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Inhibitory Effects of Methyl Jasmonate on the Germination and Ethylene Production in Cocklebur Seeds

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Abstract. Methyl jasmonate (JA-Me) inhibited the germination of cocklebur (*Xanthium pennsylvanicum* Wallr.) seeds. The inhibition of the germination of cocklebur seeds treated with JA-Me at concentrations less than 300 μM was nullified by ethylene applied exogenously, although the inhibitory effect of 1,000 μM JA-Me was not recovered completely even by high concentrations of ethylene (10,000 μL/liter). JA-Me inhibited ethylene production before seed germination. The level of 1-aminocyclopropane-1-carboxylic acid (ACC) in the cotyledonary tissues treated with JA-Me decreased but not the level of 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC). JA-Me inhibited the conversion of ACC to ethylene in the tissues. These results suggested that JA-Me inhibits ethylene production by prevention of ACC oxidation in addition to ACC synthesis. We believe that the inhibition of ethylene production by JA-Me results in the retardation of the germination of cocklebur seeds.

Key Words. Methyl jasmonate—Seed germination—Ethylene—*Xanthium pennsylvanicum*—ACC—MACC

Jasmonic acid (JA) and its derivatives including methyl jasmonate (JA-Me) have been regarded as endogenous plant growth regulators because of their ubiquitous occurrence in the plant kingdom and their pleiotropic effects on plant growth and development (Sembdner and Parthier 1993). Jasmonates applied exogenously to plants, for example, inhibit stem and root growth (Staswick et al. 1992), induce pericarp or leaf senescence (Yeh et al. 1995), prevent chlorophyll (Abeles et al. 1989) and carotenoid formation (Saniewski and Czapski 1983), and reduce photosynthetic (Maslenkova et al. 1990, Popova et al. 1988) and respiratory activity (Popova et al. 1988). Recently, a correlation of endogenous jasmonates with stress responses and involvement in the defense systems has been highlighted because of the finding of novel jasmonate-induced proteins (O'Donnell et al. 1996, Sembdner and Parthier 1993).

Exogenous jasmonates seem to have inhibitory effects on germination in many cases of nondormant seeds, for example, lettuce (Yamane et al. 1981), sunflower (Corbineau et al. 1988, 1989), amaranth (Kepczynski and Bialecka 1994), and rapeseed and flax (Wilen et al. 1991) seeds. But jasmonates break the seed dormancy of apple (Ranjan and Lewak 1992, 1995) and promote germination of the turions of *Spirodela* (Appenroth et al. 1991). Jasmonates have been identified in immature and mature seeds of various kinds of plants (Meyer et al. 1984, Sembdner and Parthier 1993). However, the physiological roles of jasmonates in seed germination remain unclear.

It is known, on the other hand, that jasmonates interact with other phytohormones in a variety of the biological activities, supporting an idea that jasmonates have been considered putative plant growth regulators (Parthier 1990). Because jasmonates are thought to play an important role in senescence and the stress response (Parthier 1990), special interest has been aroused in the effects of jasmonates on ethylene biosynthesis. Jasmonates stimulate ethylene production in ripening tomatoes (Saniewski et al. 1987, Sembdner and Parthier 1993), in tomato culture cells (Greulich et al. 1995), in olive leaf discs (Sanz et al. 1993), and in preclimacteric

Abbreviations: JA, jasmonic acid; JA-Me, methyl jasmonate; ACC, 1-aminocyclopropane-1-carboxylic acid; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid.

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fruits (Fan et al. 1997), whereas they inhibit ethylene production in rice seedlings (Yeh et al. 1995) and in detached rice leaves (Tsai et al. 1996).

In this study we examined the effects of exogenous jasmonates on germination and ethylene production in cocklebur seeds. We found that JA-Me inhibited not only the seed germination but also the production of ethylene from the seeds. We will discuss the interaction between JA-Me and ethylene in the germination of cocklebur seeds.

