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**Control of Ethylene Biosynthesis and Action
to Retard the Senescence of Harvested
Vegetables**

N. Aharoni, S.F. Yang

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CONTROL OF ETHYLENE BIOSYNTHESIS AND ACTION TO RETARD THE SENESCENCE OF
HARVESTED VEGETABLES

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A. ABSTRACT

The goal of the present project was to understand the ethylene-controlled process of detached leaf senescence, and to find methods for its retardation in order to improve the keeping quality of harvested leafy vegetables. Experiments were performed with detached leaves (spinach, chives, lettuce) or stem cuts bunches of fresh herbs (parsley, dill, watercress, mint, marjoram, basil), as well as with broccoli florets. Additionally, various non-edible leaves (rice, sorghum, ipomea and tobacco) were employed as model systems for investigation of some intrinsic mechanisms. Results show that the senescence rate of the various commodities was affected by ethylene levels and by their sensitivity to ethylene. The patterns of ethylene production, as related to changes in various senescence parameters, implicate ethylene as a stimulator of foliar senescence rather than the initiator of the process. The data indicate that wounding or detachment may initiate leaf senescence. CO_2 and the juvenile plant hormones 3-indoleacetic acid (IAA), cytokinin (CK) and gibberellic acid (GA_3), were found to retard senescence although their effect was accompanied by acceleration of ethylene production. The accumulation of respiratory CO_2 within polyethylene packages combined with a GA_3 -spray, was employed as a useful method for delaying yellowing of various leafy commodities.

Some new findings regarding the control of ethylene biosynthesis were established in the course of this study: (A) CO_2 was found to enhance the synthesis of the ethylene forming enzyme (EFE); (B) The sucrose-enhanced ethylene production was found to be derived from a sucrose-stimulated activity of an enzyme hydrolyzing IAA-amine conjugates; (C) GA_3 was found to enhance ethylene production of several leaves by increasing 1-aminocyclopropane-1-carboxylic acid (ACC) and EFE as well as by suppressing malonyl-ACC; (D) Abscissic acid (ABA) in spinach leaves (dicot) exerted a stimulatory effect on EFE activity which was CO_2 -mediated, whereas in rice leaves (monocot) ABA inhibited ethylene production; (E) Indications for a senescence-dependent metabolism of malonyl-ACC were found in spinach leaves; (F) The CO_2 -inhibited photorespiration in rice leaves was found to be responsible partially for the senescence-retarding effect of CO_2 in the light.

B. OBJECTIVES OF THE RESEARCH

The main objective of the original research proposal was to evaluate effective and harmless treatments to inhibit ethylene action in harvested green vegetables, for retardation of their rapid senescence. For this purpose the following goals were outlined:

- (a) To study the effect of ethylene on the senescence processes and keeping quality of green and leafy vegetables.
- (b) To study the effect of stress-induced ethylene production on the senescence and storability of leafy vegetables.
- (c) To investigate some intrinsic factors involved in the biosynthesis of ethylene in the senescing leaf.
- (d) To investigate the effect of plant hormones on ethylene production and action in senescing tissues.
- (e) To evaluate practical methods based on the use of naturally-occurring agents, such as plant hormones and CO₂, for regulating senescence of edible products.

Experiments were originally planned with commercially-important green and leafy vegetables. However, due to the growing interest and market value of various perishable fresh herbs in the course of the last 3 years and considering their low keeping quality, these greens were included as well in the scope of this study.

2.2 EFFECTS OF CO₂ ON LEAF SENESCENCE OF C₃ AND C₄ PLANTS

Ma. Concepcion C. Lizada and S.F. Yang

Goring and Koshuchova (1) observed that the absence of CO₂ enhanced senescence in the leaves of oat (a C₃ plant) but not of corn (a C₄ plant). They pointed to the possibility that photorespiration and its products might be involved in the senescence process. This study was carried out to determine whether this difference also holds for rice (C₃) and sorghum (C₄).

