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Isolation and Identification of a Senescence-promoting Substance from Wormwood (*Artemisia absinthium* L.)

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ABSTRACT

The senescence-promoting substance of wormwood (*Artemisia absinthium* L.) as detected by the oat (*Avena sativa* L. cv "Victory") leaf assay has been identified as (−)-methyl jasmonate, methyl (1S, 2R)-3-oxo-2-(2'-*cis*-pentenyl)-cyclopentane-1-acetate, by gas-liquid chromatography-mass spectrometry and optical rotatory dispersion. Its senescence-promoting effect was much stronger than that of abscisic acid, and even at such a low concentration as 1 to 2.5 micrograms per milliliter, it could completely eliminate the anti-senescence action of 2 micrograms per milliliter kinetin. Comparing the biological activity of the (−)- with the (±)-forms of methyl jasmonate, it seemed that only the (−)-form was biologically active.

Senescence of green plant tissues is characterized by the loss of various cell components, especially Chl (3). Cytokinins delay the senescence (21) and ABA accelerates it (2). Actinomycin D (20), chloramphenicol and thiouracil (23) are also known to accelerate the yellowing of various plant tissues. These substances can eliminate the antisenescence action of cytokinins. A high concentration of L-serine has been reported to possess a similar effect in oat leaf segments, especially in the presence of cytokinin, possibly through enhancement of protease biosynthesis (12). In barley leaf segments a high concentration of EDTA has a bleaching effect only in the light (11).

We have recently found that an extract of wormwood (*Artemisia absinthium* L.) contains a senescence-promoting factor as examined by using oat (*Avena sativa* L. cv "Victory") leaf segments. The extracts of wormwood have been known to inhibit the seed germination and growth of fennel (*Foeniculum vulgare* Mill.) (16), and the inhibition was shown to be associated with absinthin, artemetin, and other related compounds contained in this plant (15). Our preliminary studies show that the senescence-promoting factor is none of the above-mentioned compounds, and that it is a non-polar substance such as esters on the basis of its chromatographic behavior. It is not ABA-methyl ester and was thought to be a physiologically new compound.

This paper reports on the purification and identification of this substance. Comparative data on the biological activity of this factor and ABA are also presented.

MATERIALS AND METHODS

Plant Material. Leaves and stems of wormwood (*Artemisia absinthium* L.) were harvested on our campus June, 1977 through August, 1978. They were washed with tap water, weighed, and stored at −20 C before extraction.

Bioassay. The oat (*Avena sativa* L. cv "Victory") leaf was used according to Thimann's method (17). Seeds were germinated in Vermiculite and seedlings were grown under continuous white fluorescent light of about 5000 lux at 25 C for 7 days. The upper 3-cm leaf segments of first leaves were excised for the assay. Ten such leaf segments were placed on two layers of Toyo No. 2-filter paper moistened with 5 ml of test solutions in a sterilized Petri dish. The test substance or ABA was dissolved in water containing 500 μg/ml of the surfactants Tween 80: Span 80 (7:3, w/w) with or without 2 μg/ml kinetin. Controls were treated with the same medium without kinetin. The Petri dishes were kept in a dark room at 25 C. The leaf segments were sampled at 2 or 4 days after treatment and their Chl contents were determined. The initial Chl content was determined before incubation. Kinetin and ABA were purchased from Sigma Chemical Co., St. Louis, Mo.

Determination of Chl Content. The Chl content of the leaf segments was estimated as follows. The leaf segments of each sample were extracted with 10 ml of boiling 80% aqueous ethanol for 30 min. The extract volume was made up to 10 ml with 80% aqueous ethanol and the *A* of the extract was measured at 665 nm with a Hitachi model 101 spectrophotometer. The mean *A* of four to eight experiments was calculated. The results were expressed as percentages of the initial value, or the value for 2 μg/ml kinetin treatment.