Materials and Methods

Plants

Nondormant lower cocklebur (*Xanthium pennsylvanicum* Wallr.) seeds, which were harvested in the fall of 1994, after-ripened in dry storage for 5 months at room temperature, and had been stored at 8°C (Ishizawa et al. 1988), were used throughout all experiments in this study. Cocklebur seeds were washed several times with tap water, rinsed with deionized water, and then blotted with tissue paper before used. Cotyledonary segments were prepared according to the methods of Esashi and Katoh (1975). Briefly, cotyledonary segments that were 5 mm long were initially imbibed in water at 25°C for 3 h, during which time the seed coat fragments were removed easily and discarded. The segments were incubated on two layers of wet filterpaper in a 15-cm Petri dish for another 21 h at 25°C in the dark. After rinsing with distilled water and blotting with tissue paper, the segments were used to measure ethylene production.

Germination

Ten seeds were put on two layers of filterpaper wetted with 8 mL of a test solution in a 9-cm Petri dish and incubated in the dark at 25°C. For treatment with ethylene, 10 seeds were put on two layers of filterpaper wetted with 3.5 mL of a test solution in a 125-mL flask. The flasks were sealed with skirted rubber stoppers. The necessary amount of ethylene was injected with a syringe into the flask through the rubber stopper, a small hole of which was sealed with a piece of vinyl tape. For control of ethylene treatment, the flasks contained a small glass tube with 0.2 mL of 2.5 M $Hg(ClO_4)_2$, an ethylene absorbent. The criterion for judging the germination of cocklebur seeds was the protrusion of the radicle from the seed coats. Each experiment was repeated at least twice with incubation of triplicate Petri dishes or flasks.

Measurement of Ethylene Production

To measure the ethylene production from cocklebur seeds or the cotyledonary segments, 30 seeds or 20 segments were arranged on a sheet of filterpaper wetted with 1.5 mL of a test solution in a 30-mL vial. The vial was sealed with a rubber stopper. Incubations were carried out with triplicate vials. At given times after the start of incubation at 25°C in the dark, a 1-mL gas sample was withdrawn from the head space of the vial with a hypodermal syringe, and ethylene was assayed on a gas chromatograph (Shimadzu GC-8A) equipped with an active alumina column and a flame ionization detector.

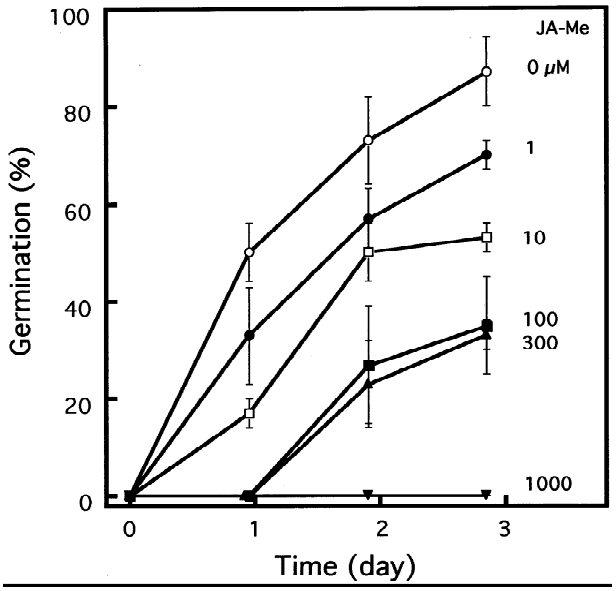

Fig. 1. Time courses of the germination of cocklebur seeds treated with different concentrations of JA-Me. Ten seeds were incubated on two layers of filterpaper wetted with 8 mL of JA-Me solution at different concentrations (0, 1, 10, 100, 300, and 1,000 μM) at 25°C in the dark. Data show the means ± S.E. of three replicates.

Extraction and Determination of ACC and MACC

Immediately after ethylene measurement, the segments from the three replicate vials were plunged separately into liquid nitrogen. The frozen tissues (about 1 g fresh weight) was extracted three times with 5 mL of 80% ethanol at 70°C for 1 h. The extracts were combined, dried under reduced pressure with a centrifuge evaporator, and redissolved in 1 mL of deionized water. Water-insoluble materials in the extract were removed with 1 mL of chloroform. The extracts were divided into two parts to determine separately the contents of ACC and MACC. For the MACC assay, MACC was hydrolyzed to ACC with 2 N HCl (0.2 mL of 4 N HCl added to 0.2 mL of the extract) at 100°C for 2 h. The ACC level in the samples was determined according to the method of Lizada and Yang (1979).