Materials and Methods

Rice was grown under the same conditions described for *I. convolvulus* (See Section 4.3), except that seeds were sown directly and allowed to germinate in vermiculite. The first and second leaves of week-old seedlings of sorghum and rice, respectively were excised at the base and used as whole leaves. Incubations were done at 25°C using cool white light, where indicated, with an intensity of 63 $\mu\text{g}/\text{m}^2\text{-sec}$. To prevent excessive moisture loss, a strip of filter paper was placed in the incubation vial and was kept saturated with water. A flow-through system was used except where indicated otherwise. Except for incubations with CO₂, a piece of filter paper with KOH was placed in a center well in incubation vials. Treatment solutions consisted of the indicated compound in a 50 mM MES buffer (pH 6) containing 0.15% Tween-20. Chlorophyll was determined by extraction in ethanol, protein by the Bradford method and ammonia by the Carlson method.

Results and discussion: At 30°C in light rice leaves exhibited a small but significant (6.1%) loss of chlorophyll after only one day of incubation. The rate of chlorophyll loss was higher in sorghum over 4 days of incubation, with a 50% loss observed only 2 days after excission. A comparative

loss in chlorophyll was not observed in rice until after 4 days of incubation.

In contrast, protein was lost initially at a higher rate in rice, exhibiting a decline of 13% (from 45.6 mg/g to 39.6 mg/g) 1 day after excission compared to only 3% (from 12 mg/g to 11.6 mg/g) in sorghum. Beyond the second day of incubation, however, rice and sorghum showed comparable rates of loss in protein. The pattern of ammonia accumulation reflected that of protein loss with rice initially showing a 3-fold increase from 16.7 mg to 48.8 mg and sorghum less than a 2-fold increase from 7.4 mg to 12.2 mg. Starting on the second day of incubation both showed comparable rates of NH_2 accumulation.

Effect of CO_2 on Senescence. CO_2 at concentrations of 1 and 2% inhibited chlorophyll loss 4 days after excission by 43% and 52%, respectively, in both rice and sorghum, contrary to what might be expected on the basis of Goring and Koshuchovas' (1) observations in corn and oats. On the other hand, the same level of CO_2 inhibited protein loss by 23% in rice but only 13% in sorghum. Increasing the level to 2% increased the inhibition in rice to 33%, but did not increase the inhibition in sorghum. Thus, senescence in sorghum is subject to inhibition by CO_2 mainly with respect to chlorophyll degradation. The effects of CO_2 on chlorophyll degradation in the dark were similar to those observed in light for both rice and sorghum. CO_2 , even at a level of 0.5%, inhibited protein loss in rice, but not in sorghum where the only significant inhibition of protein loss (11%) was observed at 2% CO_2 . Thomas and Stoddart (3) have also reported differing responses between chlorophyll degradation and protein breakdown in a mutant of *Festuca pratensis*.

Further experiments with rice and sorghum showed that CO_2 altered the pattern of ethylene production in both rice and sorghum in the dark. CO_2 (3%) caused a 4-fold increase in ethylene production in rice for the first 36 h of incubation, beyond which the leaves subjected to CO_2 had comparable levels of ethylene production as the control (ca. 2 nL/g-h). Ethylene production in sorghum was lower compared to rice, never exceeding 2 nL/g-h during the period of observation (96 h). Ethylene production was lower in CO_2 -treated leaves (0.2 nL/g-h) compared to the controls (0.8 nL/g-h) 1 day after excision and start of treatment. However, 3 days after excision, the CO_2 -treated leaves showed a level of ethylene production which was twice that of the control.

Effect of Inhibitors of Photorespiration on Senescence.

Methionine sulfoximine (MSX, 2.5 mM) an inhibitor of glutamine synthetase, increased protein and chlorophyll loss by 64 and 75% respectively, in rice leaves incubated for 3 days in the light. Acceleration of protein loss was eliminated in the presence of 0.1 M isonicotinic hydrazide (INH), a known inhibitor of photorespiration. Chlorophyll degradation, in contrast, was inhibited by 50% by the same level of INH with or without the presence of MSX. Analysis for NH_3 in the leaf and in the treatment solution showed that MSX increased the level of NH_3 to 13 times that of the control, while INH decreased the MSX-induced increase in NH_3 by 75%.

