A NEW METHOD FOR THE QUANTIFICATION OF 1-(MALONYLAMINO)CYCLOPROPANE-1-CARBOXYLIC ACID IN WATER-STRESSED PLANT TISSUES

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Abstract

Since the discovery of MACC as a major metabolite of both endogenous and exogenously applied ACC, it has become evident that the formation of MACC from ACC can act to regulate ethylene production in certain tissues. Hence, it was suggested that MACC could serve as an indicator of water-stress history in plant tissues. The accurate quantification of MACC in plant tissues is essential for the understanding of the role of MACC in the regulation of ethylene biosynthesis. Hoffman and co-workers described a method for the measurement of MACC in which it was hydrolysed by HCl to ACC, which was then assayed by chemical oxidation to form ethylene. To date, no other method has been developed for quantifying MACC. Attempts have been made by others to raise monoclonal antibodies to MACC so that an immunoassay could be developed in order to gain a deeper understanding of stress-induced ethylene production. However, to date, no further publications have been forthcoming. Here, quantification methods for MACC , employing GC-MS are described. This method is compared with widely-used indirect assay for MACC, which is based upon hydrolysis of MACC to ACC and conversion of ACC by hypochlorite reagent to ethylene which is subsequently quantified by gas chromatography.

Introduction

Apelbaum and Yang [3] found that the pathway for ethylene biosynthesis in wilted wheat leaves was the same

as that in ripening fruit [28]. Briefly, ethylene is synthesized from the amino acid methionine, which is first converted to SAM and later to the immediate precursor of ethylene: ACC. The conversion of SAM to ACC in the ethylene biosynthesis pathways is one of the key reactions controlling the production of stress ethylene. Stress reportedly induces

the synthesis of ACC synthase which is, in turn responsible for converting SAM to ACC and results in increased ethylene production [9, 28, 29]. Therefore, the need for accurate assay procedures for this substrate in plant extracts is of great importance. Two oxidative chemical assays have been developed for the quantification of ACC [4,16]. In both assays, ACC was determined by the chemical conversion of ACC to ethylene, which was subsequently released and quantified by GC. Lizada and Yang's method [16] for the determination of ACC is based on the liberation of ethylene from ACC by an oxidative reagent (NaOCl) in the presence of a mercuric salt catalyst with the evolved ethylene being measured on a GC. This method has been widely used [6,7,21,22,25,27]. However, a few investigators have identified potential problems. Knee [15] has found that certain natural amino acids other than ACC, for example, homoserine and homocysteine yield ethylene in this ACC assay system. It has also been observed that ammonia and aliphatic primary and secondary amines present in extracts greatly decrease the yield of ethylene from ACC under standard assay conditions [20]. The suggestion that phenolic compounds could interfere with the assay of ACC in avocado extracts either by forming a phenol-mercuric complex or by competing with ACC for the available NaOCl required for the oxidation of ACC to ethylene has been described.[24]. In addition, appreciable losses during oxidation have been reported [18]. These problems led to the development of more accurate and specific gass chromatographic-mass spectrometric techniques for determination of ACC [5,8,18]. Since the discovery of MACC as a major metabolite of both endogenous and exogenously applied ACC, [2, 10, 11] it has become evident that the formation of MACC from ACC can act to regulate ethylene production in certain tissues [14]. MACC is thought to be an inactive secondary product of ACC since it is not degradable in plant tissues, [28]. Therefore, it was suggested that MACC could serve as an indicator of water-stress history [10]. The suggestion that MACC might be used in liberating ACC in developing cocklebur seeds has been considered [23]. First it was found that applied MACC did not promote ethylene production in these seeds. Moreover, cocklebur seed produced high rates of ethlylene emanation during germination, but there was only a slight decrease in the MACC concentration during this period. Thus, it was concluded that MACC was not a source for the production of ethylene by these seeds. However, it is known that MACC can be converted to ACC for ethylene production in certain plant tissues [13]. Nevertheless, such observations provide cause for a re-examination of the rate of MACC in the control of ethylene biosynthesis. Therefore, the accurate quantification of MACC in plant extracts is essential to an understanding of any such role for MACC.

Hoffman et al. [11] described a method for the measurement of MACC in which it was hydrolysed by HCl to ACC, which could then be measured by chemical oxidation to ethylene. Such a quantification is indirect and shares the flaws of the assay for ACC [16]. Due to the lack of sensitivity of the existing assay for MACC, the method is only suitable where there are large differences in MACC content between the tissues compared. To date, no other method has been developed for MACC determination. It has been reported that the preparation of monoclonal antibodies to MACC was underway in order to develop an immunological assay for MACC [12].