ISOLATION OF THE SENESCENCE-PROMOTING FACTOR

Extraction and Solvent Fractionation. Leaves and stems (9.7 kg) were homogenized with a blender and extracted with 20 liters of 80% aqueous acetone three times. All the extracts were combined and concentrated at 35 C *in vacuo* to give an aqueous residue. The aqueous solution was adjusted to pH 2.5 with 2 N HCl and extracted with 4 liters of ethyl acetate three times. Since the active factor was in the neutral ethyl acetate fraction, the acidic ethyl acetate fraction was extracted with 2 liters of 5% $NaHCO_3$ solution to remove acidic materials. The neutral ethyl acetate fraction was dried over anhydrous Na_2SO_4 and then evaporated to dryness *in vacuo* to give an active crude material of 9.9 g.

PURIFICATION

Charcoal Adsorption Column Chromatography. Crude material (9.9 g) was dissolved in a small amount of 10% aqueous acetone, charged onto a column packed with charcoal (100 g of Wako charcoal for chromatography; 2.3 × 36 cm) and eluted with acetone-water. The acetone content of the mixture was increased in a series of 10% steps and the fractions eluted with 70 and 80% aqueous acetone contained the active factor (4.4 g).

Silicic Acid Adsorption Column Chromatography. Active factor eluted with 70 and 80% aqueous acetone (4.4 g) was dissolved in a small amount of benzene, charged onto a column packed with silicic acid (50 g of Wako-gel C 100; 1.0 × 42 cm) and eluted with

benzene-ethyl acetate. The ethyl acetate content of the mixture was increased in a series of 5% steps. The eluate with benzene-ethyl acetate (95:5) contained the active factor. The eluate of this fraction was evaporated to dryness and 111 mg of an active brown oil was obtained. It was dissolved in a small amount of *n*-hexane, charged onto a column packed with the same silicic acid (10 g; 1.0 × 8 cm) and eluted with *n*-hexane-ethyl acetate. The ethyl acetate content of the mixture was increased in a series of 10% steps. The eluate with *n*-hexane-ethyl acetate (90:10) contained 17 mg of the active factor.

Thin-layer Chromatography. Thin layers of silica gel GF_{254} with 0.25 mm thickness and PF_{254} with 0.5 mm thickness from Merck Co. Ltd. were used for analytical and preparative TLC, respectively. Seventeen milligrams of partially purified active factor was further purified on preparative TLC developed with (A) *n*-hexane-ethyl acetate (5:1, v/v, multiple development). The active zone of R_F 0.27-0.37 was scraped off and eluted with ethyl acetate to give 5 mg of an active yellow oil. It was finally purified by preparative TLC developed with (B) benzene-ethyl acetate (10:1, v/v). The active zone of R_F 0.30-0.34 was scraped off and eluted with ethyl acetate, giving on evaporation 1 mg of an odoriferous yellow oil.

Gas-liquid Chromatography and Combined Gas Chromatography-Mass Spectrometry. GLC was carried out on a Hitachi model 163 gas chromatograph fitted with a hydrogen flame ionizing detector. A 3 mm x 2 m glass column was packed with 5% SE-30 on 80-100 mesh Gas Chromosorb W. The carrier gas was N_2 at flow rate of 30 ml min^{-1}. Column temperature was 180 C (isothermal condition), and that of injection and detector parts was 210 C.

GC-MS was carried out with a Hitachi RMU-6L spectrometer connected with a Hitachi 063 gas chromatograph. OV-1 (2%) on silanized Chromosorb W packed in a 3 mm x 1 m silanized glass column was used. The ionizing voltage was 70 electron volts.

Measurements of Optical Rotatory Dispersion. The ORD[1] of the senescence-promoting factor (0.21 mg) in methanol (2 ml, c = 0.0105) was recorded by a JASCO ORD/CD spectrometer model J-20, with a cell of 0.1 dm in width. The operating temperature was 25 C.

RESULTS

Identification of the Senescence-promoting Substance. The senescence-promoting substance of wormwood was isolated and purified as an odoriferous yellow oil (1 mg). This active substance ran as a homogeneous spot on analytical TLC in solvent A or B and showed a single peak in GLC with 5% SE-30 at 180 C (isothermal, Fig. 1). As shown in Figure 2, the mass spectrum of the GLC peak at 3.8 min gave ions at m/e ratios of 224 (37, M^+), 206 (13), 193 (17), 177 (14), 156 (24), 151 (50), 135 (20), 133 (20), 121 (17), 109 (34), 95 (44) and 83 (100, base peak) and corresponded to the formula $C_{13}H_{20}O_3$ suggesting that this active substance is methyl jasmonate. The identification was confirmed by direct comparison of this substance with an authentic sample of (±)-methyl jasmonate. The R_F value in TLC and the retention time in GLC of the sample agreed with those of the active substance. In addition, the mass spectrum of the sample completely agreed with that of the active substance (Fig. 2).