MSX enhanced chlorophyll loss in rice even in the dark, although by only 43%, while INH affected a greater inhibition (80%) of chlorophyll loss than in the light. The NH_3 levels accumulated in MSX-treated leaves incubated in the dark (456 ug/g) were lower than those accumulated in

MSX-treated leaves incubated in the light. In the dark INH reduced MSX-induced NH_3 accumulation by only 43%.

In sorghum, MSX did not significantly affect chlorophyll degradation but increased protein breakdown by only 21%, which is about a third of the increase in rice. MSX-induced increase in NH_3 was also lower in sorghum (only 5 times that of the control).

The results of experiments using inhibitors indicate that photorespiration could be involved in foliar senescence in the light. The inhibition of senescence appears to be associated with the inhibition of the release of NH_3 , the major source of which has been postulated as the glycine-serine conversion in photorespiration (2).

CO_2 appears to inhibit senescence in the light partly through an inhibition of photorespiration. Preliminary experiments showed that NH_3 accumulation in rice is also inhibited by CO_2 in the light. Inherent differences between rice and corn in terms of protein loss during either light or dark periods, are also indicated by the effects of CO_2 .

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3. ROLE OF ETHYLENE IN PROCESSES OF LEAF SENESCENCE

3.1 ETHYLENE-ENHANCED SENESCENCE OF LEAFY VEGETABLES AND FRESH HERBS

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Abstract

One of the most common postharvest problems of green vegetables and fresh herbs is associated with their rapid senescence, a process which is thought to be regulated by ethylene, the senescing hormone. In the present work various senescence parameters as well as patterns of ethylene and CO_2 evolution were screened simultaneously in detached leaves of spinach, parsley and watercress, in order to study their senescence process. Senescence was enhanced by wounding, by application of the ethylene precursor - ACC (0.1 mM), and by gasing the tissue with 10 $\mu\text{l/L}$ ethylene. Retardation of senescence was achieved by application of inhibitors of ethylene biosynthesis (50 mM AOA) and action (60 μM Ag^+ , 11% CO_2). All three plant systems, consisting of intact leaves, leaflets or leaf discs, exhibited two surges of ethylene production during their dark incubation. The first peak occurred in response to excision or detachment, while the second one coincided more or less with commencement of senescence. A positive correlation between the level of wound-ethylene production and the rate of senescence has been found. The findings suggest that ethylene plays a significant role in accelerating senescence processes of leafy vegetables and herbs.

Abbreviations: ACC = 1-aminocyclopropane-1-carboxylic acid; AOA = aminooxy acetic acid; CHL = chlorophyll; MDA = malondialdehyde; TBA = thiobarbituric acid.

1. Introduction

The involvement of ethylene in the senescence of flowers and fruit ripening was demonstrated clearly, when the hormone was implicated in both the initiation and acceleration of this developmental process (Sacher, 1973; Halevy and Mayak, 1981). Unlike the situation in flowers and fruits, evidence is still accumulating to invoke a role for ethylene in the acceleration of foliar senescence

(Roberts et al., 1984; Thimann, 1985; Goldthwaite, 1987). So far, no solid evidence has been documented showing ethylene alone as a natural initiator of foliar senescence, but it was rather suggested that ethylene plays a regulatory role (Aharoni, 1979b; Kao and Yang, 1983). Chl degradation is the visible characteristic metabolic change of leaf senescence, and it is usually accompanied or preceded by extensive proteolysis and lipid oxidation, followed by the corresponding accumulation of free amino acids and products of lipids catabolism (Thomas and Stoddart, 1980; Thomas, 1986; Thimann, 1987). The present study constituted an attempt to establish a possible role for ethylene in promoting leaf senescence, by testing the above mentioned parameters in various leafy vegetables and herbs which senesce at different rates.