In order to gain a deeper understanding of stress-induced ethylene production, methods have been developed in order to determine both ACC and MACC in tomato extracts.

Materials and Methods

Plant Material

All plant materials used in the experiments were initially grown under standard glasshouse conditions. Tomato plants (23-28 d old) were transferred to a Saxcil growth cabinet for a further 7d prior to the start of the experimental procedure. Lighting in the cabinet was supplied as a 16h photoperiod by 28 X 80W warm white fluorescent tubes and 12 X 100 W tungsten filament bulbs. The temperature was maintained and $20 \pm 1°C$ during the light period and $18 \pm 1°C$ during the 8h dark period. The light intensity at the top of the canopy was between 15000 and 20000 Lux.

Application of Water Stress

Water deficits were created by either withholding water for periods of up to 5d from intact plants or according to Wright's method [26], when using detached leaves.

Measurement of MACC

Determination of MACC by the method of Hoffman et al. (1982a)

The amount of MACC in tomato leaf extracts was estimated from the amount of ACC determined after HCl hydrolysis [11]. A fraction containing MACC eluted from a Dowex-50 column was collected and reduced to dryness in vacuo. The residue was then dissolved in 2ml of water and transferred to tubes containing 2ml of 4N HCl. The tubes were sealed with teflon-lined caps and hydrolysis was allowed to proceed for 2h at 120°C. The resulting ACC was then measured according to the method of Lizada and Yang [16].

Determination of MACC by hydrolysis to ACC and quantification by GC-MS

Crude methanol-extracts were divided into two equal aliquots and a known amount of $[2, 2, 3, 3^{-2}H_4]$ ACC was

added as an internal standard to both. One aliquot was hydrolysed with 4M HC l at 120°C for 2h and redissolved in water after the HCl had been evaporated *in vacuo*. ACC was estimated in both aliquots using GC-MS [18] and the amount of MACC was calculated as the difference between the ACC content of the two samples (i.e. with and without hydrolysis).

Determination of MACC by GC-MS
Synthesis of unlabelled and labelled [2,2,3,3²H₄]MACC

Unlabelled MACC was chemiclly synthesized from ethyl malonyl chloride (Aldrich Chemical Co.) and AC C (Sigma Chemical Co.) according to the Satoh and Esashi method [23]. 1.67g of ethyl malonyl chloride (kindly purified by Dr. Roger Horgan) and 5ml of 4M NaOH were gradually added over 1.5 h being constantly stirred to an ice-cold solution of 1.01g ACC in 6ml of 2M NaOH. After the addition of 1ml of 4M NaOH, the solution was left at room temperature for 4h. The mixture was then partitioned against 10ml of diethyl ether. The aqueous phase was passed through a Dowex-50 (H⁺) column (bed volume 50ml) which was then eluted with water. The eluant was evaporated to an oily residue *in vacuo* at 36°C. The residue was kept at 4°C until crystallisation occurred. The residue was then dissolved in acetonitrile, which was then filtered and evaporated. The residue was then recrystallised from the acetonitrile with a yield of about 1g.

[2, 2, 3, 3-²H₄] MACC was also synthesized from [2,2,3,3-²H₄] ACC which had previously been synthesized by Dr. Roger Horgan using the method described above.

MACC Extraction

Tomato leaf tissues were weighed, frozen in liquid nitrogen, and ground with a little sand in 100% methanol (MeOH) using a mortar and pestle. The tissue was extracted three times. A known quantity of [2,2,3,3-²H₄] MACC was added to the extract prior to filtration through Whatman number 1 filter paper. The clarified extract was reduced to dryness *in vacuo* at 40°C and the residue dissolved in a small quantity of water prior to loading on to a Dowex-50X8-100 column as before. The column was eluted with a total of 100 ml water, pH adjusted to 3.0 with acetic acid. The eluate was evaporated to dryness and the residue dissolved in a small quantity of water and added to a column of Dowex 1-X8-100 (60 X 15 mm i.d., formate form, adjusted to pH 8.6 with NH₄OH). The column was washed with 100 ml water (pH adjusted to pH 8.6 with NH₄OH) and diluted with 100ml 6M formic acid. The eluate was dried and the residue redissolved in water and applied to a Seppak C18 cartridge column (Waters Associates, Massachussets, USA), which was washed with 10ml of 15% (V/V) acetonitrile in water. The latter was evaporated and methyalted twice with a concentrated

solution of Diazomethane in ether. The methylated sample was dried under a stream of nitrogen, redissolved in 300ml of water and injected on to the ODS2 Spherisorb HPLC column (150 X 4mm i.d.) (Phase Separations, Queensferry, Clwyd, UK). The column was eluted at 2ml min⁻¹, with a linear gradient from 0-15% (V/V) acetonitrile in water over a 15 min period. The fraction known to contain N-malonyl-ACC dimethyl ester was collected and taken for GC-MS analysis.