The ORD of the isolated methyl jasmonate was determined in a methanolic solution. The curve showed a negative Cotton effect with peaks at 313 and 276 nm, and zero rotation at 298 nm. The data is presented in Table I. These ORD characteristics agreed with those reported previously for (−)-methyl jasmonate, methyl (1S, 2R)-3-oxo-2-(2′-*cis*-pentenyl)-cyclopentane-1-acetate (6, 9, 10).

On the basis of the above evidence this active substance of wormwood was identified as (−)-methyl jasmonate.

[1] Abbreviation: ORD: optical rotatory dispersion.

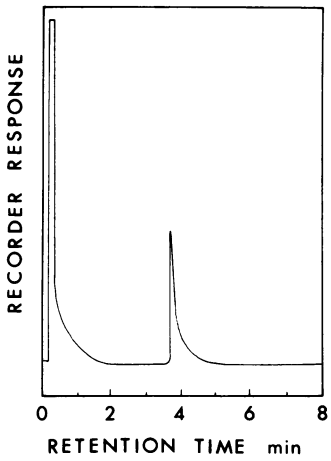

FIG. 1. Gas chromatograph of the senescence-promoting substance isolated from wormwood. GLC conditions: glass column (3 mm x 2 m) packed with 5% SE-30; N_2 flow rate, 30 ml min^{-1}; column temp., 180 C (isothermal).

Biological Activity of (−)-Methyl Jasmonate. In the bioassay, the senescence of control leaf segments proceeded rapidly soon after their excision. At 2 days, Chl content decreased to about 50% of initial value, and at 4 days to about 10%. In the presence of (−)-methyl jasmonate at 0.1 and 1.0 μg/ml, the loss of Chl in the leaf segments was even more rapid: at 2 days Chl dropped to about 30% of the initial value (Fig. 3).

The interaction of methyl jasmonate with kinetin, a senescence-retarding hormone, was examined. In the presence of 2 μg/ml kinetin about 80% of the initial Chl content was retained 4 days after treatment. The kinetin activity was suppressed by methyl jasmonate, the suppression being marked at concentrations more than 0.1 μg/ml (Fig. 4). On the concentration range between 0.1 and 2.5 μg/ml, the senescence-promoting activity of (−)-methyl jasmonate was higher than that of (±)-methyl jasmonate. In addition, the minimum concentration of the racemic mixture needed for complete decolorization of the leaf segments was about 2 to 5 times higher than that of the (−)-enantiomer.

The interaction of ABA with kinetin was also examined for comparison. Four days after treatment about 26.4 μg/ml of ABA was needed to eliminate the anti-senescence action of 2 μg/ml kinetin (Fig. 5).

DISCUSSION

The senescence-promoting substance isolated from wormwood (*Artemisia absinthium* L.) was identified as (−)-methyl jasmonate. This compound was first isolated from jasmine oil (*Jasminum grandiflorum* L.) as an odoriferous compound (6). It was also found in the essential oil of Tunisian rosemary (*Rosmarinus officinalis* L.) (4). However, no biological activity of methyl jasmonate was described in these reports. This is the first time that its biological activity as a senescence-promoting factor has been demonstrated.

