX-87H (Bio-Rad).

Transport of [1,2^{14}C]fluoroacetate

Five µCi in the form of a 5 µl aqueous solution containing 1 % di-(2-ethylhexyl)sodium sulphosuccinate (to reduce surface tension) was spread on a leaf of a seedling. The seedling had a stem with four branches which were difficult to distinguish from the stem. Therefore all five of the organs will be referred to as branches. The one branch had a soft young apical leaf and four similar but much older leaves lower down. The central leaf of the five was treated with ^{14}C-fluoroacetate. The leaves on all the other branches appeared to be similar in age and fairly old. After 24 hours the plant was divided into the different leaves, stems and roots. Two ml Soluene-350 (Packard) was added to subsamples of each plant organ and left for 60 hours at 52 °C. The radioactivity was determined with a liquid scintillation counter after 8 ml Insta Gel II, 16 ml Hionic Fluor (both from Packard) and 2 ml 0.5 M HCl were added to each sample. To determine if the measured radioactivity was from fluoroacetate or metabolites of it, fluoroacetate extracts of other subsamples of all the plant parts were analyzed by HPLC. The eluate was fractionated and the radioactivity in all the fractions was determined with a liquid scintillation counter as above without prior addition of Soluene-350.

In another experiment the 5 µCi of the ^{14}C-labelled fluoroacetate was dissolved in 1 ml water and fed to the roots of seedlings by allowing the root tip to absorb the solution from a small test tube. After 24 hours the seedlings were divided into leaves, stems and roots, put onto Agfa medical X-ray film and left in the dark for 1 week at 2 °C, whereafter the X-ray films were developed.

Results

Extraction of fluoroacetate

The retention time of fluoroacetate under the conditions described in Methods is 12.71 min. The amount of fluoroacetate recovered after each extraction is shown in Table 1. The total amount recovered after the third extraction was 96.56 %. Fluoroacetate concentrations as low as 0.1 µg/g fresh weight (standard deviation 0.012) could be determined accurately by means of HPLC. The fluoroacetate concentrations obtained for the 3 replicates of the same composite leaf sample which were separately processed was 97.01 mg/kg fresh weight with a standard deviation of 6.13.

Fluoroacetate concentration in the different plant organs

The fluoroacetate concentrations in the different plant parts are given in Table 2. Fluoroacetate was detected in all

Table 1: Recovery of fluoroacetate from *D. cymosum* (means of four replicates).

Extraction number	Recovery %
1	50.29
2	38.91
3	7.36
4	0.00
Total	96.56

Table 2: Fluoroacetate concentration in the different parts of *D. cymosum*.

Plant part	*Fresh weight (g)	Fluoroacetate concentration (mg/kg fresh weight)
Flower	8.82	362.1
Young leaf	10.92	231.9
Old leaf	11.13	97.0
Stem	13.06	84.2
Immature pericarp	152.23	72.1
Immature seed	52.70	410.4
Ripe pericarp	156.78	0.0
Ripe seed	51.77	163.5
Root	9.43	70.4

* Fresh weight of the plant part extracted.

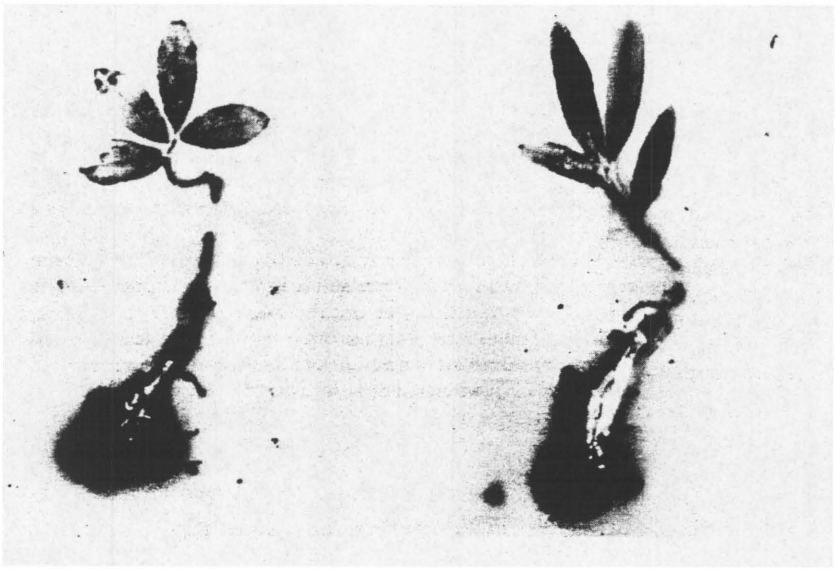

Fig. 1: Autoradiographs of *D. cymosum* seedlings fed with ^{14}C-fluoroacetate via their roots. Left: Seedling with old leaves only. Right: Seedling with young leaves only.

the plant parts assayed except the ripe pericarp. The immature seeds, mature flowers and young leaves (soft and not fully expanded) contained the highest fluoroacetate concentrations.

Transport of [1,2-^{14}C]fluoroacetate

At the end of the experiment fluoroacetate accounted for 90.3 % of the radioactivity of the seedling that was fed ^{14}C-fluoroacetate through one of its leaves. The results (Table 3) show that fluoroacetate was more readily transported from the treated leaf to the young leaf rather than the old leaves on the same branch as the treated leaf. ^{14}C-Fluoroacetate was also transported to the other branches and their leaves but in relatively small amounts. A substantial amount of ^{14}C-fluoroacetate, albeit considerably less than that which migrated into the young leaf, was transported to the roots. A high percentage of the radioactivity fed to the plant (55.43 %) could not be accounted for at the end of the experiment. It therefore seems as though the plant is capable of breaking down fluoroacetate to volatile products which are lost from the plant.

The autoradiographs showed that fluoroacetate can be taken up by the roots and transported to the leaves of the plant (Fig. 1). From these results and those in Table 3, it is quite evident that fluoroacetate can be readily transported from the roots to the leaves and vice versa. The rate of fluoroacetate uptake by the roots and transport to the leaves was found to be more or less the same for plants which had young leaves only and plants which had old leaves only (Fig. 1).

Table 3: Distribution of (1,2-^{14}C) fluoroacetate in *D. cymosum* after application to one leaf on branch no. 1.

Plant part		Specific acitivity in dpm per g.d.w.
Treated leaf		446,681
Branch 1	young, not fully expanded leaf	152,667
	leaf above treated leaf	4,674
	leaf below treated leaf	4,264
	oldest leaf on branch	4,000
	branch	7,859
Branch 2	leaf	5,400
	branch	5,738
Branch 3	leaf	4,744
	branch	2,306
Branch 4	leaf (average of two)	6,155
	branch	2,786
Branch 5	leaf (average of four)	6,554
	branch	3,066
Root	basal half	18,167
	apical half	16,467

Discussion

The method described for the determination of fluoroacetate in *D. cymosum* is fast, sensitive and accurate. Derivatization of fluoroacetate is not necessary and the present method is more sensitive than the HPLC-method described by Ray et al. (1981). Fluoroacetate concentrations as low as 0.1 µg/g fresh plant material can be detected. This is the same level of sensitivity that can be obtained by gas chromatography (Okuno et al. 1982) but less sensitive than the 0.005 µg/g that can be detected by the gas chromatographic-mass spectrometric method of Vartiainen and Kauranen (1984). The instrumentation for the latter method is, however, expensive and not standard equipment in most laboratories.

The different plant organs of field-grown plants were found to differ greatly in their fluoroacetate concentration. The fluoroacetate concentration of the young leaves was the same as the 232 mg/kg fresh weight reported by Tannock (1975 b) for young leaves in December. On the other hand, the old leaves analyzed in the present study yielded fluo-

roacetate concentrations that were considerably lower than the 160 mg/kg fresh weight found by Tannock in December. Tannock assayed only young and old leaves for fluoroacetate. The values he obtained differed considerably for different times of the year. So for instance fluoroacetate concentration of old leaves sampled in March 1974 was only 21 mg/kg fresh weight.

The high fluoroacetate concentration of the young leaves can possibly be due to their ability to mobilize the toxin from other organs and accumulate it. The young leaves might also be relatively more active than most other organs in synthesizing fluoroacetate. On the other hand, it is possible that the leaves can metabolize fluoroacetate to volatile products that are easily lost from the plant and that old leaves are more active in this regard than young leaves.

The results of the experiment in which radioactive fluoroacetate was fed to the roots of the plant indicate that if fluoroacetate is available in the rooting medium, the plant has the potential to absorb it through its roots and transport it to the other plant parts. One can only speculate on the origin of fluoroacetate in the soil as reported by Hall and Cain (1972). There seems to be two possibilities for the origin of this fluoroacetate. One is that fluoroacetate is synthesized in the soil by micro-organisms and the other is that fluoroacetate is released during decomposition of plant material. The latter case presupposes that the plant must be able to synthesize fluoroacetate.

Acknowledgements

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CHAPTER 3

FLUOROACETATE PRODUCTION BY *DICHAPETALUM CYMOSUM*

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Fluoroacetate Production by *Dichapetalum cymosum*

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Summary

Field-grown *Dichapetalum cymosum* plants which appeared normal were invariably found to be infested with bacteria. It has not yet been ascertained whether the bacteria can synthesize fluoroacetate but seedlings of *D. cymosum*, when grown from surface sterilized seed in an aseptic environment, appear to synthesize fluoroacetate. A callus culture of *D. cymosum* was established which appears to be internally devoid of microorganisms. The culture grows well in the absence of fluoride and then does not contain fluoroacetate. Fluoride (0.78–6.24 mM) does not affect the growth of the callus but results in the production of up to 1227 mg fluoroacetate per kg fresh callus.

Key words: Dichapetalum cymosum, callus culture, endophytic bacteria, fluoride, fluoroacetate, tissue culture.

Introduction

Dichapetalum cymosum (Hook) Engl. is a perennial plant that normally produces only one set of leaves per year during spring. Removal of the leaves does not result in the production of a new flush before the next spring. Marais (1944) identified the toxic principle of the plant as fluoroacetate. However, no work appears to have been done on the enzymes which enable the plant to synthesize and/or degrade fluoroacetate. Although young leaves that are soft and relatively rich in fluoroacetate (Tannock 1975, Meyer and Grobbelaar 1989) would appear to be suitable material from which to extract the enzymes involved in the metabolism of fluoroacetate, such leaves unfortunately are available only during a short period each year. For this reason attempts were made to create a callus tissue culture of *D. cymosum* which could be used as a suitable enzyme source throughout the year.

During the initial attempts to establish a callus culture, it became evident that *D. cymosum* plants are normally infested with bacteria. As a result, the question arose as to whether it is the plant and/or its endophyte(s) that produces the fluoroacetate found within the plant. By use of the aseptic callus culture that was eventually obtained, the problem has since been partially resolved.

Materials and Methods

Materials

Vegetative material of *D. cymosum* was usually collected from plants growing on the northern outskirts of Pretoria on the farm, Derdepoort. Although the plants flowered profusely each spring, fruit was never observed at this locality. Fruits were occasionally available in small numbers on the farm, Bultfontein, near Verena about 110 km northeast of Pretoria.

Establishment of callus culture

The plant material was surface sterilized by first scrubbing it with a soft brush under running tap water, after which it was submerged in 80 % ethanol, rinsed with water and submerged in a sodium hypochlorite solution before finally being rinsed with water. In different trials the ethanol treatment was varied between 2 and 10 min and was combined with a series of sodium hypochlorite treatments in which the sodium hypochlorite concentration was varied from 0.35 % to 3.5 % and the time of exposure to sodium hypochlorite was varied from 5 to 30 min.

