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Short Communication

Identification and Metabolism of 1-(Malonylamino)cyclopropane-1-carboxylic Acid in Germinating Peanut Seeds[1]

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ABSTRACT

Peanut seeds (*Arachis hypogea* L. Yue-you 551) contain 50 to 100 nanomoles per gram conjugated 1-aminocyclopropanecarboxylic acid (ACC). Based on paper chromatography, paper electrophoresis, and gas chromatography-mass spectrometry, it was verified that the major ACC conjugate was *N*-malonyl-ACC (MACC). Germinating peanut seeds converted [2-^{14}C]ACC to ethylene 70 times more efficiently than *N*-malonyl-[2-^{14}C]ACC; when ACC was administered, most of it was metabolized to MACC. Germinating peanut seeds produced ethylene and converted L-[3,4-^{14}C]methionine to ethylene; this ethylene biosynthesis was inhibited by aminoethoxyvinylglycine. These data indicate that MACC occurs in peanut seeds but does not serve as the source of ethylene during germination; ethylene is, however, synthesized from methionine via ACC.

Adams and Yang (1) have established that ethylene is produced by the following pathway: methionine → SAM[2] → ACC → C_2H_4. Recently, MACC has been identified as a major conjugate of ACC, the immediate precursor of ethylene, in both wheat leaves (5) and buckwheat epicotyls (2). Inasmuch as many bound forms of plant hormones were present in seeds (3) and seeds also produce ethylene during their germination (6), we have examined the level of conjugated ACC in seeds of *Arachis hypogea* L., *Lactuca sativa* L., *Pisum sativum* L., *Vigna radiata* L., and *Phaseolus vulgaris* L. and found that peanut seeds contain a significant amount of conjugated ACC. In the present study, we investigated the level of MACC and whether or not MACC is the source of ethylene during peanut seed germination.

MATERIALS AND METHODS

Materials. Spanish-type peanut seeds, *Arachis hypogea* L. (Yue-you 551) were obtained from Economic Crops Institute, Guangdong Province, China. Seeds were used intact or dissected by hand into the embryonic axis and cotyledons after soaking for 1 h. L-[3,4-^{14}C]Methionine and *S*-adenosyl[3,4-^{14}C]methionine were purchased from Research Products International and ACC from Calbiochem. [2-^{14}C]ACC was prepared enzymically from *S*-adenosyl[3,4-^{14}C]methionine (35 μCi/μmol) with ACC synthase prepared from red tomato fruits which had been sliced (1–2 cm in thickness) and incubated for 12 h according to the procedures described by Boller *et al.* (4) and Yu *et al.* (8). *N*-Malonyl-[2-^{14}C] ACC was isolated by paper chromatography and paper electrophoresis from wheat leaves which had been administered [2-^{14}C] ACC as described by Hoffman *et al.* (5). MACC was synthesized as described previously (5). AVG was a gift from J. P. Scannell (Hoffmann-LaRoche).

Feeding Experiments. Each peanut seed, with seedcoat intact, was placed in a 16- × 100-mm test tube containing 50 μl of radioactive solutions (see Table II for specific radioactivity) and oriented with the radicle end facing downward by loosely placing the wide end of a 1-ml disposable pipet tip around the peanut top. The tubes were capped with rubber serum stoppers. After the radioactive solution was absorbed, an additional 200 μl of water was added by syringe. Seeds germinated and radicles elongated to approximately 5 mm within 30 h after imbibition began. The ethylene accumulated during a 30-h imbibition period was transferred to an evacuated 25-ml scintillation vial and measured as described by Yu and Yang (9). The seeds were then removed, rinsed twice with 0.5-ml aliquots of water, and ground with a mortar and pestle with 5 ml 80% ethanol. The homogenate was centrifuged and the extract concentrated, chromatographed, and electrophoresed as described below. Radioactivity in the paper chromatogram and electrophoretogram was detected with a radioscanner. The relative radioactivity for metabolites in each scan was estimated by cutting out and weighing the paper comprising the area under each peak. Radioactivity taken up by the seeds was calculated from the difference between the radioactivity initially supplied and that remaining in the tube plus in the water rinse.