Results

Effects of JA-Me on Germination of Cocklebur Seeds

After-ripened and nondormant lower cocklebur seeds start to germinate easily when incubating with water at 25°C in the dark. Fig. 1 shows the time courses of the germination of cocklebur seeds treated with various concentrations of JA-Me. JA-Me at concentrations over 1 μM inhibited germination significantly. Rates of germination slowed down with increasing the concentrations of JA-Me and reached zero at 1,000 μM JA-Me. But this severe inhibition was reversible because it was relieved gradually by washing the seeds with water after the treatment with 1,000 μM JA-Me for 3 days (data not shown).

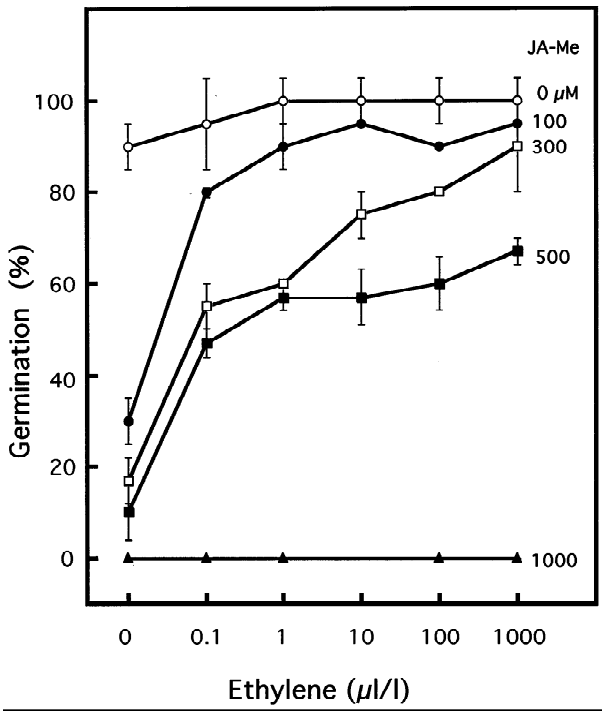

Fig. 2. Counteraction of ethylene on the inhibition of cocklebur seed germination by JA-Me. Ten seeds were incubated on two layers of filterpaper wetted with 3.5 mL of JA-Me solution at different concentrations (100, 300, 500, and 1,000 μM) in a 125-mL flask that was sealed with a rubber cap for 2 days at 25°C in the dark. A necessary amount of ethylene was injected into the flask through a rubber stopper. For control without ethylene, a small glass tube with 0.2 mL of 2.5 M $Hg(ClO_4)_2$ was included in the flask to absorb the ethylene. Data show the means ± S.E. of three replicates.

On the other hand, JA also inhibited germination at a similar range of concentrations (data not shown). We studied further the action of only JA-Me.

Counteraction of Ethylene on JA-Me Inhibition in Cocklebur Seed Germination

We examined whether any plant hormone has the ability to relieve the retardation of cocklebur seed germination by JA-Me. Abscisic acid at concentrations greater than 10 μM had inhibitory effects on the germination. Gibberellic acids (0.1 or 0.01 mM), indoleacetic acid (0.1 or 0.01 mM), and 6-benzylaminopurine (1 or 10 μM) had no effect, but ethylene was found to recover the inhibition of seed germination by JA-Me. To understand how ethylene counteracts the action of JA-Me, the effects of ethylene at various concentrations on the germination of cocklebur seeds treated with different concentrations of JA-Me for 2 days were examined (Fig. 2). Ethylene had no significant effect on the germination of cocklebur seed, untreated with JA-Me, but 1 μL/liter ethylene completely overcame the inhibition by 100 μM JA-Me. When JA-Me of more than 300 μM was applied, higher concentrations of ethylene were necessary for complete recovery from the inhibition. Fig. 2 indicates that the inhibition of the germination by 1,000 μM JA-Me was not overcome even by ethylene of 1,000 μL/liter. But the germination of the seeds treated with 1,000 μM JA-Me gradually started after a prolonged period of incubation with 10,000 μL/liter ethylene and reached around 50% after 4 days of the incubation, showing that the inhibition of 1,000 μM JA-Me is partially recovered by exogenous ethylene (data not shown).