2. Materials and methods

Experiments were performed with fully expanded leaves of spinach (Spinacia oleracea L.), parsley (Petroselinum crispum Mill.), and watercress (Nasturtium officinale R. Br.), which were freshly harvested from the field. Parsley or watercress bunches were stored in water-containing flasks and enclosed into 2-L jars fitted with inlet and outlet ports. The jars were sealed and connected to gas cylinders containing either 10 μ l/L ethylene (for parsley) or 11% CO₂ (for watercress). A flow-through system was used with a flow rate maintained at 50 ml/min. Control treatments were established similarly but were flushed with air from air cylinders. The jars were incubated for 6-9 days in the dark at 30°C (parsley) or 20°C (watercress) and changes in ethylene production, chl, amino acids, protein and total aldehydes content were determined daily. To test the effect of various agents on senescence, samples of eight parsley leaflets were incubated on moistened filter paper in petri dishes. Where indicated, 0.1 mM ACC or 50 mM AOA were included. Pretreatment of leaflets with 60 μ M AgNO₃ was done according to Aharoni et al. (1979a). The petri dishes were incubated in the dark at 25°C for 9 days, and ethylene production rates were monitored daily by enclosing the treated leaflets in 50-ml flasks for 2 h. Leaflets were subsequently assayed for chl and amino acids content. To test the wounding effect, samples consisting of 4 detached leaves or 8 leaf discs of spinach were enclosed in 0.5-L jars or in 50-ml flasks, respectively. Discs were excised and treated as described previously (Philosoph-Hadas et al., 1985). Both jars and flasks were incubated in the dark at 25°C for 6-9 days and the changes in ethylene production, respiration rate, chl and amino acid contents were assayed daily.

Ethylene and CO₂ levels were determined by gas chromatographs as detailed by Philosoph-Hadas et al. (1986). Chl and amino acids were extracted from leaves

into boiling 80% ethanol for 30 min and quantitated spectrophotometrically according to Arnon (1949) or by the ninhydrin method (Yemm and Cocking, 1954), respectively. Protein was extracted from leaves by 1 N NaOH (Goldthwaite, 1974) and estimated by the method of Bradford (1976). Saturated aldehydes were extracted from 1-g tissue with 20% TCA and assayed with the TBA test described by Kosugi and Kikugawa (1985). Treatments were tested in triplicate and the data presented are expressed as the mean with its standard deviation.

3. Results

3.1. Wounding-induced senescence

A comparative study with detached intact spinach leaves and excised discs was conducted to check the effect of increased endogenous ethylene production, induced by excision, on the rate of senescence. While the initiation of chl loss and amino acids accumulation in intact spinach leaves did not occur before the 5th day of incubation (Fig. 1A), the corresponding changes of these parameters in spinach leaf discs could be detected already between 1-3 days (Fig. 2, inset). The climacteric-like rise in ethylene production of intact leaves and leaf discs paralleled these changes (Figs. 1B, 2). Respiration peaked in intact leaves one day before ethylene did (Fig. 1B), while in leaf discs it peaked in parallel to the ethylene rise (Fig. 2). In addition to the typical climacteric-like rise of ethylene, leaf discs exhibited a very sharp burst of wound ethylene, 4 h following excision (Fig. 2). Intact leaves exhibited, in response to detachment, a very moderate ethylene rise (10-fold lower), which peaked on the first day of incubation (Fig. 1A).

3.2. Ethylene-enhanced senescence

In addition to wounding, ethylene-enhanced senescence was obtained by gasing the tissue with 10 μ l/L ethylene (Figs. 3,4) or by application of ACC, the immediate precursor of ethylene (Fig. 5). Figure 3 demonstrates that although exogenous ethylene application suppressed endogenous ethylene production in parsley leaves, it enhanced their senescence rate, as exhibited by the promoted chl loss (Fig. 3, inset) and increased accumulation of amino acids (Fig. 4). Treatment of parsley leaflets with ACC, which stimulated endogenous ethylene production during the first 5 days (Fig. 5B), similarly promoted chl breakdown (Fig. 5A) and accumulation of amino acids (Fig. 6) from the fifth or the first day, respectively. The significant differences in amino acid content between treated (Figs. 4,6) and control leaves preceded chl differences (Figs. 3,5) by 1 to 4

days, indicating that amino acids is a more sensitive parameter for detecting senescence.