The surface-sterilized material was cut into small segments which were incubated either in liquid medium or on medium solidified with 1 % agar. When liquid medium was used, the apparatus devised by Caplin and Steward (1952) was used to aerate the cultures. The tubes rather than the nipple flasks devised for use with the apparatus were employed in the studies. Ten ml medium was used per tube.

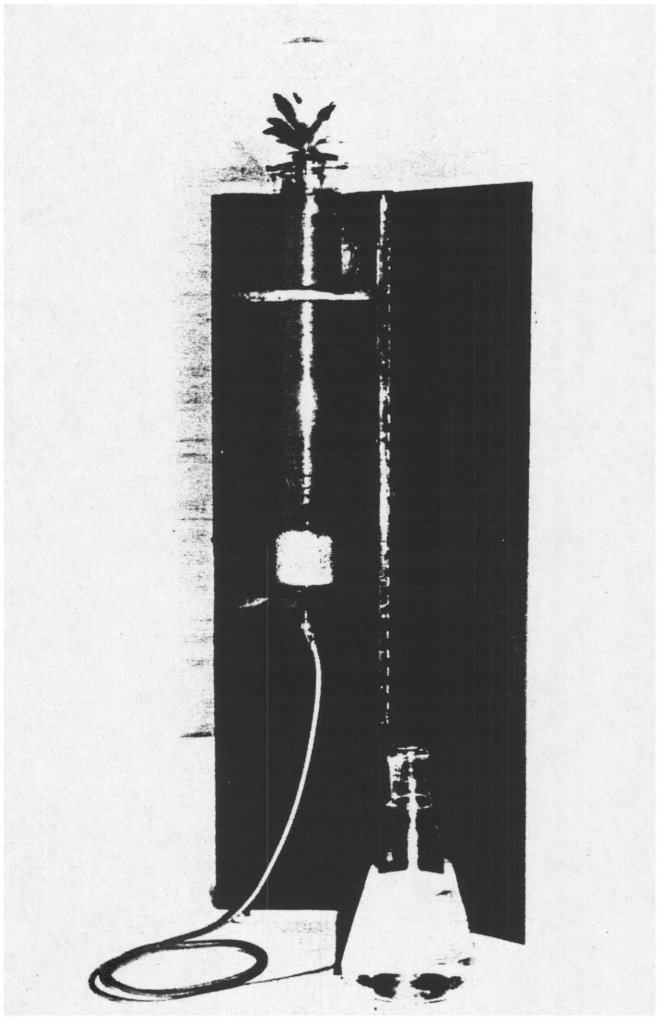

Fig. 1: Apparatus for growing *Dichapetalum cymosum* seedlings in soil for extended periods under aseptic conditions. The spherical detachable glass bulb at the top has a double collar at its base, one of which fits inside and the other outside the rim of the vertical glass column which contains the sterilized soil. A conical flask containing sterile water is connected to the lower end of the column by means of a rubber tube.

Biosynthesis of fluoroacetate by callus

Callus was grown aseptically in nipple flasks (Caplin and Steward 1952) which each contained 150 ml of Schenk and Hildebrandt (1972) liquid medium without cytokinins or auxins. The medium was enriched with NaF at 6.24, 3.12, 1.56 and 0.78 mM in different treatments. The callus was harvested after three weeks.

Test for asepsis of callus

To test whether the callus material is intracellularly devoid of the bacteria which normally infests *D. cymosum* tissue, samples of callus were aseptically homogenized in a mortar with a pestle. Samples of the homogenate were plated on nutrient agar and yeast extract agar which in both cases was supplemented with 1% D-ribose. The plates were incubated at 27 °C. [In parallel studies with the *D. cymosum* endophyte (Meyer et al. 1990) it was established that ribose significantly enhances the growth of bacteria on these media].

Biosynthesis of fluoroacetate by D. cymosum seedlings under aseptic conditions

Ripe but firm *D. cymsoum* fruits (30) were surface sterilized by washing under running tap water, immersing in 80% ethanol (5 min), rinsing with water, immersing in 3.5% sodium hypochlorite (15 min) and finally rinsing five times in water.

The fruits were dissected under aseptic conditions to extract the seeds (usually one per fruit) which were immediately planted, one per 350 ml screw-capped honey jar, about 1 cm deep in moist sand. After the seeds germinated and their taproots were about 5 cm long, six of them were aseptically transferred to the specially designed apparatus illustrated in Fig. 1. During the transplantation, the bulky cotyledons of three of the seedlings were aseptically removed. The glass columns holding the soil into which the seedlings were planted had an internal diameter of 11 cm and a length of 34 cm above the sintered glass plate. The diameter of the glass bulb covering the top of the column was 22 cm. The conical flask connected to the bottom of the column by means of rubber tubing contained 5 litres of water. The soil in the column was moistened by lifting the conical flask to the desired height for a while.

The soil was from the natural habitat of *D. cymosum*, and the column with soil was previously sterilized by autoclaving for 3 h at 120 °C on three occasions at intervals of three days. The other components of the apparatus, as well as the honey jars with sand, were autoclaved once for 30 min at 120 °C. The column and conical flask were kept in a light-tight wooden box from which the glass bulb projected. The whole apparatus was kept under shade-cloth (60% transmittance) in a glasshouse whose temperature was maintained at 27 °C from 06^{00} h to 18^{00} h daily and at 21 °C for the remainder of the time.

The seedlings were harvested 15 months after they were transplanted. Their roots were carefully washed free of soil after which the plants were subdivided into leaves, stems and roots. The fluoroacetate of the different organs of each plant was extracted separately and determined quantitatively by the method of Meyer and Grobbelaar (1989).

Samples of the soil and water from the culture apparatus were tested for the presence of micro-organisms. The material was plated separately onto nutrient agar and yeast extract agar and the petri dishes incubated for several weeks at 27 °C.

Average fluoroacetate per ungerminated seed

The fresh mass of each of 80 of the 119 seeds collected in the particular season was determined. The fluoroacetate content of a 51.77 g sample of the seed, taken at random, was determined by the method of Meyer and Grobbelaar (1989).

Results

Establishment of callus culture

Initially, leaves, aerial stems and roots were used as starting material. After employing various surface sterilization procedures, the plant material was incubated in White (1943) medium supplemented with different combinations of 2,4-D (0–20 ppm) and deproteinized coconut milk (0–20%). In all cases in which the plant tissue survived the sterilization treatment, the cultures were found to be contaminated with micro-organisms within a day or two. From the agar plates it was clear that the contaminants originated from the plant tissue and that in most cases it appeared to consist of a single bacterial type.

Immature fruits, when subjected to the same treatments as applied to the vegetative tissue described above, yielded aseptic cultures in most cases. Consequently, the sterilization procedure described above under «Materials and Methods» for fruit used in studies on the biosynthesis of fluoroacetate by *D. cymosum* seedlings under aseptic conditions was decided upon for all future work on fruit.

However, in some cases the pericarp material developed a callus which was short-lived. Although tissue from the developing seed remained alive for much longer, it did not give rise to a callus in any of the trials.

In all later trials only immature fruits were used as starting material. The tissue was tested on Murashige and Skoog (1962) medium (MS medium) and also against a combination of the inorganic components of the MS medium and the organic components minus NAA used by Button et al. (1971) (BBC medium). In other treatments these two media were supplemented with 1 or 5 ppm 2,4-D or 1 or 5 ppm NAA. Only liquid cultures were set up.

In several of the treatments, the pericarp tissue rapidly produced copious amounts of callus which, however, soon perished despite renewed attempts to keep it alive by subculturing onto various media. In several cases, seed tissue on the MS-BBC medium produced slow-growing callus. Similar calli also developed in a few cases on the complete MS medium both without auxin and enriched with 1 ppm 2,4-D.

After about one year the growth rate of the calli had deteriorated to such an extent that three other culture media were tested both in the dark and in light (combination of one 55 watt Thorn «cool white» fluorescent tube to three 15 watt incandescant bulbs yielding an irradiance of about 1000 lux at the cultures). The media tested were those of Gamborg et al. (1968), Murashige and Tucker (1969) and Schenk and Hildebrandt (1972), all without auxins and cytokinins.

The Schenk and Hildebrandt (1972) medium gave the best growth results. Although there was no significant effect of light, in all subsequent studies callus was grown in light, subculturing on Schenk and Hildebrandt (1972) medium without auxins and cytokinins. This has been done for the past several years without any apparent decline in the growth rate of callus.

Biosynthesis of fluoroacetate by callus

The results of this experiment are presented in Table 1. Because only one of the treatments was replicated, it was not possible to compare the results statistically. However, the results strongly suggest that NaF, at the concentrations tested, does not significantly affect the growth (fresh mass production) of callus. The addition of NaF at all concentrations did, however, result in the production of considerable amounts of fluoroacetate relative to that which is normally encountered in *D. cymosum* plants (Tannock 1975, Meyer and Grobbelaar 1989). For the NaF concentrations employed, the amount of fluoroacetate produced also appears to be directly related to the NaF concentration administered.

No bacteria or fungi grew on the ribose-enriched nutrient agar or yeast extract agar plates onto which homogenized callus was introduced.

Table 1: The effect ot sodium fluoride on fresh mass (FM) and fluoroacetate (FA) concentration in *Dichapetalum cymosum* callus.

mM NaF	Replicate	FM (g)	FA cont. (mg/kg FM)	
0.00	1	2.75	0.0	
0.78	1	4.21	97.0	Mean = 112
	2	5.25	97.7	
	3	3.10	100.2	
	4	4.16	153.0	
1.56	1	3.78	354.7	
3.12	1	2.33	1079.7	
6.24	1	4.01	1226.7	

Table 2: Mean fresh mass (FM) and fluoroacetate (FA) content of whole *Dichapetalum cymosum* seedlings grown for 15 months with and without cotyledons in aseptic containers.*

	Cotyledons removed			Cotyledons intact		
	FM (g)	FA content (mg/kg FM)	(mg/plt)	FM (g)	FA content (mg/kg FM)	(mg/plt)
Leaves	0.414	128.90	0.052	9.651	96.66	0.945
Stems	0.377	89.97	0.033	2.958	64.82	0.190
Roots	2.280	54.81	0.102	12.457	21.93	0.273
Total	3.070		0.188	25.065		1.408

* In all cases, the value for the «minus cotyledon seedlings» differed significantly from the corresponding value of the «intact seedlings» at a 5 % level of probability.

Biosynthesis of fluoroacetate by D. cymosum seedlings under aseptic conditions

Removal of the cotyledons from the seedlings shortly after germination severely retarded their growth, as can be seen from the fresh-mass data (Table 2). Despite the significantly higher fluoroacetate concentration in the organs of these depauperated seedlings, their total fluoroacetate content was significantly lower than for the intact seedlings whose cotyledonous remains were not included in the fluoroacetate determinations.

The soil and water samples taken from the apparatus in which the seedlings were grown did not reveal the presence of any microbial contaminants.

Discussion

The results strongly suggest that all the vegetative organs of field-grown *D. cymosum* are normally infested by one or more types of bacteria. Although it appears that the bacteria are also able to infect the pericarp and to a lesser extent the seed, most seeds seem to be internally aseptic before the fruit becomes fully ripe. Preliminary attempts to locate bacteria within the plant tissue by transmission electron microscopy have not yet enabled us to ascertain whether the bacteria are located intercellularly or intracellularly.