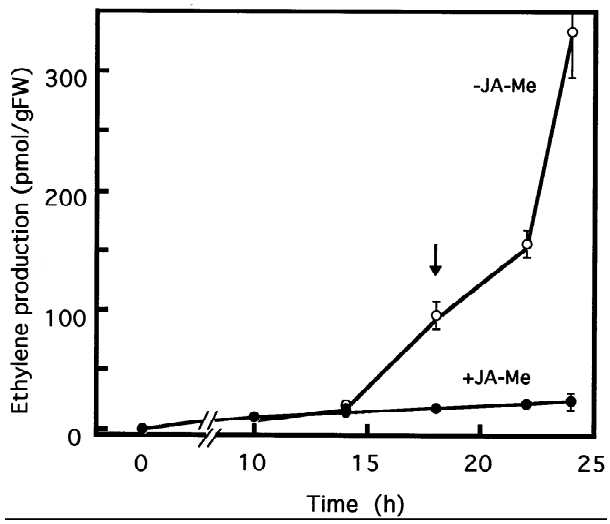

Fig. 3. Time courses of ethylene production from germinating cocklebur seeds treated with (●) or without (○) 1,000 μM JA-Me. Thirty seeds were incubated in a 30-mL vial containing water or 1,000 μM JA-Me at 25°C in the dark. Accumulated ethylene in the sealed vials during a different period of incubation was determined by gas chromatography. Data are the means ± S.E. of three replicates. The *arrow* shows the initial time of the start of germination.

Inhibitory Effects of JA-Me on Ethylene Production by Cocklebur Seeds

Satoh et al. (1984a) reported that endogenous ethylene produced by cocklebur seeds before germination is involved in the stimulation of the seed germination. It is plausible, therefore, that the inhibition of the germination by JA-Me comes from the inhibition of ethylene production. Next, we examined whether JA-Me affects ethylene production during seed germination.

Fig. 3 shows time courses of ethylene production accompanied by the germination of cocklebur seeds. Ethylene production started to increase after 18 h of incubation at 25°C in the dark, just when some seeds started

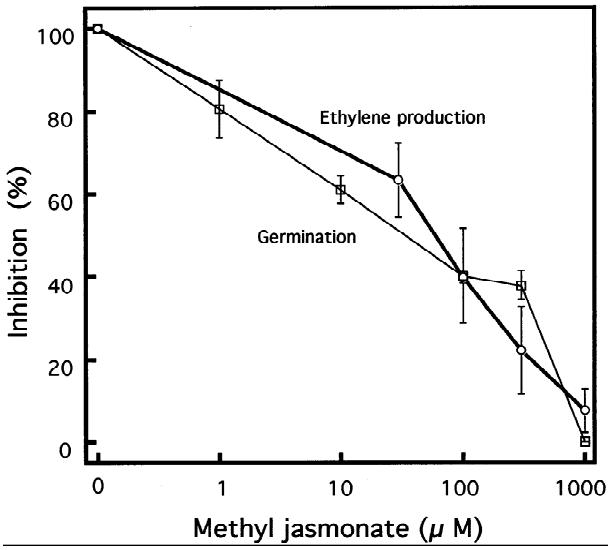

Fig. 4. Dose-response curves of JA-Me for the inhibition of ethylene production from cocklebur seeds (○) and their germination (□). Thirty seeds were incubated in a 30-mL vial containing various concentrations of JA-Me at 25°C in the dark. After 1 day of incubation, accumulated ethylene in the sealed vial was determined. Percentages of inhibition against ethylene produced from the seeds incubated with water were calculated. *Bars* show S.E. of two replicates. Percentages of inhibition of germination rates after 3 days of incubation were calculated from the data in Fig. 1.