Determination of ACC Conjugate. Quantification of the ACC conjugate was carried out by hydrolyzing it to ACC in 2 N HCl at 100°C for 3 h; after neutralization with KOH, the resulting solution was assayed for ACC according to the method of Lizada and Yang (7). Ethylene was determined by gas chromatography.

Isolation of MACC. Peanut seeds (30 g fresh weight) were ground in an Omni mixer with 70 ml of 80% ethanol. After extraction at 70°C for 3 h, the homogenate was centrifuged and the supernatant was concentrated *in vacuo* at 35°C. The residual aqueous solution made up to 6 ml was partitioned against 1 ml of chloroform. Two ml of aqueous extract were passed, in series, through a column (1 × 20 cm) of ion exchange resin Dowex 50 (H^+ form) and a column (1 × 20 cm) of Dowex 1 (OH^- form). Anions were eluted from the Dowex-1 column with 5 bed-volumes (50 ml) of 6 N formic acid. The formic acid was evaporated and the residue was chromatographed on Whatman 3 MM paper using 1-butanol:acetic acid:H_2O (4:1:1.5, v/v) as developing solvent. The zone corresponding to authentic *N*-malonyl[2-^{14}C]ACC, run on a parallel strip and detected by a radioscanner, was eluted with 50% ethanol. The eluate was concentrated *in vacuo* at 45°C and elec-

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[2] Abbreviations: SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine.

trophoresed on Whatman 3 MM paper using 20 mM phosphate buffer (pH 7.0). The zone corresponding to authentic MACC was eluted and concentrated as above. The aqueous eluate was passed through a small column of Dowex 1 (formate form) and eluted with 5 bed-volumes (2.5 ml) of 6 N formic acid. The eluate was evaporated, methylated with diazomethane, and subjected to GC-MS analysis as described (5) using a temperature program of 4°C/min.

RESULTS AND DISCUSSION

Levels of Free ACC and Conjugated ACC in Peanut Seed. Peanut seeds were soaked in water for 1 h and hand dissected into embryonic axis and cotyledon which represented 3% and 97% of the total weight, respectively. Table I indicates that 1 h after imbibition the embryonic axis contained 0.04 nmol ACC/g whole seed or 1.3 nmol ACC/g axis, while the cotyledons contained 0.7 nmol/g whole seed or /g cotyledons. Thus, although cotyledons contained the majority of ACC, the concentration of ACC in both seed parts was similar. The distribution of conjugated ACC in both embryonic axis (1.2 nmol/g whole seed or 40 nmol/g embryonic axis) and cotyledon (54 nmol/g whole seed or cotyledon) was also similar; the axis and cotyledon contained 30 and 77 times, respectively, more ACC conjugate than ACC. More than 90% of the total conjugated materials was in the cotyledon.

After 48 h imbibition, we observed that the level of ACC in the embryonic axes increased over 25-fold to 1.1 nmol/g whole seed, whereas the level in the cotyledon increased less than 30%. Thus ACC accumulates in the embryonic axes during germination. This result is consistent with the observation of Ketring and Morgan (6) that ethylene is produced primarily by the embryonic axes. During this period, the level of conjugated ACC increased in both axis and cotyledon parts (Table I). These results are inconsistent with the view that conjugated ACC present in the unimbibed seed liberates free ACC during the process of germination.

Identification of the Conjugate as MACC. To characterize the ACC conjugate, a portion of the ethanol extract was concentrated and partitioned with chloroform as described under "Materials and Methods." The aqueous extract was subjected to paper chromatography as described previously (7). The chromatogram was cut into 5-cm strips which were individually eluted with water and assayed for conjugated ACC. The majority of activity (93%) was found in the region corresponding to [^{14}C]MACC (run on a parallel strip) as shown in the upper panel of Figure 1. The eluate from the zone containing conjugate was electrophoresed on paper and the electrophoretogram was cut into 4-cm sections which were similarly eluted with 2 N HCl and assayed for conjugate. Again, nearly all conjugate (87%) was found in the zone corresponding to MACC. For further characterization of the ACC conjugate as MACC, the ethanol extract was partially purified by ion exchange resins, paper chromatography, and paper electrophoresis and the zone corresponding to MACC was eluted and derivatized for GC-MS analysis as described in "Materials and Methods." The existence of MACC dimethyl ester was established by its characteristic mass spectrum (5). Based on these data, we conclude that MACC is the major conjugate of ACC.