Whether the bacterial endophyte(s) of *D. cymosum* can produce fluoroacetate has not yet been investigated. After Hall and Cain (1972) found substantial amounts of what appears to be fluoroacetate in the soil in which *D. cymosum* grows naturally, they speculated that soil micro-organisms might be responsible for the biosynthesis of the toxin and that the plant may even acquire its fluoroacetate from the

soil. They do not consider it likely that the fluoroacetate in the soil could have been derived from the decomposition of *D. cymosum* leaf litter, as they believe that any such fluoroacetate would be readily biodegraded. A weakness in their argument is that they admit that other more readily biodegradable organic acids are normal and detectable constituents of soil.

In the present study, however, seedlings of *D. cymosum* grown in an aseptic external environment for more than one year appear to be able to synthesize fluoroacetate. The average fluoroacetate content of the batch of 119 seeds from which those used in the experiment were drawn, was 163.5 mg/kg fresh seed. The fresh mass of the individual ungerminated seeds ranged from 1.1 g to 5.7 g with a mean value of 3.117 g. Using these mean values, the average fluoroacetate content per ungerminated fresh seed amounts to 0.5 mg. The corresponding value for the largest seed in the batch is 0.9 mg. The fluoroacetate content of the intact seedlings, therefore, was more than one and a half times that which could occur in the heaviest recorded ungerminated seed in the batch. Therefore, the seedlings must have synthesized fluoroacetate; this of course is assuming that the fluoroacetate concentration does not vary with seed mass. Unfortunately, however, it was not established whether the seedlings were internally aseptic and consequently one can not rule out the possibility that at least part of the fluoroacetate in the seedlings was derived from bacteria residing within the seedling's tissues.

The seedling leaves, which were old when the plants were harvested, had a fluoroacetate concentration of 97 and 129 mg/kg fresh mass for the seedlings with and without cotyledons, respectively. This appears to be quite normal as Tannock (1975) recorded values ranging from 30 to 215 (mean 131) mg/kg fresh mass for similar but field-grown material. Meyer and Grobbelaar (1989) reported a value of 97 mg fluoroacetate per kg for old fresh leaves from field-grown plants.

Whatever doubt may exist about the ability of *D. cymosum* seedlings to synthesize fluoroacetate, there can be no question about the results obtained with aseptic *D. cymosum* callus. It has clearly been demonstrated that the callus can grow normally for several years in the absence of fluoride and be devoid of fluoroacetate. However, when fluoride is provided, fluoroacetate is formed. The fluoroacetate concentration in such tissues can equal or exceed that normally found in the vegetative organs of field grown *D. cymosum* plants (Tannock 1975, Meyer and Grobbelaar 1989). In this case the presence of bacteria within the tissue can be ruled out.

Compared to field-grown plants, the *D. cymosum* seedlings grown in an aseptic environment in this study appeared quite normal. The bacterial endophyte(s) consequently do not appear to affect the plant morphologically to a noticeable degree.

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CHAPTER 4

FLUOROACETATE-METABOLIZING PSEUDOMONAD ISOLATED FROM *DICHAPETALUM CYMOSUM*

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Fluoroacetate-Metabolizing Pseudomonad Isolated from *Dichapetalum cymosum*

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A pseudomonad was isolated from the fluoroacetate-producing plant *Dichapetalum cymosum* (Hook) Engl. and identified as *Pseudomonas cepacia*. We established that this isolate was capable of growing in fluoroacetate-enriched solutions without any reduction in growth rate. Our isolate of *P. cepacia* was capable of defluorinating 2.69 μg of fluoroacetate per 10^9 cells per h. Fluoroacetate was degraded to CO_2 at a rate of 23.53 ng/10^9 cells per h.

Pseudomonads have been isolated from a variety of materials, including soils, water, spoiled food, and diseased plants and animals (13). Goldman (4) isolated a pseudomonad from soil which is capable of cleaving the fluorine-carbon bond of fluoroacetate. The enzyme which brought about the defluorination was purified and named haloacetate halidohydrolase (5). The great stability of the fluorine-carbon bond of fluoroacetate is indicated by its ability to withstand boiling and treatment with concentrated sulfuric acid (15). Sodium fusion at 500°C or refluxing in 30% NaOH is necessary for the complete liberation of fluorine as fluoride from fluoroacetate (12). Kelly (6), Tonomura et al. (18), and Walker and Lien (19) have also reported the ability of soil pseudomonads and other bacteria to metabolize fluoroacetate.

Dichapetalum cymosum causes sudden death to animals and has been recognized as a hazard to livestock in southern Africa (17). Marais (11) isolated the toxic component of the plant and identified it as fluoroacetate. While attempting to establish a callus culture of *D. cymosum*, it became evident that the plants were invariably infested with bacteria. Although the bacteria appears to infect the pericarp and to a lesser extent the seed, most seeds appear to be aseptic internally before the fruit has become fully ripe (5a). This observation led to the present study.

MATERIALS AND METHODS

Materials. Stems (approximately 2 cm in diameter) and seeds of *Dichapetalum cymosum* (Hook) Engl. were sampled near Pretoria on three occasions between August and December 1988 and in the Ellisras district (about 250 km northwest of Pretoria) in November 1988. [1,2-^{14}C]fluoroacetate (22 Ci/mol) was obtained from Amersham International plc, Amersham, England.

Isolation of bacteria. Six ripe seeds which had freshly been excised from the fruit and four stems of *D. cymosum* were thoroughly washed with distilled water and surface sterilized for 20 min in 0.35% sodium hypochlorite [Gillett (Pty) Ltd]. The following procedure was conducted under aseptic conditions: Sodium hypochlorite was removed by washing the seeds and stems with water, and the outer 2 mm of the seeds and stems was removed with a razor blade. The remaining material was twice more sterilized and peeled as described above. The remaining chunks (approximately 3 mm by 3 mm) were incubated in nutrient agar [Biolab (Pty) Ltd] in petri dishes at 29°C.

Identification of bacterial isolates. Cultures isolated from the seeds and stems were tentatively identified by using the API 20 NE system (API System S.A., Lyon, France). This identification was further substantiated by polyacrylamide gel electrophoresis of soluble cellular proteins according to the modification by Van Zyl and Steyn (E. Van Zyl and P. L. Steyn, Syst. Appl. Microbiol., in press) of the method of Kersters and De Ley (7). *Pseudomonas cepacia* NCPPB 1962, obtained from the National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Fisheries and Food, Hatching Green Harpenden, Hertfordshire, England, was used as the reference strain.

Growth of bacteria in fluoroacetate-enriched nutrient solution. The bacteria were grown for 72 h at 29°C in 0.5% liquid yeast extract [Biolab (Pty) Ltd] which was enriched with 1% D-ribose and fluoroacetate in the following concentrations: 10, 25, and 50 mM. The A_{600} of the cultures was measured, and the density of the bacteria in the culture was determined from a calibration curve of absorbance versus cell number. The bacterial density was 8.5×10^5 cells per ml at the time of inoculation. Each treatment was repeated three times.

Cleavage of the C—F bond of fluoroacetate. The bacterium was grown in a solution containing 0.5% liquid yeast extract [Biolab (Pty) Ltd], 1% D-ribose, and 50 mM fluoroacetate. The bacterial density at the time of inoculation was 4.3×10^3 cells per ml. Uninoculated medium served as a control. The fluoride concentration of the solutions was measured periodically by means of a fluoride-specific ion electrode (Orion Research Inc). This experiment was repeated three times.

Metabolism of [1,2-^{14}C]fluoroacetate by bacterial isolate. The bacteria were grown in 0.5% liquid yeast extract enriched with 1% D-ribose for 72 h at 29°C, after which the culture was divided into two aliquots, one of which was autoclaved for 15 min at 120°C. Samples (3 ml each) of the autoclaved and unautoclaved cultures were transferred to nylon Conway dishes. Three milliliters of fresh medium was added to a third Conway dish. One microcurie of [1,2-^{14}C]fluoroacetate was added to the solution in each Conway dish, and 0.5 ml of 0.1 M NaOH was pipetted into the center well of each Conway dish before the dishes were sealed with high-vacuum grease (Dow Corning) and Perspex plates. After 48 h of incubation at 29°C, 0.4 ml of each NaOH

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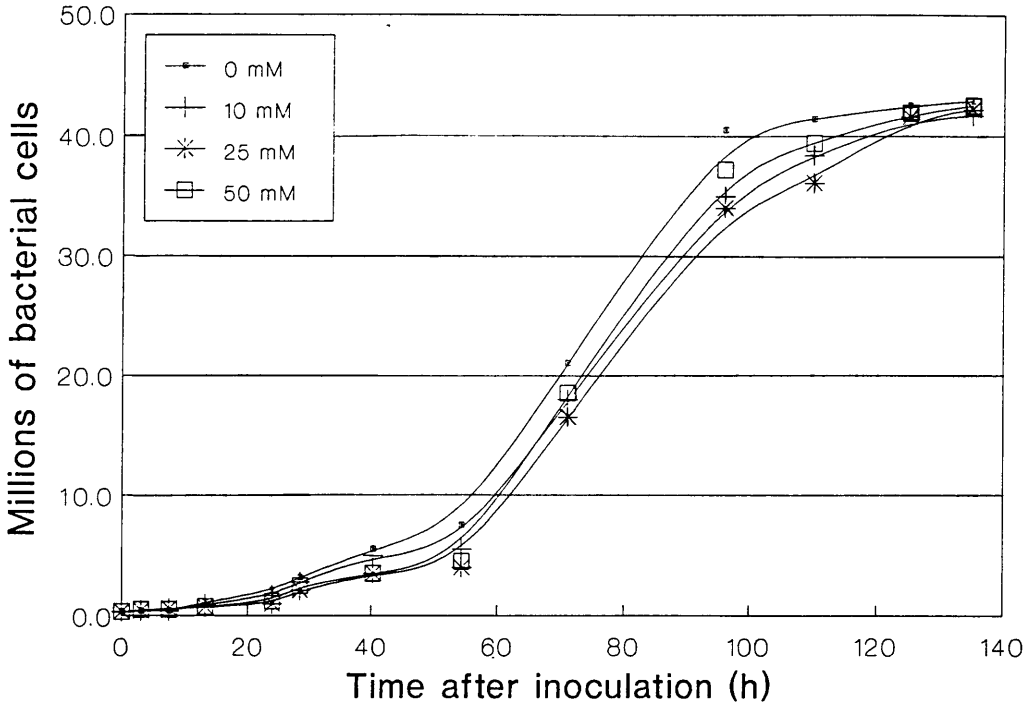

FIG. 1. Number of cells of *P. cepacia* per milliliter of fluoroacetate-enriched medium. Means of three replicates.

solution was mixed with 10 ml of Insta Gel II [Packard (Pty) Ltd] and the radioactivity was determined with a liquid scintillation counter. To verify that the radioactivity trapped in the center wells was in the form of $^{14}CO_2$, samples of the NaOH solutions were mixed with an equal volume of 1 M $Ba(OH)_2$ to precipitate the radioactive carbonate and filtered through Whatman no. 42 filter paper with suction. The radioactivity in the filtrates was determined with a liquid scintillation counter.

RESULTS

Isolation and identification of bacteria. Bacterial colonies developed after 3 and 4 days on plates on which stems and seeds, respectively, were incubated. All four stems and three of six seeds used in this study were infested with bacteria. All isolates were identified as *Pseudomonas cepacia* according to the API 20 NE system (99.9%). This preliminary identification was confirmed by polyacrylamide gel electrophoresis of soluble cellular proteins when compared with those of reference strain *P. cepacia* NCPPB 1962.