A Kratos MS-25 mass spectrometer linked to a Carlo-Erba 4200 gas chromatograph was used in all MACC determinations. The GC employed a SGE BPL 25m x 0.22 mm (i.d) column operating with a helium pressure of 0.8kg cm⁻² and a temperature program from 35-80°C (ballistic) and then to 250°C. (8°C min⁻¹). The temperature of the source, separator and inlet of the MS were 190, 230 and 240°C respectively. An ionising voltage of 70ev and scan speed of 1s decade⁻¹ was employed. For selected ion monitoring (SIM), the M⁺ at m/z 215 and m/z 219 were monitored. As for ACC determination, data were acquired using a DS55 data system, operating under the following conditions: dwell time 140ms, setting time 100ms, at a sampling rate of 20khz. Mass spectrum of N-malonyl ACC dimethyl ester, [2,23,3-²H₄]MACC dimethyl ester and of N-malonyl ACC dimethyl ester extracted and derivatised from plant tissue are shown in Figure 1 (a)-(c).

Results and Discussion
Determination of MACC

Using Hoffman et al. method [11], the efficiency of hydrolysis of 5.35 nmol.g⁻¹ fw of authentic MACC added to an extract from the third leaf of unstressed plants was determined. Conversion of the resulting ACC to ethylene ranged from 16.7% to 39.7% (Table 1.1). The variable efficiency in the three replicates was reflected by the concentrations calculated, which varied from 0.29 to 1.15 nmol.g⁻¹ fw. The results of MACC determinations from
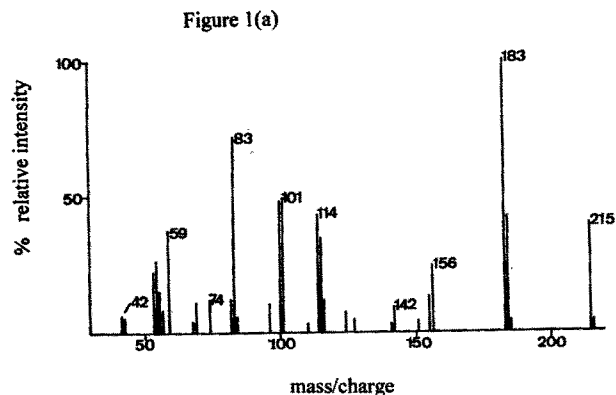

Figure 1(a)

Figure 1(a). Mass spectrum of authentic N-malonyl ACC dimethyl ester

Figure 1(b)
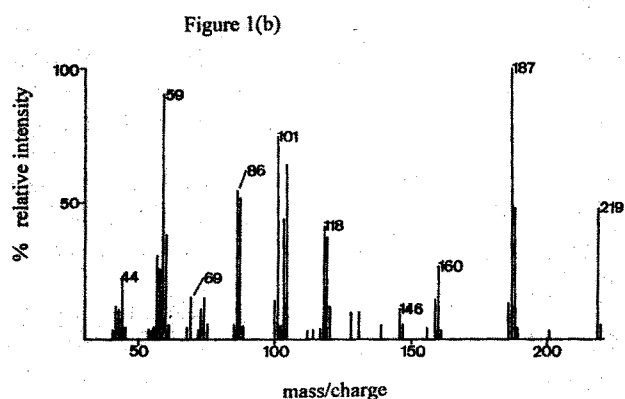

Figure 1(c)

mass/charge

mass/charge

Figure 1(b). Mass spectrum of [2,2,3,3-^{2}H$_4$) N-malonyl ACC dimethyl ester
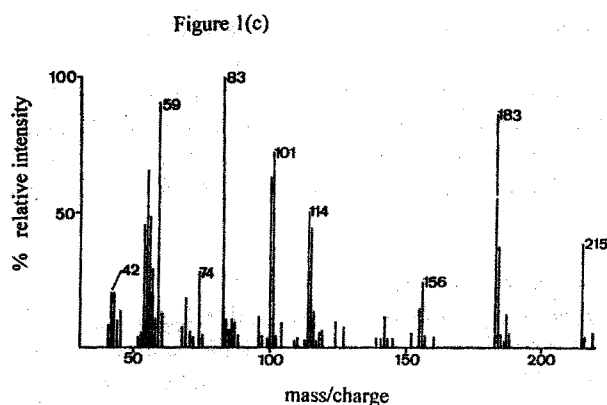
Figure 1(c). Mass spectum of N-malonyl ACC dimethyl ester derivatized and extracted from wheat leaf tissue. An internal standard of [2, 2, 3-2H4] MACC was added to the extract and therefore, ions from the deuterated compound are also present