Table I. *Levels of Free and Conjugated ACC in Peanut Seed*

Six dry seeds, weighing 3 g, were imbibed in water for 1 or 48 h at 25°C prior to hand dissection. Radicle protrusion occurred between 18 and 22 h. The amount of ACC or conjugated ACC present in different seed parts was expressed per gram of whole seed prior to imbibition. Data presented are typical results from a single experiment repeated three times.

Seed Part	ACC	Conjugated ACC
	nmol/g whole seed	
Imbibed for 1 h		
Embryonic axes	0.04	1.2
Cotyledon	0.7	54
Imbibed for 48 h		
Embryonic axes	1.1	6.0
Cotyledon	0.9	181

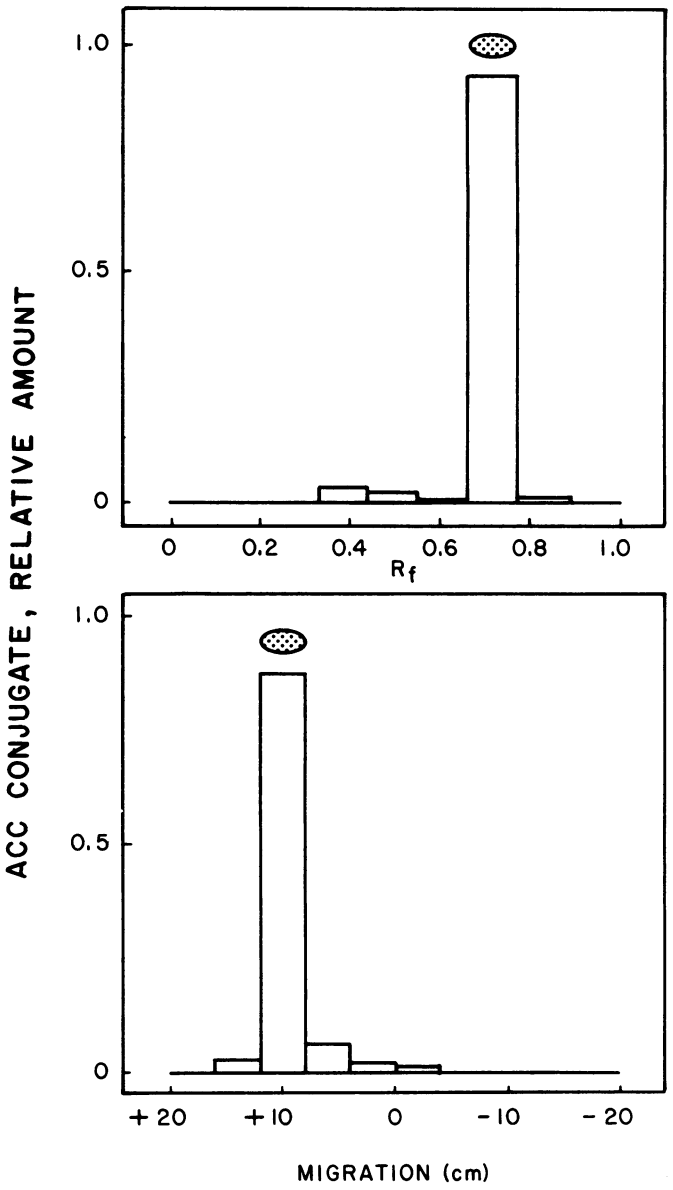

FIG. 1. Histograms of the mobility of ACC conjugates in paper chromatography (upper panel) and paper electrophoresis (lower panel). The aqueous extract of peanut seeds after chloroform extraction was subjected to paper chromatography. An aliquot of each eluate from different zones was hydrolyzed in 2 N HCl for 3 h, and the resulting ACC was assayed by converting it to ethylene as described by Lizada and Yang (6). The eluate from the paper chromatogram containing ACC conjugate (R_F of 0.66–0.77) was subjected to paper electrophoresis, and the content of conjugated ACC was similarly assayed. The spots indicate the mobility of *N*-malonyl-[2-^{14}C]ACC which was run on separate but parallel papers.