Growth of *P. cepacia* in fluoroacetate-enriched nutrient solution. The addition of fluoroacetate to the nutrient medium had very little effect on the growth of *P. cepacia* even at the relatively high concentration of 50 mM (Fig. 1). The abundance of the bacteria in the different treatments did not differ significantly at the 1% probability level.

Cleavage of the C—F bond of fluoroacetate. In both treatments, a small amount of fluoride was produced during the first 20 h (Fig. 2), after which no additional fluoride was produced in the control treatment. The *P. cepacia* culture entered its logarithmic growth phase only after 85 h probably because of the small inoculum density. The culture started to produce fluoride at this stage and continued to do so for the next 52 h at a constant rate of abut 2.69 $\mu g/10^9$ cells per h.

Metabolism of [1,2-^{14}C]fluoroacetate by *P. cepacia*. No radioactivity was found in the filtrates of samples treated with $Ba(OH)_2$, suggesting that all the radioactivity was in the form of CO_2. If the CO_2 that was produced by the live bacteria originated from both carbons of fluoroacetate, then the live *P. cepacia* degraded the fluoroacetate at a rate of 6.423 ng/h. A very small but similar amount of CO_2, equivalent to approximately 0.027 ng of fluoroacetate per h, was produced in the two control treatments. The bacterial density was 1.08×10^8 cells per ml when the [^{14}C]fluoroacetate was added. $^{14}CO_2$ was produced from [1,2-^{14}C]fluoroacetate at 336 dpm in nutrient medium alone, 316 dpm in medium with dead *P. cepacia*, and 71, 507 dpm in medium with live *P. cepacia*. (Results are the means of three experiments).

DISCUSSION

P. cepacia is a soil-inhabiting bacterium which causes root rot of onions (2) and may also be an opportunistic pathogen of humans (3). It seems, however, to have no noticeable effect on the morphology of *D. cymosum*, from which it was isolated. *P. cepacia* is capable of living inside *D. cymosum* probably because it can metabolize fluoroacetate by cleaving the C-F bond. Because a normal tricarboxylic acid cycle operates in all strains of pseudomonads examined (13), it is possible that the carbon of the acetate resulting from the defluorination of fluoroacetate is released as CO_2 through the bacterial Krebs cycle.

P. cepacia is able to utilize a greater number of compounds as sole sources of carbon and energy than any other bacterium investigated in this regard to date (2, 14, 16). The extraordinary nutritional versatility of *P. cepacia* and its ability to colonize both plant and animal tissues represent a degree of adaptability not encountered in most bacteria (9). Such adaptability presumably demands a large number of different biochemical reactions and implies the operation of well-developed, and possibly novel, regulatory mechanisms.

Relatively high fluoroacetate concentrations (231.9 mg/kg

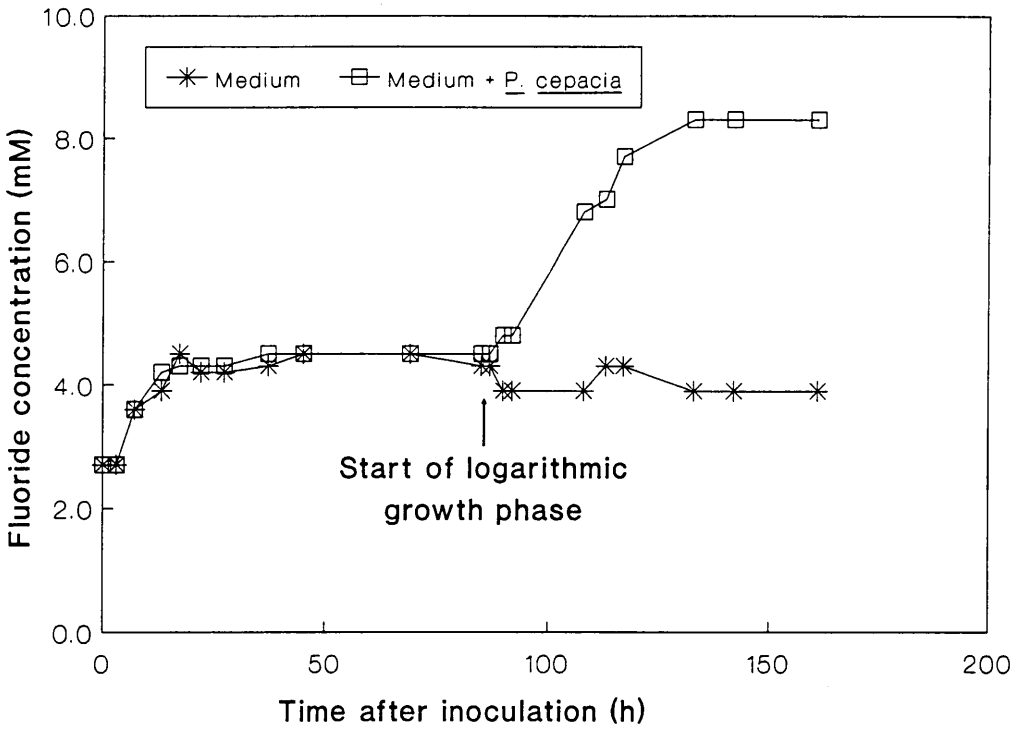

FIG. 2. Release of fluorine in the form of fluoride from fluoroacetate by *P. cepacia* culture initially containing 10^9 cells per ml.

of fresh young leaves) are present in *D. cymosum* (11a). The presence of *P. cepacia* in *D. cymosum* may contribute to the degradation of fluoroacetate. *D. cymosum* appears to have a functional Krebs cycle whose aconitase is inhibited by fluorocitrate (1, 10). Because the conversion of fluoroacetate to fluorocitrate apparently is a unique property of certain cell organelles (8), it is conceivable that despite a relatively high overall fluoroacetate concentration in *D. cymosum*, the concentrations in key organelles are kept low, thereby precluding the poisoning of the plant. The possible clustering of *P. cepacia* around such key organelles could considerably assist the plant in preventing fluoroacetate from entering the organelles. On the other hand, it would appear that *D. cymosum* itself also has the ability to degrade fluoroacetate (5a), and this activity could be concentrated in or around the key subcellular organelles. This is, however, speculation and is worthy of further investigation.

With gene manipulation, it might be possible to enable rumen bacteria to break down fluoroacetate. This exciting prospect also needs further investigation.

ACKNOWLEDGMENTS

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CHAPTER 5

FLUOROACETATE DEGRADATION BY *DICHAPETALUM CYMOSUM*

FLUOROACETATE DEGRADATION BY *DICHAPETALUM CYMOSUM*

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Summary

The fluoroacetate-containing plant, *Dichapetalum cymosum* is capable of releasing CO_2 from fluoroacetate. Fluoroacetate is more readily degraded by the old than the young leaves of the plant. An aseptic callus culture of *D. cymosum* is also capable of degrading fluoroacetate albeit at a much lower rate than the leaves of the plant. Inoculation of the callus with *Pseudomonas cepacia* isolated from the plant, increased the rate of CO_2 release from fluoroacetate about five fold.

Key words: *Dichapetalum cymosum*, fluoroacetate, *Pseudomonas cepacia*.

Introduction

Fluoroacetate, the toxic principle of *Dichapetalum cymosum* (Hook.) Engl. was isolated by Marais in 1944. The oral lethal dose of this toxic compound is 0.06 - 0.20 mg/kg for dogs, 0.15 - 0.62 mg/kg for cattle and 0.25 - 0.50 mg/kg for sheep (Kellerman et al. 1988). Animals convert fluoroacetate to fluorocitrate which inhibits aconitase competitively, thereby blocking the Krebs cycle (Morrison and Peters 1954, Fanshier et al. 1964, Villafranca and Platus 1973). Plants are less sensitive to fluoroacetate than animals. To inhibit the aconitase of pig heart and sycamore (*Acer pseudoplatanus* L.) to the same degree with fluorocitrate, a 2 000-fold higher fluorocitrate concentration is required in the latter case (Treble et al. 1962).

Relatively high fluoroacetate concentrations (231.9 mg/kg fresh mass) are present in young *D. cymosum* leaves (Meyer and Grobbelaar 1990). In the old leaves the fluoroacetate concentration is however much lower (97.0 mg/kg fresh mass). Meyer and Grobbelaar (1990) found that foliarly applied fluoroacetate is more readily accumulated by young than old leaves. The question now arises as to whether fluoroacetate is more readily metabolized by the old than the young leaves of *D. cymosum*. When Eloff and Grobbelaar (1972) fed fluoroacetate-2-^{14}C to young and old leaves of *D. cymosum*, they found that per leaf more radioactivity was incorporated into the carbohydrates and amino acids of the old than the young leaves.

The aim of this study was to establish whether *D. cymosum* can

degrade fluoroacetate to CO_2. The investigation was complicated by the discovery that all vegetative parts of *D. cymosum* are apparently infested with a bacterium. This endophyte was subsequently identified as *Pseudomonas cepacia* by Meyer et al. (1990). One billion (10^9) cells of the isolated *P. cepacia* was found to be capable of defluorinating 2.69 mg of fluoroacetate per hour and of degrading 23.59 ng of fluoroacetate per hour to CO_2. For this reason the rate of fluoroacetate degradation by young and old *D. cymosum* leaves as well as aseptic *D. cymosum* callus with and without *P. cepacia* was compared.

Materials and Methods

Materials

Young and old leaves were sampled from approximately one and a half year old *D. cymosum* plants which were grown from seed and cultivated in a glasshouse (27°C day and 21°C night temperature). The aseptic callus culture of *D. cymosum* was initiated from seed by Grobbelaar and Meyer (1990). *P. cepacia* was isolated from stems of *D. cymosum* (Meyer et al. 1990). [1,2-^{14}C]Fluoroacetate (22 Ci/mole) was obtained from Amersham International plc, England.

Methods

Fluoroacetate breakdown by leaves

The following procedure was carried out on disks (1 cm diame-

ter) of young, old, and autoclaved leaves (15 min at 120°C) as well as Whatman no.42 filter paper. Six disks were used per Conway dish and 60 µl in all, off a solution containing 1 µCi [1,2-^{14}C]fluoroacetate and 2% di-(2-ethylhexyl) sodium sulphosuccinate as wetting agent, was spread over the disks of each Conway dish. The dishes were sealed with Perspex plates using high-vacuum grease (Dow Corning) after 0.5 ml 0.1 M NaOH was added to the centre wells. The radioactivity in the NaOH solution was determined after 6, 12, 24 and 48 hours of incubation at 29°C in white light with an irradiance of about 1000 lux (one 55 Watt Thorn "cool white" fluorescent tube and three 15 Watt incandescent bulbs).

Fluoroacetate breakdown by *D. cymosum* callus with and without *P. cepacia*

The following procedure was conducted under aseptic conditions: The *D. cymosum* callus culture was grown in Schenk and Hildebrandt liquid medium (1972) without auxins and cytokinins, as described by Grobbelaar and Meyer (1990). The bacteria were grown at 29°C in 0.5% liquid yeast extract enriched with 1% D-ribose. Six pieces of callus were dipped into the bacterial culture and placed in a Conway dish. A solution (60µl) containing 1µCi of [1,2-^{14}C]fluoroacetate was used per Conway dish. The fluoroacetate was spread on the calli with bacteria and 0.5 ml of 0.1 M NaOH was placed in the centre well of the Conway dish before the dish was sealed with a Perspex plate using high-vacuum grease (Dow

Corning). The same procedure was carried out on calli which were not dipped into the bacterial culture and on calli that were autoclaved for 15 min at 120°C. The radioactivity in the NaOH solution was determined after 6, 12, 24 and 48 hours of incubation under the same conditions as was used for the leaf disks.