leaf extracts of 3d-stressed plants (Table 1.2) show that the conversion efficiency was again variable but perhaps even lower than in the control tissues. Using [2,2,3,3^{2}H4]MACC, it was shown that the MACC was totally hydrolysed to ACC. Therefore, the low and variable efficiency recorder must be due to the conversion of ACC to ethylene. Table 1.3 shows the concentration of MACC in leaf extracts of unstressed and 2d-stressed plants. The concentrations of MACC from control tissues assayed by Hoffman et al. method [11] are approximately half those measured by either GC-MS directly or by GC-MS analysis of ACC after

hydrolysis of the MACC. A similar result was recorded from MACC measurements in stressed leaf extracts. There was little difference between the concentration of MACC detected in control and 2d-stressed leaf tissue.

Lizada and Yang [16] reported that the efficiency of conversion of authentic ACC to ethylene in their assay was 80%, although they reported that the efficiency was reduced to 60% when the amount of standard ACC was low (<5 pmol). In this study, the efficiency of the conversion of authentic ACC to ethylene was similarly tested and the values obtained were in the range of 59 to 68%. Other

Table 1.1. Determination of MACC extracted from the 3rd leaf of 3d-stressed and control plants by the Hoffman et al. method, [11]. After excision leaves were frozen in liquid nitrogen and extracted in cold methanol, the extract was divided into two aliquots and applied to Dowex-50 ion exchange columns. The columns were eluted with water and the eluate containing MACC reduced to dryness *in vacuo*. The residue was dissolved in water and hydrolysed in 4M HCl at 110°C for 3h. On cooling, the hydrolysed mixture was diluted with water and reduced to dryness *in vacuo*. The residue was applied to Dowex-50 and the NH$_4$OH eluate collected and reduced to dryness *in vacuo*. The residue was dissolved in water and the ACC estimated using Lizada and Yang's method [16].

Replicate Number	MACC added to extract nmol g^{-1}fw	Ethylene evolved nmolg^{-1}fw	% Conversion of Acc to ethylene	MACC in extract nmol g^{-1}fw
1	0	0.845	-	-
	5.35	1.737	16.7	0.29
2	0	0.763	-	-
	5.35	2.89	39.7	1.15
3	0	0.496	-	-
	5.0	2.176	31.4	0.68

Table 1.2. Determination of MACC from 3d-stressed plants

Replicate Number	MACC added to extract nmol g⁻¹ fw	Ethylene evolved nmolg⁻¹fw	% Conversion of Acc to ethylene	MACC in extract nmol g⁻¹ fw
1	0	0.685	-	-
	5.35	1.23	10.2	0.126
2	0	0.495	-	-
	5.35	1.46	18.1	0.26
3	0	0.427	-	-
	5.0	1.83	26.2	0.48

researchers have reported similar efficiency values conversion ranging from 60-65% [19] and 60 and 80% [24]. All these efficiency values were determined in assay mixtures in the absence of plant extracts. Therefore, it was concluded that the assay itself was sufficiently efficient to estimate ACC from tomato plant tissue. The efficiency of ACC oxidation was monitored for each leaf extract by the addition of 5 nmol g⁻¹ fw of authentic ACC to replicate extracts which were then subjected to identical analytical procedures as far as was possible.

The yield of ethylene from ACC in extracts of both stressed and unstressed tomato leaves proved to be both low and variable. Such variability has also been reported in carnation petal extracts where the yield of ethylene from

Table 1.3. Amount of MACC in extracts of unstressed and 2d-stressed leaves as determined by the Hoffman method and indirect/direct GC-MS quantification. 10g of leaf tissue from both sets of plants were extracted in cold methanol. Extracts were divided into 5 equal aliquots. Two aliquots were used to estimate MACC by the Hoffman method et al.,[11]. To one portion, a known amount of authentic MACC was added prior to application on to a Dowex-50 ion exchange column. Aliquots 3 and 4 were used for indirect measurements of MACC. A known amount of [2,2,3,3²H₄] ACC was added to both extracts and one was subjected to acid hydrolysis in 4M HCl for 2h at 110°C. ACC was measured as described previously. ACC measured from the hydrolysed portion was subtracted from the ACC measured in the other portion to calculate the amount of MACC in the sample. The final aliquot was then subjected to direct MACC measurement by GC-MS.