Metabolism of MACC. Inasmuch as peanut seeds contain a large amount of conjugated ACC, which is mostly MACC, and since they produce ethylene during germination, we investigated whether this ethylene was derived from MACC. Radiolabeled ACC and MACC of equal activity and specific radioactivity were fed to peanut seeds, and the ethylene produced over the next 30-

Table II. *Conversion of ACC, MACC, and Met to C_2H_4 by Peanut Seeds*

Each peanut seed (0.5 g) was administered radioactive substrate and enclosed in tubes for 30 h at 20°C. One seed additionally received 15 nmol AVG. Data presented are typical results from a single experiment repeated three times.

Substrate	Radioactivity Uptake	C_2H_4	
	%	*nCi*	*nl*
[2-^{14}C]ACC (56 nCi, 28 nmol)	97	2.7	170
Malonyl-[2-^{14}C]ACC (56 nCi, 28 nmol)	94	0.04	94
L-[3,4-^{14}C]Met (221 nCi, 4 nmol)	73	1.8	75
L-[3,4-^{14}C]Met + AVG (221 nCi, 4 nmol)		0.15	1.3

h period was collected in $Hg(OCl_4)_2$ and subsequently counted. Approximately 70 times more [^{14}C]ethylene was produced from [^{14}C]ACC than from [^{14}C]MACC (Table II). This difference could not be due to a different uptake rate, because a similar uptake rate was observed in both treatments. The lower conversion rate of [^{14}C]MACC to [^{14}C]ethylene could have resulted in part from the higher endogenous MACC level present in the germinated peanuts. However, this effect could not be very great, because germinated peanut contained, according to the data of Table I, about 100 nmol MACC in 0.5 g of seeds, whereas the amount of exogenous MACC supplied was 28 nmol in Table II. Thus, the specific radioactivity of MACC would be reduced to about one-fourth due to the endogenous MACC. This reduction in specific radioactivity was too small to account for its ineffectiveness as an ethylene precursor. We have therefore concluded that MACC was not an effective precursor of ethylene in this tissue. When the seeds fed with [^{14}C]ACC were analyzed after imbibition for 30 h under the conditions specified in Table II, more than 90% of the applied [^{14}C]ACC was converted to [^{14}C]MACC as revealed by relative peak area of the radiochromatograms from three separate experiments. In contrast, the seeds fed with [^{14}C]MACC showed almost no free [^{14}C]ACC in the tissue. It appears that during germination ACC is rapidly conjugated, whereas hydrolysis of the conjugate to ACC is insignificant. During germination, it has been observed that a rapid increase in ethylene production occurred at the time of radical protrusion which is followed by a rapid decline in ethylene production (6). The conjugation of ACC into MACC in germinated seeds may be partly responsible for the decline in ethylene production following radicle protrusion.

If the precursor of ethylene, ACC, is not formed via hydrolysis of ACC conjugates, it could be produced from methionine via SAM. To test this, we supplied peanut seeds with methionine of high specific radioactivity, since much of the applied methionine would be utilized in protein synthesis. Table II indicates that, indeed, germinating peanut seeds have the ability to convert L-[3,4-^{14}C]Met to [^{14}C]C_2H_4. We therefore examined the effect of AVG, a pyridoxal phosphate inhibitor which blocks the conversion of methionine to ethylene, but does not block the conversion of ACC to ethylene, on conversion of L-[3,4-^{14}C]Met to ethylene. AVG was extremely effective in inhibiting both radioactive and total ethylene production, consistent with the notion that ethylene is derived from methionine (Table II). Although AVG drastically reduced ethylene production, germination was not delayed and radicle length was not inhibited in the AVG-treated seeds compared to seeds receiving only radioactive methionine. Based on the observations that (a) MACC was not metabolized in germinating peanut while ACC was metabolized to ethylene and MACC, (b) germinating seeds converted methionine to ethylene, and (c) ethylene production was inhibited by AVG, we conclude that the ethylene produced during germination is derived from methionine via ACC. Thus, MACC does not appear to be an active storage form of ACC in this tissue. This view is consistent with our observations that, when compared to ACC, MACC is only poorly converted into ethylene in apple fruit, wheat leaf, and mung bean hypocotyl tissue (NE Hoffman, unpublished results). Thus, MACC is the major ACC conjugate which releases only very slowly free ACC for the synthesis of ethylene.

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