Determination of radioactivity

The NaOH was rinsed from the Conway dishes and made to 2 ml with water. One ml was mixed with 10 ml Insta Gel II (Packard) and the radioactivity determined with a liquid scintillation counter. To verify that the radioactivity trapped in the centre wells was in the form of $^{14}CO_2$, samples of the NaOH solutions were mixed with an equal volume of 1 M $Ba(OH)_2$ to precipitate the carbonate. After filtration through Whatman no.42 filter paper using suction, the radioactivity of the filtrates was determined with a liquid scintillation counter.

Results

No radioactivity was found in the filtrates of samples treated with $Ba(OH)_2$, suggesting that all the radioactivity was in the form of CO_2. The $^{14}CO_2$ produced by the autoclaved leaves during 48 hours of incubation, gave a count of 672 dpm/g dry weight whilst that produced by the autoclaved callus and filter paper during the same period gave values of 698 and 571 dpm/g dry weight respectively.

D. cymosum is capable of degrading fluoroacetate to CO_2 (Fig. 1). In the 6 hour as well as the 12 hour treatment the amount of $^{14}CO_2$ produced by the young and old leaves did not differ significantly at the 5% probability level. However, during 48 hours, the old leaves produced nearly three times the amount of $^{14}CO_2$ than did the young leaves (282 963 dpm/g dry weight compared to 104 643 dpm/g dry weight respectively).

The amount of CO_2 produced during 48 hours was similar for old leaves (282 963 dpm/g dry weight) and callus that had been inoculated with bacteria (291 383 dpm/g dry weight). During the same period the aseptic callus produced a relatively small amount of $^{14}CO_2$ (65 261 dpm/g dry weight). During each of the four incubation periods, the aseptic callus was the least active and the inoculated callus the most active of the four materials tested in degrading fluoroacetate to CO_2.

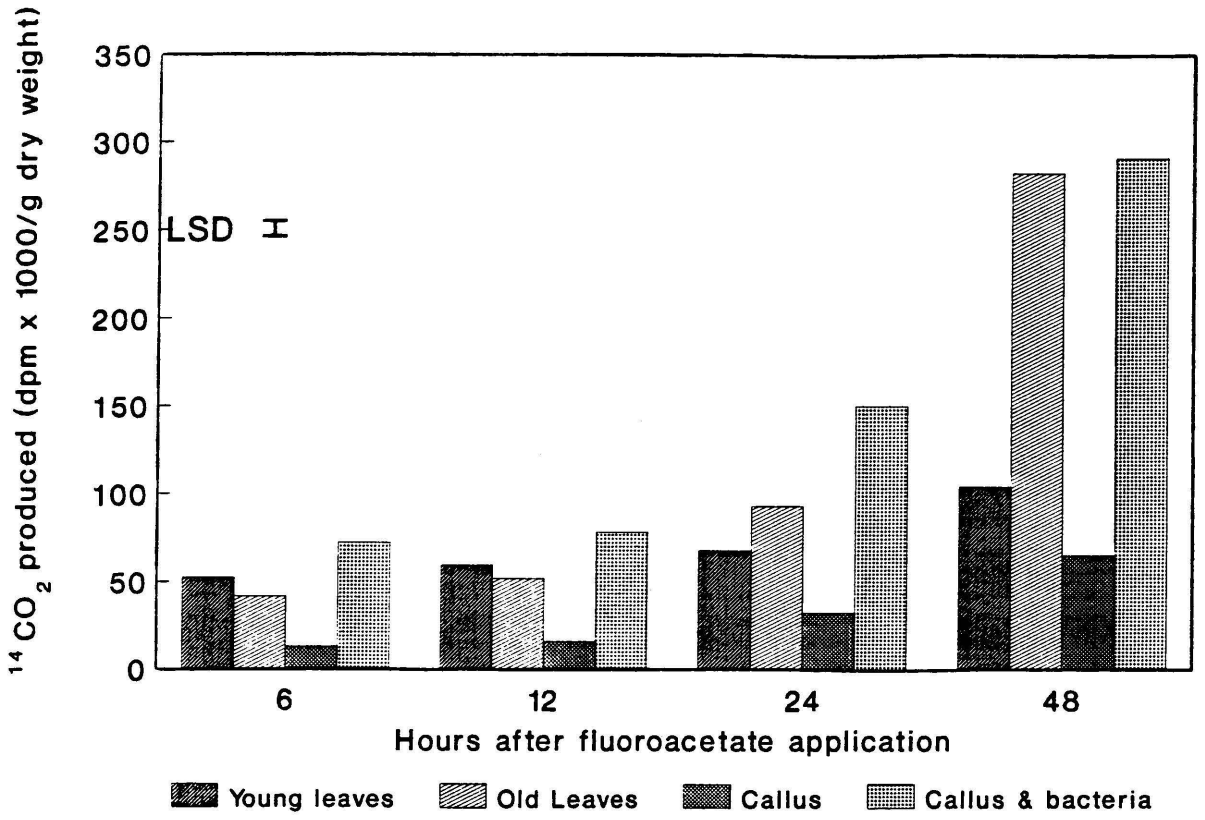

Fig. 1: Production of $^{14}CO_2$ from [1,2-^{14}C]fluoroacetate by young and old *D. cymosum* leaves, callus and callus inoculated with *P. cepacia* isolated from *D. cymosum*. LSD was calculated on the 5% level of significance according to the method of Tukey (Steel and Torrie 1960). Four replicates were used per treatment.

Discussion

The old leaves of *D. cymosum* are less toxic than the young leaves for two possible reasons: Firstly fluoroacetate is more readily degraded by the old than the young leaves and secondly fluoroacetate is more readily accumulated by young than old leaves (Meyer and Grobbelaar 1990). The number of *P. cepacia* cells in old and young leaves are unfortunately not known and if this differs substantially, it might explain the difference in the fluoroacetate concentration of the young and old leaves.

From the results of callus treated with radioactive fluoroacetate, it is clear that the plant is capable of degrading fluoroacetate to CO_2 without the presence of bacteria. However much less breakdown occurred in the callus than in the leaves. The difference is probably due to the presence of bacteria in the leaves of *D. cymosum* because the rate of fluoroacetate breakdown was approximately five times higher in callus that was inoculated with bacteria than in aseptic callus during all four treatment periods. From an earlier report (Meyer et al. 1990) it is known that the bacterial cells are capable of degrading fluoroacetate at a high rate to CO_2.

Oxygen uptake by *Parinarium capense* leaf disks is inhibited about 40% by 50 mM fluoroacetate. This concentration of fluoroacetate also causes the citrate concentration to double in this plant (Louw et al. 1970). No inhibition of oxygen uptake or accumulation of fluorocitrate occurs at a fluoroacetate concentration of 50 mM in *D. cymosum* (Louw et al. 1970). The same

authors however found that aconitase isolated from *D. cymosum* is about as sensitive to inhibition by fluorocitrate as aconitase from *P. capense*. It is therefore unlikely that fluoroacetate is metabolized to fluorocitrate via fluoroacetyl coenzyme A by *D. cymosum* as would appear to be the case in *P. capense*. A normal tricarboxylic acid cycle operates in all strains of pseudomonads examined (Palleroni 1986) and Balinsky and Schneiderman (1964) and Eloff (1972) reported the functioning of this cycle in *D. cymosum*. Although these two organisms might have another means of degrading fluoroacetate to CO_2, the available results do not make it mandatory to exclude the involvement of the tricarboxylic acid from the process. *P. cepacia* is capable of defluorinating fluoroacetate at a high rate. (Meyer et al. 1990) and it is possible that *D. cymosum* can carry out the same process. The carbon of the acetate resulting from the defluorination of fluoroacetate could then conceivably be released as CO_2 by the tricarboxylic acid cycle.

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CHAPTER 6

FLUOROACETYL-COENZYME A HYDROLASE-LIKE ACTIVITY IN *DICHAPETALUM CYMOSUM*

FLUOROACETYL-COENZYME A HYDROLASE-LIKE ACTIVITY IN *DICHAPETALUM CYMOSUM*

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Summary

Fluoroacetyl-CoA was synthesized from sodium fluoroacetate via its anhydride. Porcine heart citrate synthase and a crude mitochondrial enzyme extract from *Dichapetalum cymosum* were used as catalysts. With fluoroacetyl-CoA as the only substrate and the *D. cymosum* crude mitochondrial extract as enzyme source, CoASH release took place at a rate of 0.0295 µmoles/min/mg protein, indicating the presence of an enzyme in *D. cymosum* which could be fluoroacetyl-CoA hydrolase. This hydrolase could not use acetyl-CoA as a substrate. The presence of this enzyme in *D. cymosum* probably explains why this plant is not poisoned by its production of fluoroacetate.

Key words: Citrate synthase, *Dichapetalum cymosum*, fluoroacetyl-coenzyme A, fluoroacetyl-coenzyme A hydrolase.

Introduction

Only fluoroacetyl-CoA (Brady 1955, Marcus and Elliott 1956, Srere 1972) and propionyl-CoA (Wiegand and Remington 1986) can replace acetyl-CoA as a substrate for the reaction catalyzed by citrate synthase. When fluoroacetyl-CoA and oxalacetate are used as substrates, the highly toxic fluorocitrate is formed. Fluorocitrate inhibits aconitase competitively, thereby blocking the Krebs cycle (Morrison and Peters 1954, Fanshier et al. 1964, Villafranca and Platus 1973). Fluoroacetyl-CoA can be synthesized *in vivo* and *in vitro* from fluoroacetate by acetyl-CoA synthetase (Brady 1955, Marcus and Elliott 1956, Mathews and van Holde 1990).

Fluoroacetate is relatively nontoxic to plants when compared to animals (Peters 1957, Treble et al. 1962). A possible explanation for this is to be found in the work of Kuhn, Knauf and Stumpf (1981) who reported that the enzyme, acetyl-CoA synthetase is not present in the mitochondria of spinach (*Spinacia oleracea*) but is localized solely in its chloroplasts. If the mitochondria of plants, unlike those of animals, do not contain acetyl-CoA synthetase, they will not be able to synthesize fluorocitrate from fluoroacetate via fluoroacetyl-CoA. Consequently fluoroacetate would not be as toxic to plants as it is to animals. If however, fluoroacetyl-CoA can be synthesized outside the mitochondria in plant cells and can then be transported into the mitochondria, the aconitase inhibiting fluorocitrate would be formed. Brooks and Stumpf (1966) and Liedvogel and Stumpf (1982)

did however, find that acetyl-CoA synthesized in the chloroplasts can not be transported across the chloroplast and mitochondrion membranes. It is therefore unlikely that fluoroacetyl-CoA would be transported across these membranes. The relative resistance of plants towards fluoroacetate could therefore possibly be explained by their inability to convert it to fluorocitrate in their mitochondria. It must however be emphasized that the work of Stumpf and his co-workers was carried out on spinach only and that their findings do not necessarily apply to plants in general and *D. cymosum* in particular.