Treatments	MACC added to extract g⁻¹ fw	Ethylene evolved in asay nmol g⁻¹ fw	% Conversion of applied MACC to ethylene	MACC in extract extract in nmol g⁻¹ fw	MACC in extract (indirect GC-MS nmol g⁻¹ fw)	MACC in extract (direct GC-MS nmol g⁻¹ fw)
Control 1	0	0.935	-	-	1.05	1.17
	5.35	1.946	19	0.37		
Control 2	0	0.865	-	-	1.86	1.98
	5.35	2.690	34.1	0.92		
Stressed 1	0	1.243	-	-	1.97	2.33
	5.35	2.496	23.4	0.58		
Stressed 2	0	1.090	-	-	1.73	1.65
	5.0	1.982	16.7	0.33		

ACC varied between different experiments from 11 to 77% [24]. Lizada and Yang [16] tested the efficiency of the assay for conversion of authentic ACC to ethylene in the presence of plant extracts by adding 10-20nmol ACC per g of tissue to the extract prior to the assay. They reported that the efficiency of conversion of ACC to ethylene was 73-75%. However, they took no account of the losses of ACC occurring during the extraction and purification processes. In this study, the recovery of authentic ACC from methanol extracts which were subsequently assayed was between 58.8 and 70.9%. This percentage was reduced to only about 20% after the ACC was eluted from a Dowex-50 column with 2N NH₄OH. Clark (personal communication), working in the same laboratory, found that residual ammonia left over from the concentration of extracted ACC after ion exchange could interfere with the assay. It has also been reported that 100mM of ammonia in assay mixtures containing 2.5nM, 1mM HgCl, and 47mM NaOCl reduced recovery from 60-70% to 8% [29].

The relatively low ethylene yields obtained in this investigation were apparently not due to destruction of ethylene produced in the assay, as the incubation of a known amount of ethylene with the reaction mixture containing no ACC did not affect the ethylene concentration measured. If ethylene degradation during the assay does not occur, then ACC may have remained unreacted in the assay solution. When an aliquot of the readdition mixture was assayed for ACC content by the method of McGaw et al. [18], about 17 to 24% of the ACC applied remained in the reaction mixture, indicating incomplete oxidation of ACC. The lack of full recovery of ACC either in the form of ethylene or as remaining ACC could be due to the formation of other compounds not detectable by this GC-MS method used.

It has been reported that the poor conversion of ACC to ethylene in avocado plant extracts can be improved by increasing the concentration of NaOCl or HgCl2 in the assay mixture [21,24]. However, in the experiments reported here, doubling of the concentration of NaOCl had little effects on increasing the conversion to ethylene in extracts of apical tissues. Although many manipulations can be tested to optimize the assay, it appears that, at least with regard to tomato tissue, the assay is so variable that the value of such an approach is questionable.

The efficiency of the conversion of MACC to ACC and subsequent conversion of ACC to ethylene by Hoffman et al. method [11] in tomato extracts was estimated. Although variable, the results indicated that the average efficiency of the conversion of MACC to ACC and subsequent conversion to ethylene was slightly lower in the extracts of 3d-stressed plants compared to that of controls. Nevertheless, the concentration of MACC has been

calculated to be lower in stressed tissue than in controlled tissue using Hoffman et al. method [11]. As compounds present in plant extracts can interfere with the assay for ACC, it may be that stressed plants accumulate substances which are themselves responsible for this interference, thus accounting for this reduction. MACC extraction and quantification were assessed using three different techniques. Using aliquots of the same extract, it was obvserved that the amount of MACC estimated using the Hoffman et al. method [11] was much lower than that measured directly using GC-MS or by using GC-MS to measure ACC after hydrolysis of MACC. Analyses of MACC by GC-MS showed that, in general, the MACC content was higher in stressed tissue than in controls. This appears to be in contrast to the results obtained when MACC was measured by the Hoffman method [11], as compounds present in plant extracts appear to interfere with the assay of ACC. The major problem with the Hoffman method is that it ulimately uses the method of Lizada and Yang [16] for estimating ACC, the limitations of which have already been described.

The efficiency of hydrolysis of MACC to ACC was checked by adding known amounts of [2,2,3,3²H₄] MACC with the observation that no detectable MACC remained after hydrolysis. Thus, low recovery using the Hoffman method is likely to be the result of poor conversion efficiency during the conversion of ACC to ethylene. The results obtained here would indicate that the Hoffman method is unsuitable for quantitative purposes and consequently, the indirect assay was not used for the determination of MACC content in tomato plants. In light of the high efficiency of hydrolysis, either direct or indirect GC-MS are suitable methods for quantifying MACC.

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