3.3. Retardation of senescence by inhibitors of ethylene biosynthesis and action

If ethylene enhances senescence, it is conceivable that ethylene inhibitors will retard this process. Indeed, treatment of parsley leaflets with AOA, which inhibits ethylene biosynthesis on the step of ACC formation (Yang and Hoffman, 1984), and with Ag^+ , which blocks ethylene action although stimulating its synthesis (Aharoni et al., 1979a), led to retention of a high level of chl during 9 days of incubation (Fig. 5A) with no typical climacteric ethylene rise obtained in control leaflets (Fig. 5B). The retarded senescence rate was also exhibited by a moderate increase of amino acid content in Ag^+ - and AOA-treated leaflets as compared with the rapid increase in control ones (Fig. 6). Since parsley leaflets senesce rather slowly (Fig. 5A), we checked the effect of another inhibitor of ethylene action, CO_2 , on senescence of watercress leaves, which senesce much more rapidly. Indeed, the results depicted in Figure 7 (inset) show that CO_2 was very effective in retaining a high chl level as well as in slowing down the decrease in proteins (Fig. 8A) and the increase in amino acids (Fig. 8B), MDA (Fig. 9A) and total aldehydes (Fig. 9B) content during 5 days of senescence. Figure 7 further indicates that the rapid senescence of watercress leaves (60% chl loss within 2 days) may be attributed to the upsurge of wound-ethylene production following detachment.

4. Discussion

The present study demonstrated that intact leaves, leaflets or leaf discs from a variety of species exhibit two peaks of ethylene production during dark senescence under experimental conditions. The first ethylene-rise occurs in response to either excision or detachment, while the second one, appearing in a climacteric-like pattern, is associated with the senescence process. We have shown that leaf senescence, as measured by yellowing, loss of protein and accumulation of amino acids and total aldehydes, was promoted by ethylene applied or induced in various manners (Figs. 2-9). On the other hand, inhibitors of ethylene biosynthesis and action retarded considerably these metabolic changes associated with senescence (Figs. 5-9). This implies that ethylene is involved in the regulation of leaf senescence (Aharoni, 1979b; Gepstein and Thimann, 1981; Roberts et al., 1984).

Aharoni and Lieberman (1979b) have suggested that the climacteric-like rise of ethylene production did not initiate leaf senescence because that rise occurred a considerable time after commencement of chl loss. Our

findings further support this observation and also suggest that increased levels of ethylene do not seem to be the primary event in the induction of the senescence syndrome, although such changes may well be part of, or contribute to, a more complex induction system (Thomas and Stoddart, 1980). Nevertheless, it is possible that the remarkable burst of wound-ethylene production following excision may promote senescence of the detached tissue, since it preceded all the metabolic changes associated with this process (Figs. 2,3,4,7-9). Hence, the wounding effect seems to induce senescence through the followings: (a) increased ethylene production; (b) increasing the tissue sensitivity to ethylene (Kao and Yang, 1983); and/or (c) triggering other metabolic processes (Thomas, 1986).

The results suggest that the increased ethylene production obtained by excised leafy tissues, is an important factor which determines the storability of perishable commodities such as yellowing-susceptible fresh herbs. The use of ethylene inhibitors may therefore provide us with an efficient method for delaying senescence of these leafy produce.

Acknowledgement

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Figures 1 & 2 - time course of changes in chl, amino acids ethylene production and respiration in intact leaves (1) and leaf discs (2) of spinach.

Figures 3 & 4 - Effect of exogenous ethylene on ethylene production rates, chl (3) and amino acids content (4) in parsley leaves.

Figures 5 & 6 - Effect of ACC, AOA and Ag^+ on chl, ethylene production rates (5) and amino acids content (6) in parsley leaflets.

Figures 7 & 8 - Effect of CO_2 on chl and ethylene production rates (7), and on protein and amino acids content (8) in watercress leaflets.

Figure 9 - Effect of CO₂ on MDA and total aldehydes content in watercress leaves in the course of senescence.

3.2 EFFECTS OF WOUNDING AND ETHYLENE ON SENESCENCE OF DETACHED LEAFY TISSUES

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FOOTNOTES

- 1) Supported by grant No. I-773-84 from BARD, The United States-Israel Binational Agricultural Research and Development Fund. Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. No. 2809-E, 1989 series.
- 2) Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycin; CHL, chlorophyll.