Fluoroacetate was isolated from *Dichapetalum cymosum* (Hook.) Engl. by Marais in 1944. Relatively high fluoroacetate concentrations (232 mg/kg fresh mass) are present in young *D. cymosum* leaves (Tannock 1975, Meyer and Grobbelaar 1990). The following remark was made by Treble, Lamport and Peters in 1962: "...the mystery remains why *D. cymosum* and *Acacia georginae* which make fluoroacetate are not poisoned by their own product". This might partly be explained by the reasoning in the previous paragraph but although plants seem to be more resistant to fluoroacetate than animals (Peters 1957, Treble et al. 1962), there are reports of plants being sensitive to it (Peters 1957). Oxygen uptake by *Parinarium capense* leaf disks is inhibited about 40% by a fluoroacetate concentration of 50 mM. This concentration of fluoroacetate also causes the citrate concentration to double in this plant (Louw et al. 1970). These findings suggest that unlike the results obtained by Stumpf and his co-workers (Kuhn, Knauf and

Stumpf 1981) with spinach, fluoroacetate can be taken up by the mitochondria of *P. capense* which apparently can convert it to fluorocitrate. No inhibition of oxygen uptake or accumulation of fluorocitrate occurs in *D. cymosum* by foliarly applied fluoroacetate at a concentration of 50 mM (Louw et al. 1970). These authors also reported that aconitase isolated from *D. cymosum* is about as sensitive to inhibition by fluorocitrate as aconitase from *P. capense*. Eloff and Von Sydow (1971) also showed that *D. cymosum* aconitase and oxygen uptake are inhibited by fluorocitrate. They concluded that the tolerance of *D. cymosum* to high concentrations of fluoroacetate may be ascribed to the fact that the 'lethal synthesis' of fluorocitrate does not take place in the plant, most probably because citrate synthetase has different affinities for fluoroacetyl-CoA and acetyl-CoA. It was the aim of this study to investigate the metabolic fate of fluoroacetyl-CoA in *D. cymosum*.

Materials and Methods

Materials

An aseptic callus culture of *D. cymosum*, initiated from seed by Grobbelaar and Meyer (1990), was used as a source of crude mitochondrial enzyme extract. Citrate synthase from porcine heart (170 units/mg protein) was purchased from Sigma Chemical Company.

Isolation of mitochondria

D. cymosum callus (45.713 g fresh mass) was rinsed with water

and immediately chilled. All subsequent steps, unless noted otherwise, were carried out at 1-4°C. The callus was ground in a solution containing 8.0 g polyvinylpyrrolidone, 50 mM potassium phosphate buffer (pH 7.6), 0.4 M sucrose, 5 mM EGTA and 0.1% bovine serum albumin (Day and Hanson 1977) with mortar and pestle. The slurry (150 ml final volume) was filtered through four layers of nylon cloth and the filtrate centrifuged at 2 000 g for three min. The supernatant was centrifuged at 20 000 g for three min, the pellet suspended in 0.4 M sucrose and again centrifuged at 20 000 g for three min. The crude mitochondrial pellet was gently re-suspended in a small volume of 0.4 M sucrose, transferred to a centrifuge tube containing 40 ml 25% Percoll in 0.25 M sucrose and 5.0 mM Tris-HCl (pH 7.5) and centrifuged at 35 000 g for 30 min. The mitochondrial layer was removed with a Pasteur pipette, mixed with 50 ml of a solution that contained sucrose (0.25 M), potassium phosphate buffer (50 mM, pH 7.2), ß-mercaptoethanol (5.0 mM) and bovine serum albumin (0.05 g) and centrifuged for 10 min at 9500 g.

Rupturing of mitochondria

The mitochondrial pellet was sonicated 20 times for 10 sec at a time at 20 kcycles/sec, with intervals of 5 sec between successive sonications. The suspension was centrifuged for 10 min at 37000 g and the supernatant used as a crude mitochondrial enzyme source. Protein in the crude mitochondrial enzyme preparation was determined by the method of Bradford (1976).

Preparation of fluoroacetic anhydride

Concentrated sulfuric acid (10 ml) was added to 1.50 g sodium fluoroacetate. After thorough mixing, 0.57 g crude fluoroacetic acid was obtained by distillation under reduced pressure. A solution of the fluoroacetic acid and dicyclohexylcarbodiimide (1.50 g) in ether was stirred at room temperature for 6 h. The dicyclohexylurea that formed, was removed by filtration and the filtrate evaporated to yield fluoroacetic anhydride as residue.

Preparation of fluoroacetyl-CoA

The trilithium salt of CoASH (21.7 mg) was dissolved in 10 ml carbonate buffer (0.2 M, pH 7.5). A slight excess of fluoroacetic anhydride in 10 ml ether was added to the aqueous solution, the pH quickly adjusted to 6.1 with 2 M hydrochloric acid and the solution shaken vigorously for 15 min. The aqueous phase was extracted three times with ether and the remaining traces of ether removed in a stream of nitrogen. The resulting aqueous solution was used in subsequent enzyme assays.

Enzyme assay

All assays were performed at 25°C in a total volume of 2 ml contained in a spectrophotometer cuvette with a 1 cm light path. The release of CoASH was monitored through its reaction with 5'5'-dithiobis (2-nitrobenzoic acid) (DTNB), to form a mercaptide which absorbs light at 412 nm (Srere et al. 1963). All reaction mixtures contained the following (final concentrations): Tris-HCl

(0.2 M, pH 8.0), DTNB (0.1 mM) in potassium phosphate (0.1 M, pH 7.0) and water. Oxalacetate (100 µM), acetyl-CoA (50 µM) and fluoroacetyl-CoA (50 µM) were used singly or in certain combinations (Table 1) as substrates. The amount of enzyme used was adjusted so that the rate of increase in optical density did not exceed 0.050 for the period between 15 and 30 sec after the start of the reaction. Three replicates were used per treatment.

Results

Fluoroacetyl-CoA and acetyl-CoA were found to be unstable and 50 µM solutions of them decomposed at rates of 0.276 nmoles/min and 0.114 nmoles/min respectively, at 25°C and pH 8.0. The relevant enzyme activity rates in Table 1 have been corrected for these non-enzymatic hydrolyses.

Citrate synthase from porcine heart was unable to hydrolyse acetyl-CoA or fluoroacetyl-CoA when these were used singly as substrates (Table 1). The rate at which it liberated CoASH when acetyl-CoA plus oxalacetate were used as substrates (78.1000 µmoles/min/mg protein) was approximately 87 times higher than the rate at which CoASH was liberated when fluoroacetyl-CoA plus oxalacetate were used as substrates (0.9008 µmoles/min/mg protein).

The crude enzyme preparation of *D. cymosum* could not hydrolyse acetyl-CoA when it was provided as the sole substrate. Remarkably however, when fluoroacetyl-CoA was used as the sole substrate,

Table 1: The CoASH-liberating activity of porcine citrate synthase and a crude mitochondrial enzyme extract from *D. cymosum*. Averages of three replicates. All treatments which showed enzyme activity differed from each other at the 5% level of significance according to the method of Tukey (Steel and Torrie 1960). The following substrates were used in different combinations (final concentrations): 100 µM oxalacetate (OAc), 50 µM acetyl-CoA (AcCoA) and 50 µM fluoroacetyl-CoA (FAcCoA). 0.2 M Tris-HCl (pH 8.0) and 0.1 mM DTNB (5'5'-dithiobis (2-nitrobenzoic acid)) in 0.1 M potassium phosphate (pH 7.0) were present in all reaction mixtures. The final volume of all reaction mixtures was 2 ml.

Enzyme preparation	Substrates	Activity µmoles/min/mg protein
Porcine citrate synthase	OAc	0.0
	AcCoA	0.0
	FAcCoA	0.0
	OAc + AcCoA	78.1000
	OAc + FAcCoA	0.9008
D. cymosum crude mitochondrial extract	OAc	0.0
	AcCoA	0.0
	FAcCoA	0.0295
	OAc + AcCoA	0.0391
	OAc + FAcCoA	0.0109

CoASH liberation took place at a rate of 0.0295 µmoles/min/mg protein. No CoASH release from fluoroacetyl-CoA could be detected when the enzyme preparation was heated to 100°C for five minutes before it was used in the assay. The rate of CoASH liberation when oxalacetate and fluoroacetyl-CoA were simultaneously used as substrates (0.0109 µmoles/min/mg protein), was approximately one third of the rate that was obtained when fluoroacetyl-CoA was used alone. When oxalacetate and acetyl-CoA were used simultaneously as substrates, the highest rate of CoASH liberation with the crude enzyme preparation (0.0391 µmoles/min/mg protein) was obtained.

Discussion

Brady (1955) also found fluoroacetyl-CoA to be unstable and reported a decomposition rate of 16% per day in the frozen state at pH 7.0. This author also found acetyl-CoA to be more stable than fluoroacetyl-CoA.

With porcine citrate synthase the rate of the reaction between oxalacetate and fluoroacetyl-CoA, was about 1% of the rate between oxalacetate and acetyl-CoA. This activity is slightly more than Brady's (1955) 0.5%, but approximately the same as that reported by Marcus and Elliott (1956).

When however, the *D. cymosum* crude mitochondrial extract was used as the enzyme source, the apparent rate of the reaction between oxalacetate and fluoroacetyl-CoA was approximately 28% of the rate between oxalacetate and acetyl-CoA. This interesting

observation can be explained by the fact that the *D. cymosum* enzyme extract was capable of liberating CoASH from fluoroacetyl-CoA without the addition of oxalacetate. The rate of CoASH release from fluoroacetyl-CoA hydrolysis was about 75% of that when acetyl-CoA and oxalacetate were supplied together as substrates. CoASH release from fluoroacetyl-CoA took place approximately three times faster in the absence of oxalacetate than in its presence. This indicates that the fluoroacetyl-CoA hydrolase-like enzyme is inhibited by oxalacetate. It is remarkable that the enzyme responsible for this reaction is so specific that it does not utilize acetyl-CoA as a substrate. Acetyl-CoA hydrolase activity of 0.0023 µmoles/min/mg protein was found in the matrix of spinach mitochondria by Liedvogel and Stumpf (1982). Apart from that report and a paper by Murphy and Stumpf (1981) on acetyl-CoA hydrolytic activity, no other information appears to be available on the existence of this enzyme in plants. Its presence in the mitochondria of animals has however been reported (Benson 1976 and Namboodiri et al. 1979).

The relative affinity of *D. cymosum* citrate synthase towards fluoroacetyl-CoA and acetyl-CoA remains unknown. This possible difference in affinity as speculated upon by Eloff and Von Sydow (1971), was obscured by the unexpected activity of the intriguing fluoroacetyl-CoA hydrolase-like enzyme. More light may be shed upon this when the citrate and fluorocitrate concentrations, instead of the reaction of the released CoASH with DTNB, are monitored although the most convincing elucidation of the problem

will necessitate experiments with purified citrate synthetase from *D. cymosum*.

In the experiment described here, the oxalacetate had to inhibit the fluoroacetyl-CoA hydrolase-like enzyme by more than 63% if it was involved in any citrate synthase activity when fluoroacetyl-CoA was used as one of the substrates. The maximum citrate synthase activity would have been obtained when the fluoroacetyl-CoA hydrolase-like enzyme was inhibited 100%. In the latter case the citrate synthase activity with fluoroacetyl-CoA as one of the substrates would have been 0.0109 µmoles/min/mg protein which represents 28% of the activity that was obtained with oxalacetate plus acetyl-CoA as joint substrates.