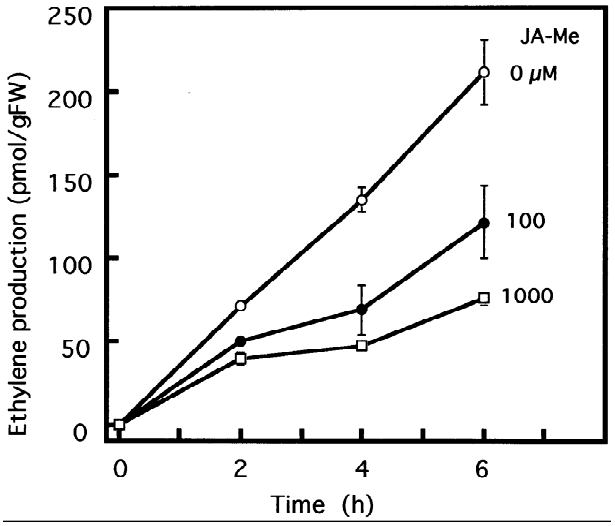

Fig. 5. Time courses of ethylene production from cotyledonary segments of cocklebur seeds. Twenty segments were incubated in a 30-mL vial containing 1.5 mL of JA-Me at different concentrations (0, 100, and 1,000 μM) at 25°C in the dark. Data show the means ± S.E. of three replicates.

germinating. The surge of ethylene production occurred after 24 h of incubation because half of seeds had germinated (see Fig. 1). Ethylene production was prevented completely by 1,000 μM JA-Me. In Fig. 4, the dose-response curve of JA-Me for the inhibition of ethylene production from cocklebur seeds is compared with its dose-response curve for inhibition of the germination. The similarity between two dose-response curves of JA-Me supported the idea that the inhibition of the ethylene production is one of the causes of germination inhibition by JA-Me.

Mechanism of JA-Me Inhibition in Ethylene Biosynthesis of Cocklebur Seeds

We chose cotyledonary segments separated from cocklebur seeds as a simpler experimental material to analyze the mechanism of inhibitory actions of JA-Me on ethylene production from cocklebur seeds. Fig. 5 shows the time courses of ethylene production from the cotyledonary segments. In the absence of JA-Me, ethylene production from the segments increased linearly until 6 h of incubation. The significant inhibition of ethylene production appeared after 2 h of treatment with 100 μM JA-Me, suggesting that JA-Me directly inhibited a step of ethylene biosynthesis. Ethylene production almost stopped during an incubation period between 2 and 4 h in the presence of 1,000 μM JA-Me, and the inhibition was apt to be relieved after 6 h of incubation.

We further examined changes in the level of ACC and MACC in the cotyledonary segments treated with or without 1,000 μM JA-Me for 3 and 6 h (Table 1). The level of ACC in the cotyledonary segments was kept almost constant throughout an experimental period for 6 h in the absence of JA-Me. The ACC level in the segments treated with JA-Me decreased significantly 3 h after the incubation and became one eighth of that in control segments 6 h after the incubation. On the other hand, the level of MACC in the segments increased 3 h after the start of incubation and then decreased despite the presence or absence of JA-Me. JA-Me had no effects on the level of MACC.

Next, we examined the effect of JA-Me on the activity of enzymes involved in ACC metabolism. First we tried to examine the effects of JA-Me on ACC synthase. However, the activity of ACC synthase could not be detected because of its low activity in the cotyledonary tissues. Then, ethylene production from the cotyledonary tissues pretreated with 3 mM ACC was determined in order to examine the effect of JA-Me on the activity of ACC oxidase (Table 2). This activity of ethylene production from tissues treated with ACC has been considered as in vivo activity of ACC oxidase. Although the inhibition by JA-Me of ethylene production from the segment not treated with ACC started after 2 h of incubation (see Fig. 5), JA-Me even at 1,000 μM hardly inhibited ACC conversion to ethylene until 4 h of the treatment (Table 2).

Table 1. Effects of JA-Me on ethylene production and level of ACC and MACC in cotylendonary segments of cocklebur seeds. The cotyledonary segments were treated with or without 1,000 μM, JA-Me for 3 or 6 h at 25°C in the dark. Data show the means ± S.E. of three replicates.

Incubation time (h)	JA-Me treatment	Ethylene[a]	ACC[a]	MACC[a]
0	−	0	0.80 ± 0.10	2.33 ± 0.05
3	−	0.15 ± 0.01	1.00 ± 0.12	3.40 ± 0.40
	+	0.09 ± 0.01	0.85 ± 0.02	3.49 ± 0.60
6	−	0.22 ± 0.03	0.80 ± 0.06	2.90 ± 0.13
	+	0.10 ± 0.01	0.12 ± 0.05	2.80 ± 0.05

[a] Results are presented as nmol/g fresh weight.