The ORD of methyl jasmonate isolated in this study is levorotatory, while naturally occurring ABA is dextrorotatory (13). Milborrow showed that (+)- and (−)-ABA possessed an equal degree of inhibitory activity for the growth of wheat coleoptile (14). Sondheimer *et al.* (18) reported that there were great differences in the inhibition of the root-related growth of germinating barley seeds between these enantiomorphs, while they were almost equally active in the inhibition of the shoot growth. The isolated methyl jasmonate (levorotatory) is more active in counteracting kinetin than synthetic methyl jasmonate (racemate) (Fig. 4). Moreover, natural methyl jasmonate has been shown to be about 2 to

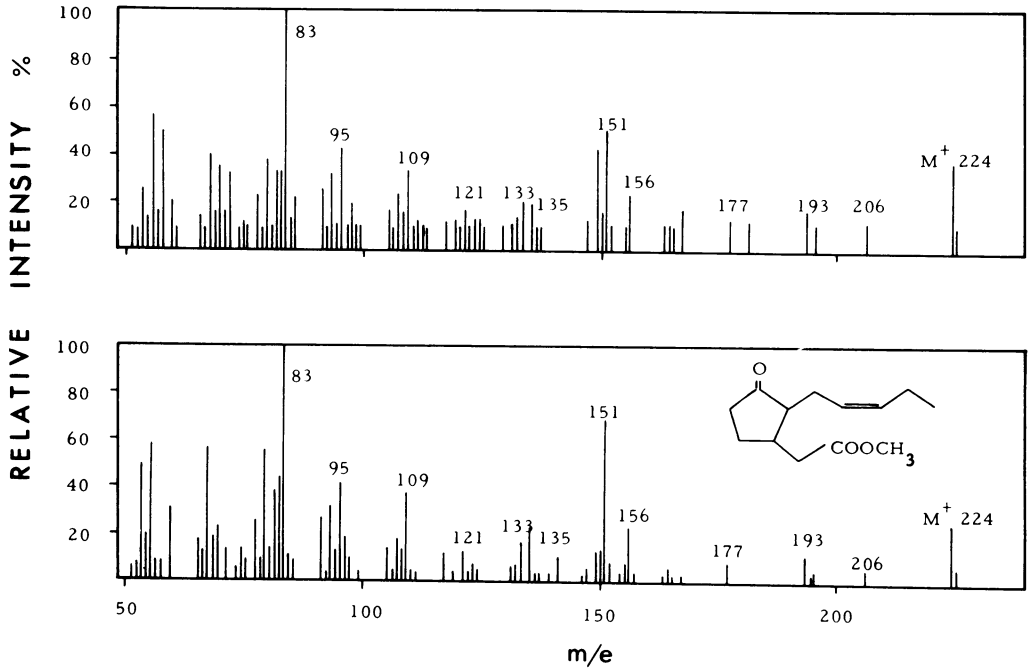

FIG. 2. Mass spectra of the senescence-promoting substance isolated (upper) and authentic (±)-methyl jasmonate (lower).

Table I. *Specific Rotation of Methyl Jasmonate Isolated from Wormwood*

Methyl jasmonate isolate (0.21 mg) was dissolved in 2 ml methanol. A cell of 0.1 dm in width was used and the operating temperature was 25 C.

Wave Length	Specific Rotation [α]
nm	*degrees*
313	−1905
298	0
276	+1905

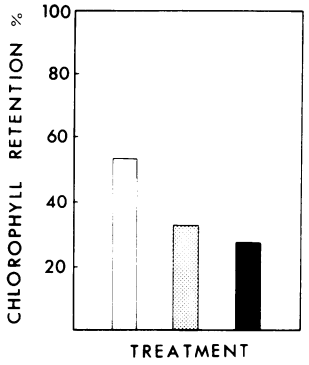

FIG. 3. Effect of (−)-methyl jasmonate on the senescence of oat leaf segments at 2 days after treatment. Chl retentions are described as percentages of initial values. □: control; ▨: 0.1 μg/ml (−)-methyl jasmonate; ■: 1.0 μg/ml (−)-methyl jasmonate.