The ability of *D. cymosum* to degrade fluoroacetate by an as yet unknown mechanism (Meyer and Grobbelaar, in press) and its symbiotic relationship with the fluoroacetate metabolizing *Pseudomonas cepacia* (Meyer et al., 1990), explains to some extent why *D. cymosum* is not poisoned by its own fluoroacetate. Should some of its fluoroacetate enter its mitochondria and be converted to fluoroacetyl-CoA, its immediate breakdown by the fluoroacetyl-CoA hydrolase-like enzyme could act as an additional precaution to prevent it from blocking the tricarboxylic acid cycle. The efficiency of this strategy will however depend largely on the effectiveness with which oxalacetate inhibits the process and the relative affinity of citrate synthase for acetyl-CoA and fluoro-acetyl-CoA.

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CHAPTER 7

THE OCCURRENCE OF N-METHYL ALANINE AND N-METHYL SERINE IN *DICHAPETALUM CYMOSUM* AND ITS ENDOPHYTE *PSEUDOMONAS CEPACIA*

THE OCCURRENCE OF N-METHYL ALANINE AND N-METHYL SERINE IN DICHAPETALUM CYMOSUM AND ITS ENDOPHYTE PSEUDOMONAS CEPACIA

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Summary

An investigation into the occurrence of N-methyl alanine (NMA) and N-methyl serine (NMS) in Dichapetalum cymosum leaves, an aseptic callus culture of D. cymosum, as well as Pseudomonas cepacia isolated from D. cymosum, was done by means of thin layer chromatography. NMA was found only in the leaves of the plant, whilst NMS was found both in the bacterial isolate and the leaves of the plant. Neither NMA nor NMS was found in the aseptic callus culture of D. cymosum. It therefore seems as though the synthesis of NMA and the occurrence of NMS in the plant, result from the symbiosis between D. cymosum and P. cepacia.

Key words: Amino acids, Dichapetalum cymosum, N-methyl alanine, N-methyl serine, Pseudomonas cepacia.

Introduction

Two novel amino acids, N-methyl-L-alanine (NMA) (Eloff and Grobbelaar, 1967) and N-methyl-L-serine (NMS) (Eloff and Grobbelaar, 1969), were isolated from Dichapetalum cymosum (Hook.) Engl.. These amino acids occur in high concentrations in D. cymosum, NMA representing up to 26.6% and NMS up to 7.9% of the total nitrogen content of certain parts of the plant (Eloff, 1972). The concentration of both amino acids decrease in aging leaves suggesting that they may be metabolically important (Eloff, 1980). Both amino acids have been shown to occur in all the plant parts of D. cymosum (Eloff, 1972). Since NMA is the amino acid with the highest concentration of the free amino acids, it has been suggested that NMA may be an important nitrogenous storage compound in the plant (Eloff 1980).

During an attempt to initiate a callus culture of D. cymosum, it was found that the plants are infested with bacteria (Grobbelaar and Meyer, 1990). The bacteria were subsequently identified as Pseudomonas cepacia (Meyer et al., 1990). The question then arose as to whether NMA and/or NMS are formed by the plant or by its endophyte P. cepacia. To answer this question a comparative study was made on the occurrence of NMA and NMS in the leaves of D. cymosum, an aseptic callus culture of D. cymosum and a culture of P. cepacia isolated from D. cymosum.

Material and Methods

Material

Leaf samples of D. cymosum were collected in the field near Pretoria in February 1990. The aseptic callus culture of D. cymosum was initiated from seed by Grobbelaar and Meyer, (1990). The P. cepacia used was isolated from D. cymosum (Meyer et al., 1990).

Methods

The D. cymosum callus culture was grown on the Schenk and Hildebrandt (1972) liquid medium without auxins and cytokinins, as described by Grobbelaar and Meyer (1990).

Samples of leaves (24.12 g fresh weight) and callus (63.30 g fresh weight) were homogenised in 70% ethanol with an ultra turrax homogeniser. The homogenates were filtered through Whatman no.42 filter paper with suction and the filtrates concentrated until free of ethanol on a rotary evaporator at 65°C, extracted with chloroform and diethyl ether and the aqueous residue concentrated further to a final volume of 2 ml.

The P. cepacia isolate was cultured on nutrient agar (Biolab) which was enriched with 1% sucrose and 0.2 M sodium fluoroacetate. After six days the bacteria were carefully scraped from the agar plates and suspended in 70% ethanol. The suspension was heated in a water bath for one hour at 80°C whereafter it was held in an ultrasonic bath for 10 minutes. Celite (BDH Chemicals) was added to the suspension to facilitate filtration through Whatman no.42 paper with

suction. The filtrate was concentrated with suction using a rotary evaporator until free of ethanol and the aqueous residue extracted with diethyl ether before concentrating it further to a small volume.

In all three cases the concentrated extracts were passed through a Dowex 50W X 4 (200-400 dry mesh) (H^+) column and the column washed with water to separate the organic acids and non-ionic compounds from the adsorbed amino acids (Hirs et al., 1952). The amino acids were displaced from the column with 2 M NH_4OH and the eluate concentrated on a rotary evaporator until free of ammonia. NMA and NMS were identified in the concentrate by co-chromatography with NMA (Sigma Chemical Company) and the leaf extract of D. cymosum (which contains both NMA and NMS) on silica-gel thin layer plates (Whatman). The solvents used were butan-1-ol : acetic acid : water (9:1:2.9 v/v/v), phenol : water (2.6:1 m/v), propan-1-ol : water (7:3 v/v) and 96% ethanol : water (7:3 v/v). The amino acids were revealed by spraying a solution of 0.2% ninhydrin and 1% acetic acid in methanol on the thin layer plates and heating at 80°C.

Results and Discussion

After using varying quantities of plant, bacteria and callus extracts in two dimensional chromatography, it is concluded that NMA occurs in the leaves only, whilst NMS occurs in both the leaves and the bacteria (Figure 1). Neither of the two amino acids were found in detectable amounts in the aseptic callus culture.

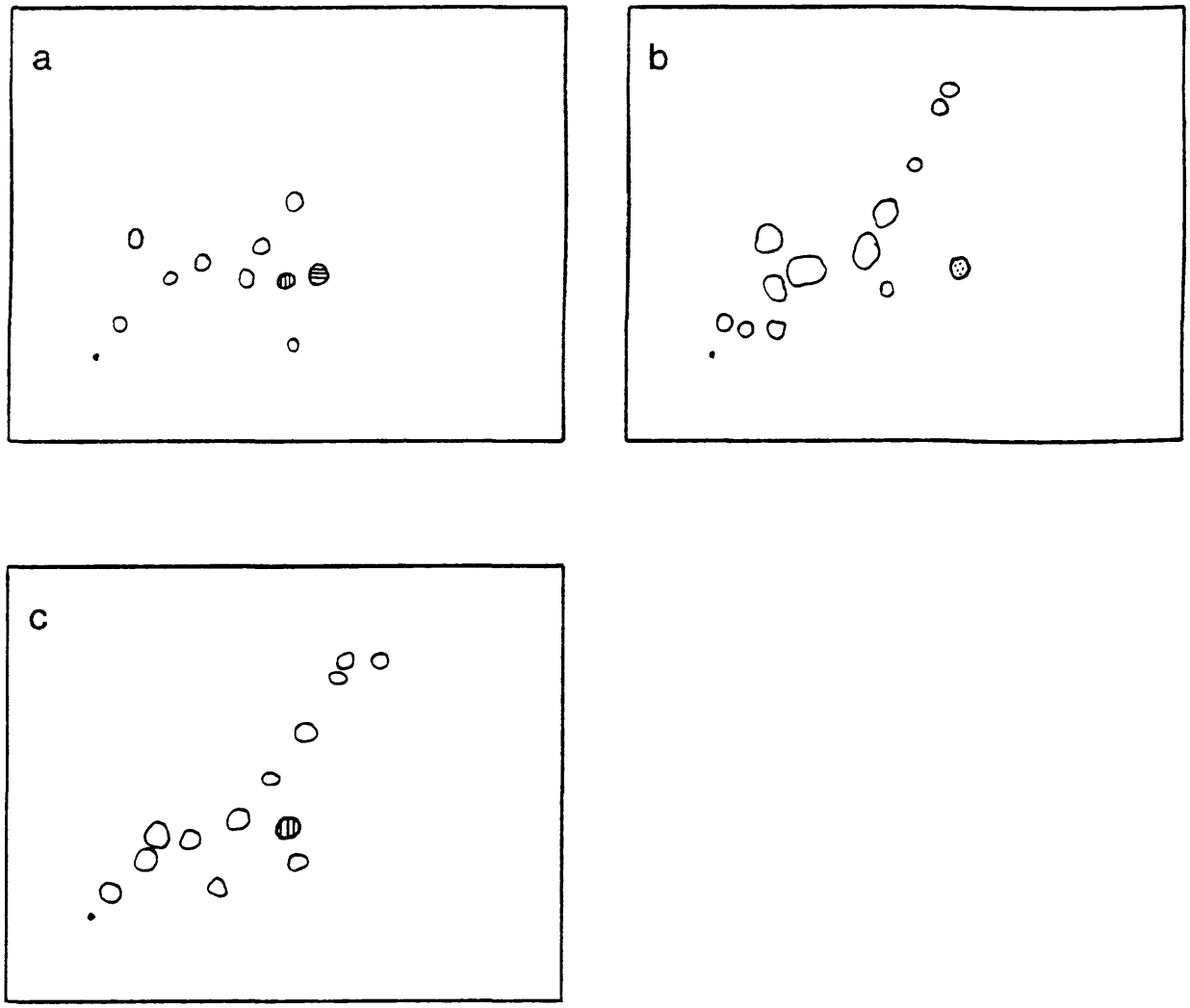

Figure 1. Representations of chromatographs of the free amino acids of D. cymosum leaves (a), D. cymosum callus culture (b) and P. cepacia (c). The amino acids were separated on silica-gel thin layer plates and the solvents used were butan-1-ol : acetic acid : water (9:1:2.9 v/v/v) vertically and phenol : water (2.6:1 m/v) horizontally. Shaded spots represent the positions of NMS (|||), NMA (≡) and proline (∴). The characteristic yellow colour of proline, when reacted with ninhydrin, distinguished it clearly from NMA which yields a red-brown colour with ninhydrin.

Due to the absence of NMS in the callus, the presence of this amino acid in the leaves appears to be due to the bacteria in the leaves which in the free-living state also contains this amino acid. To test this hypothesis D. cymosum plants would have to be grown aseptically and its leaves analyzed for NMS.

Because NMA does not occur in the callus or bacteria, its presence in the leaves suggests that its biosynthesis requires the co-operation of the plant and the bacteria living in symbiosis. On the other hand, it is possible that the synthesis of NMA and/or NMS by the plant requires a high degree of differentiation. Its biosynthesis might be limited to one or more specific organs such as the roots or leaves from whence it might be transported to all the other parts of the plant.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

Origin of fluoroacetate in *D. cymosum*

The synthesis of fluoroacetate by aseptic *D. cymosum* seedlings as well as by a callus culture of this plant, prove unambiguously that this plant can synthesize fluoroacetate in the absence of its endophyte *P. cepacia*. Unpublished results showed that an isolated culture of *P. cepacia* was not able to synthesize fluoroacetate when NaF was added to the nutrient medium. When fluoroacetate was applied to the rooting system of *D. cymosum*, this plant was capable of absorbing it. Hall & Cain's (1972) report of high fluoroacetate concentrations in the soils in which *D. cymosum* grows, thus indicates that the origin of fluoroacetate in *D. cymosum* could be from the soil as well as from the plant itself, as was shown in this study. In the absence of a more plausible explanation, it would appear highly likely that the fluoroacetate of the soil originates from decaying *D. cymosum* tissues, especially leaf litter and sloughed off periderm from both the roots and the stems. It is however still unknown where fluoroacetate is formed in the plant and by which biosynthetic route.