Table 2. Effects of JA-Me on ethylene production from cotylendonary segments of cocklebur seeds pretreated with ACC. Fifteen cotyledonary segments were incubated with 3 mM ACC for 3 h and then transferred into a sealed vial of 30 mL and incubated further with JA-Me at different concentrations (0, 30, 100, and 1,000 μM) at 25°C in the dark. Data show the means ± S.E. of three replicates.

	Ethylene (nmol/g fresh weight)		
JA-Me (μM)	2 h	4 h	6 h
0	1.15 ± 0.06	2.80 ± 0.15	4.90 ± 0.25
30	1.00 ± 0.10	2.40 ± 0.35	4.20 ± 0.60
100	1.15 ± 0.03	2.60 ± 0.20	4.20 ± 0.25
1,000	1.19 ± 0.10	2.50 ± 0.15	3.60 ± 0.30

Discussion

JAs have been reported to inhibit the seed germination of several plants: *Agrostemma* (Sembdner and Gross, 1986), *Lactuca* (Yamane et al. 1981), *Amaranthus* (Kepczynski and Bialecka 1994), and sunflower seeds (Corbineau et al. 1988). In all of these cases, high concentrations of JA or JA-Me (higher than 100 μM) are necessary to inhibit germination. Therefore, Sembdner and Parthier (1993) pointed out that a physiological effect of JAs on seed germination is doubtful. In the case of cocklebur seeds, significant inhibition of the germination was detected even at 1 μM JA-Me (Fig. 1). Inhibition of cocklebur seed germination by JA-Me was nullified by exogenous ethylene (Fig. 2). Such a recovery effect had been found in the germination of *Amaranthus* seeds (Kepczynski and Bialecka 1994). In the case of *Amaranthus* seeds, gibberellins are also effective in recovering inhibition but not in cocklebur seeds. In both cases, ethylene could not recover completely the inhibition of seed germination induced by high concentrations of JA-Me, suggesting that the high concentrations have a toxic effect. The interaction between JAs at low concentrations and ethylene suggested that JA acts as an endogenous regulator in the germination of cocklebur seeds.

Satoh and Esashi (1984a) investigated differences in ethylene production between dormant and nondormant lower seeds of cocklebur. They reported that ethylene production from nondormant seeds is higher than that from dormant seeds, that cocklebur seeds are most sensitive to ethylene before the radicle protrusion, and that some inhibitors of ethylene biosynthesis prevent germination. From these results they concluded that ethylene evoked from nondormant lower seeds of cocklebur before radicle protrusion is important for starting germination. In this study we observed that ethylene production from the nondormant seeds between 14 and 18 h after incubation started to increase but not in the seeds treated with JA-Me (Fig. 3). This result suggested that JA-Me inhibits the ethylene production necessary for the germination. This conclusion was supported by the observation that the inhibition of germination by JA-Me was overcome by exogenous ethylene (Fig. 2) and the dose of JA-Me which inhibits ethylene production is similar to that which prevents germination (Fig. 4).

It is well established that ACC is a precursor of ethylene (Satoh and Esashi 1984a), and MACC is one of metabolites of ACC in germinating cocklebur seeds (Satoh and Esashi 1984b). JA-Me decreased the level of ACC in cotyledonary tissues of cocklebur seeds (Table 1) but had no effect on the level of MACC, suggesting that JA-Me prevents the induction of ACC synthase or inhibits its activity. In addition, JA-Me partially inhibited the conversion of ACC to ethylene, that is, the activity of ACC oxidase (Table 2). But this inhibition occurred after prevention of ethylene production by JA-Me (Fig. 5). These results suggest that the inhibition of ACC synthase rather than ACC oxidase is the main cause of the prevention of ethylene production by JA-Me in cocklebur seeds. This is the first finding to show that JA-Me directly affects the pathway of ethylene biosynthesis in seed tissues.

Prevention of ethylene production in cocklebur seeds is reported to be one of the causes of staying in a primary dormancy (Satoh and Esashi 1984a). On the other hand, some reports detect JAs in immature seeds of some plants (Sembdner and Parthier 1993). It has not yet been determined what kind of JAs are contained in cocklebur seeds and how the JA level changes in a process of seed maturation and seed germination. It is worth investigating whether endogenous JA-Me is involved in the dormancy of cocklebur seeds by analyzing the level of JA-Me during a process of seed maturation and germination.

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