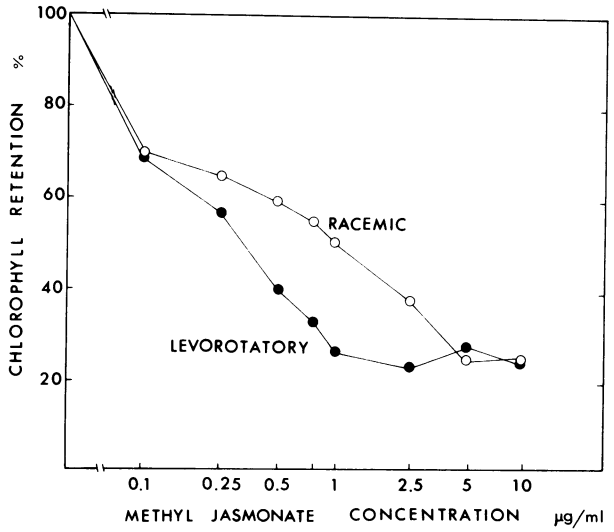

FIG. 4. Effect of (−)- or (±)-methyl jasmonate on the senescence of oat leaf segments in the presence of 2 μg/ml kinetin at 4 days after treatment. Chl retentions are described as percentages of 2 μg/ml kinetin values. ●: (−)-methyl jasmonate isolated; ○: authentic (±)-methyl jasmonate.

5 times more effective than synthetic methyl jasmonate in the complete decolorization of leaf segments.

Exogenous ABA can accelerate senescence of excised plant tissues as manifested by pigment loss (2). In the present study ABA has also been shown to promote leaf senescence and to eliminate the anti-senescence action of kinetin (Fig. 5). The effective concentration of ABA is physiologically low enough to suggest that it acts as a hormone. Other compounds which are known to promote senescence as described in the "Introduction" are either metabolic inhibitors or those which require high concentrations to manifest ABA-like activity. Levorotatory methyl jasmonate can accelerate leaf senescence both in the presence and absence of kinetin and its activity at low concentrations is much stronger than that of ABA.

Compounds which are structurally related to methyl jasmonate are known to be widely distributed in the plant kingdom. The free acid of methyl jasmonate (jasmonic acid) has been isolated from the culture filtrates of *Lasiodiplodia theobromae* as a growth inhibitor of green plants (1). It has also been isolated from the leaves and galls of chestnut (*Castanes crenata* Sieb et Zucc.) (19), and the immature seeds of *Phaseolus vulgaris* L. (24) and has been reported to have a similar effect. In addition, 5'-hydroxyjasmonic acid and the lactone of this compound have been found in the

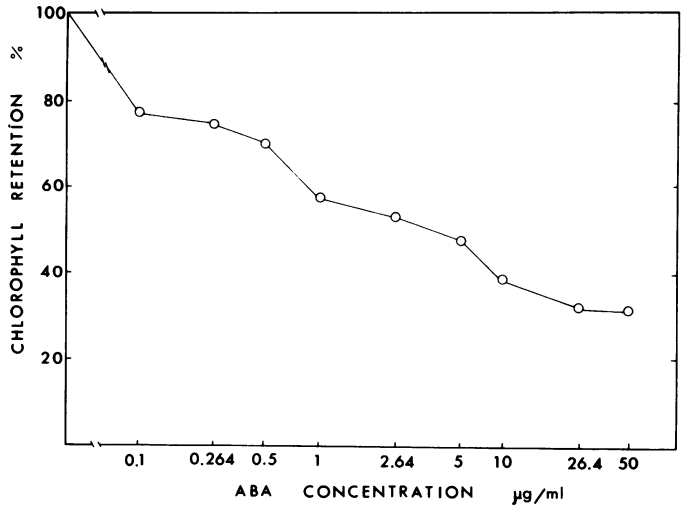

FIG. 5. Effect of (±)-ABA on the senescence of oat leaf segments in the presence of 2 µg/ml kinetin. Chl retentions are described as percentages of 2 µg/ml kinetin values.

essential oil of jasmine (*J. grandiflorum* L.) (7), and *N*-jasmonoyl- and *N*-dihydrojasmonoyl-isoleucin have been shown to occur in the fermentation medium of *Gibberella fujikuroi* (5). Cucurbic acid and its derivatives which have recently been isolated from seeds of pumpkin (*Cucurbita pepo* L.) as plant growth inhibitors have structures closely related to that of jasmonic acid (8).

Considering the wide occurrence of jasmonic acid and its related compounds in the plant kingdom together with its ABA-like activity in promoting leaf senescence at low concentrations, these substances might be physiologically significant natural regulators. Since methyl jasmonate is a volatile compound, it could be a volatile hormone in regulating the senescence and stomata aperture of oat leaf segments as has been suggested by Thimann *et al.* (22).

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