The occurrence of N-methyl alanine and N-methyl serine in *D. cymosum* and *P. cepacia*

The two novel amino acids, N-methyl alanine and N-methyl serine, isolated by Eloff & Grobbelaar (Eloff & Grobbelaar 1967;

1969) from *D. cymosum* leaves, could not be detected in the *D. cymosum* callus culture. Of the two amino acids only N-methyl serine could be detected in a culture of the plant's endophyte, *P. cepacia*. Because N-methyl alanine does not occur in the callus or the plant's isolated endophyte, it therefore seems as though the synthesis of it in *D. cymosum* leaves is a result of the symbiosis between *D. cymosum* and *P. cepacia*.

Because N-methyl serine was found in both the leaves of the plant and in the isolated endophyte, but not in the aseptic callus culture, it would appear that the leaves are incapable of synthesizing it and that its presence in the leaves is due to *P. cepacia* which normally inhabits the leaves.

It must however, be kept in mind that the biosynthesis of these amino acids by *D. cymosum* might require a high degree of tissue differentiation and that in the whole plant, it might be synthesized in a specific organ (organs) and subsequently be transported to the other parts of the plant.

Degradation of fluoroacetate

D. cymosum can synthesize as well as degrade fluoroacetate. The aseptic callus culture of *D. cymosum* is capable of releasing CO_2 from fluoroacetate. The mechanism by which this occurs is however still unknown. The bacterium *P. cepacia* isolated from *D. cymosum* seems to play an important role in the degradation of fluoroacetate. Contamination of the callus with *P. cepacia* increased the rate of CO_2 release from fluoroacetate about five fold. An

isolated culture of this bacterium could also release CO_2 from fluoroacetate and was also capable of defluorinating fluoroacetate. The defluorination process proceded at a rate that was about 105 times faster on a molar basis than the rate of CO_2 release. By using today's modern gene manipulation techniques this characteristic feature of *P. cepacia* might be transferred to rumen bacteria and thereby possibly enable them to breakdown fluoroacetate.

The fluoroacetate concentration in the older leaves of *D. cymosum* is less than in the young leaves, probably because the rate of fluoroacetate breakdown is higher in the older than in the young leaves of the plant. This does not seem to be the only reason for the difference in the fluoroacetate content of the young and old leaves. This study also showed that fluoroacetate is more readily transferred from older to young leaves of *D. cymosum*, than vice versa.

Tolerance of *D. cymosum* to fluoroacetate

Although the fluoroacetate concentration in the young leaves of *D. cymosum* is approximately 2300 times higher than the LD_{50} value for dogs, this remarkable plant seems to tolerate these high concentrations of fluoroacetate without any apparent ill effect. Fluoroacetate is toxic to animals because of its conversion to the aconitase inhibitor fluorocitrate, in the tricarboxylic acid cycle (Morrison & Peters 1954; Treble *et al.* 1962; Fanshier *et al.* 1964; Villafranca & Platus 1973). Balinsky & Schneiderman

(1964) and Eloff (1972) provided evidence for the operation of a tricarboxylic acid cycle in *D. cymosum* and Louw (1970) reported that this plant's aconitase is also inhibited by fluorocitrate. Fluoroacetate is metabolically active (Eloff 1968; Eloff & Grobbelaar 1972) and therefore one can assume that it is not restricted to the cell's vacuole. All these facts made the question about the plant's survival, in the presence of high concentrations of fluoroacetate, all the more intriguing. This study revealed the presence of an enzyme in the mitochondria of *D. cymosum* which catalyses the release of CoASH from fluoroacetyl-CoA and appears to be fluoroacetyl-CoA hydrolase. Through the activity of this enzyme, the chance for the formation of fluorocitrate in the mitochondria of *D. cymosum* would appear to be considerably reduced. This fluoroacetyl-CoA hydrolase-like enzyme could not use acetyl-CoA as substrate and therefore seems to be very specific for fluoroacetyl-CoA.

The degradation of fluoroacetate by *D. cymosum* and its endophyte *P. cepacia* as well as its defluorination by *P. cepacia*, shows that the fluoroacetate concentration can be regulated in the plant. Together these attributes can probably adequately explain the remarkable way in which *D. cymosum* is able to tolerate the high fluoroacetate concentration that sometimes occur within its tissues.

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CHAPTER 9

SUMMARY

FLUOROACETATE METABOLISM OF *DICHAPETALUM CYMOSUM*

by

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A fast and sensitive method was developed for the determination of fluoroacetate in *Dichapetalum cymosum* using high-performance liquid chromatography. The highest concentrations of fluoroacetate were found in the immature seeds, flowers and young leaves of the plant. The young leaves are more toxic than the older leaves, probably because the rate of fluoroacetate degradation is higher in old leaves than in younger leaves. Foliarly applied fluoroacetate is also more readily accumulated by the young than by the older leaves of *D. cymosum*.

Fluoroacetate can be taken up by the roots of *D. cymosum* and be transported to the leaves. Whether this happens to a significant extent under natural conditions is unknown. It was, however,

demonstrated in this study that aseptically grown *D. cymosum* seedlings, as well as an aseptic callus culture of the plant, is capable of producing fluoroacetate.

A pseudomonad was isolated from *D. cymosum* and identified as *Pseudomonas cepacia*. It was established that an isolate of this bacterium could grow in fluoroacetate enriched solutions without any reduction in growth rate. This bacterium is capable of defluorinating fluoroacetate and also of liberating CO_2 from fluoroacetate. It seems as though the synthesis of N-methyl alanine and the occurrence of N-methyl serine in *D. cymosum*, is a result of the symbiosis between the plant and its endophyte.

An aseptic callus culture of *D. cymosum* is capable of degrading fluoroacetate albeit at a much lower rate than the leaves of the plant. By contaminating the callus with *P. cepacia*, isolated from the plant, the rate of CO_2 release from fluoroacetate was increased about five fold.

A *D. cymosum* crude mitochondrial enzyme extract can release CoASH from fluoroacetyl-CoA at a rate of 29.5 nmoles/min/mg protein, indicating the presence in the extract of an enzyme which is probably fluoroacetyl-CoA hydrolase. This enzyme could not use acetyl-CoA as a substrate.

The presence of the fluoroacetyl-CoA hydrolase-like enzyme in *D. cymosum* together with the ability of the plant and its endophyte to degrade the fluoroacetate, helps to explain why *D. cymosum* is not poisoned by the high fluoroacetate concentrations which occur in the plant at times.

CHAPTER 10

OPSOMMING

FLUOROASETAATMETABOLISME VAN *DICHAPETALUM CYMOSUM*

deur

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'n Vinnige en sensitiewe metode vir die bepaling van fluoroasetaat in *Dichapetalum cymosum*, met behulp van hoë druk vloeistof chromatografie, is ontwikkel. Die hoogste fluoroasetaatkonsentrasies kom in die ontwikkelende sade, blomme en jong blare van die plant voor. Die jong blare is giftiger as die ouer blare, waarskynlik omdat fluoroasetaat teen 'n hoër tempo in die ou blare as in die jonger blare afgebreek word. Fluoroasetaat wat aan blare toegedien is, is ook in 'n groter mate deur die jong blare as deur die ouer blare van die plant geakkumuleer.

Fluoroasetaat kan deur *D. cymosum* se wortels geabsorbeer word en na die blare vervoer word. Tot watter mate dit in die natuur geskied is egter onbekend. Daar is in hierdie studie aangetoon dat *D. cymosum* saailinge wat asepties gekweek is, sowel as 'n

aseptiese kalluskultuur van die plant, wel in staat is om fluoroasetaat te sintetiseer.

'n Pseudomonad is uit *D. cymosum* geisoleer en dit is as *Pseudomonas cepacia* geidentifiseer. Daar is vasgestel dat 'n isolaat van hierdie bakterium in staat is om in fluoroasetaat-verrykte voedingsoplossings te groei, sonder enige afname in groeitempo. Hierdie bakterium besit die vermoë om fluoroasetaat te defluorineer en ook om dit af te breek met die vrystelling van CO_2.
Dit wil voorkom asof die sintese van N-metielalanien en die voorkoms van N-metielserien in *D. cymosum*, 'n gevolg is van die simbiotiese verwantskap tussen die plant en sy endofiet.

'n Aseptiese kalluskultuur van *D. cymosum* kan fluoroasetaat afbreek maar dit geskied teen 'n baie laer tempo as in die blare van die plant. Die vrystelling van CO_2 vanuit fluoroasetaat neem toe met 'n faktor van vyf, indien die kalluskultuur met *P. cepacia* besmet word.

'n *D. cymosum* ru-mitokondriale ensiemekstrak kan KoASH teen 'n tempo van 29.5 nmol/min/mg proteien vanuit fluoroasetaat-KoA vrystel. Dit wil dus voorkom asof daar 'n ensiem in die ekstrak voorkom wat waarskynlik fluoroasetiel-KoA hidrolase is. Hierdie ensiem is nie in staat om asetiel-KoA as substraat te benut nie.

Die teenwoordigheid van die fluoroasetiel-KoA hidrolase-tipe ensiem in die plant tesame met die vermoë van die plant en sy endofiet om fluoroasetaat af te breek, verklaar waarskynlik in 'n groot mate waarom *D. cymosum* bestand is teen die hoë fluoroasetaatkonsentrasies wat soms in die plant voorkom.

CHAPTER 11

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CHAPTER 12

CURRICULUM VITAE

Jacobus Johannes Marion Meyer was born in Pretoria on 20 March 1958. He attended the Louis Leipoldt primary school in Verwoerdburg and the Menlopark high school in Pretoria.

He entered the University of Pretoria in 1976 majoring in Botany and Chemistry and obtained the B.Sc. degree in 1979. The B.Sc. (Hons.) degree in Plant Physiology was conferred on him by the same university in 1980. He also served as temporary junior lecturer in Plant Physiology in the Department of Botany, during 1980.

During 1981 and 1982 he received military training and was commissioned to the rank of lieutenant in the Air Force.

He enrolled for the M.Sc. degree in Plant Physiology, at the University of Pretoria in 1983. His thesis dealt with the isolation, identification and biosynthesis of certain free amino acids in *Acacia mellifera* (Vahl) Benth. subsp. *detinens*. Pipecolic acid and 4-hydroxypipecolic acid were isolated from this plant. Their chemical structures were confirmed by mass spectroscopy and nuclear magnetic resonance spectroscopy. He demonstrated the biosynthesis in *A. mellifera* subsp. *detinens* of pipecolic acid from lysine and of 4-hydroxypipecolic acid from pipecolic acid.

The interaction between the herbicide bromacil and the insecticide dimethoate was studied by him at the Department of Agricul-

ture and Fisheries from 1984 to 1985.

He worked in the laboratory of Prof. W. Koch at the University of Munich (Germany) in 1986 on the influence of air pollution on the physiology of indigenous German trees.

He enrolled for the Ph.D. degree at the University of Pretoria in 1987 whilst acting as research assistant to Prof. N. Grobbelaar. In 1989 he was appointed lecturer in plant physiology in the Department of Botany at the University of Pretoria, a position he still holds.

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