ABSTRACT

The effects of wounding and ethylene on senescence of various detached leafy systems were examined with parsley bunches and leaflets as well as with intact leaves and leaf discs of spinach. Senescence was enhanced by application of ethylene or 1-aminocyclopropane-1-carboxylic acid (ACC) and was retarded by inhibitors of ethylene biosynthesis and action. Evidence is presented showing that the bursts of both wound- and climacteric-like ethylene promoted senescence of detached leaves. This ethylene-enhanced leaf senescence was dependent on: (a) ethylene production rates in the tissue; (b) the tissue's sensitivity to ethylene; (c) the sensitivity to ethylene of the various senescence-associated processes; and (d) the degree of wounding. The results suggest that wounding might be regarded as the primary event in the induction of the senescence syndrome in detached leaves, while ethylene is implicated as a regulator of the rate of the process.

INTRODUCTION

The involvement of ethylene in the senescence of flowers and in fruit ripening was demonstrated clearly when the hormone was implicated in both the initiation and acceleration of this developmental process (10, 20). Unlike the situation with these plant organs, no solid evidence has been documented showing ethylene alone as a natural initiator of foliar senescence (19, 21), although it was well documented that ethylene participates in the regulation of leaf blade senescence (1, 2, 5, 7, 12, 14, 17, 18, 25). Senescence of detached leaves is made visible by the characteristic loss of Chl, which is usually accompanied or preceded by extensive proteolysis, RNA hydrolysis and lipid oxidation. These metabolic changes are closely related to the corresponding accumulation of free amino acids and products of lipids catabolism, which are taken often as reference parameters for following the progress of the senescence process (14, 21-24).

Various detached leaves exhibit under experimental conditions a rapid ethylene burst following excision and a subsequent climacteric-like rise in ethylene production (1, 2, 6, 15, 17). This ethylene climacteric rise was found to precede (5) or to coincide (2, 6, 7, 17, 18) with the period of most rapid Chl loss. While the initial ethylene rise is considered as an excision-related response (12), the second climacteric rise, which is associated with advanced senescence, was found also in attached leaves that senesce on the plant (2). The present study constituted an attempt to distinguish between the roles of these two ethylene rises and to examine the possible physiological significance of wound-ethylene in promoting the senescence of excised leaves. For these purposes, some of the above mentioned senescence parameters were tested in various dicot leafy systems which senesce at different rates.

MATERIALS AND METHODS

Plant Materials and Treatments. Experiments were performed with mature, green, fully expanded leaves of spinach (Spinacia oleracea L.) and parsley (Petroselinum crispum Mill.), which were freshly harvested from the field. Leaves were rinsed with distilled water and surface-

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sterilized with 0.5% (v/v) sodium hypochlorite as described previously (1, 2, 16). Parsley bunches were distributed at random in groups of 12 stems and placed in 100-mL flasks containing 50 ml distilled water plus 50 μ g/mL chloramphenicol. The flasks were then enclosed in 2-L jars, which were sealed and connected through fitted inlet and outlet ports, to gas cylinders containing either air or 10 μ L/L ethylene. A flow-through system was used with a flow rate maintained at 50 mL/min. The parsley bunches in the jars were incubated for 7 days in the dark at 30°C and changes in their endogenous ethylene production, ACC, Chl, amino acids and protein levels were determined daily with samples of ten excised leaflets. To avoid possible contamination during the incubation period the stem bottoms were cut and the solution in the flasks was renewed every second day.

To test the effect of various agents on senescence, parsley leaflets were pretreated by floating under cool-white light for 3 h in open petri dishes containing 20 mL H₂O, 0.1 mM AVG or 0.1 mM ACC (1). Pretreatment of leaflets with 60 μ M AgNO₃ was done similarly but for 30 min (1). Samples of eight pretreated leaflets (0.5 g) were then enclosed in petri dishes on a filter paper moistened with 2 mL of distilled water. Where indicated, 0.1 mM ACC or 0.1 mM AVG was included. The petri dishes were incubated in the dark at 25°C for 9 days, and ethylene production rates were monitored daily by enclosing the treated leaflets in 50-mL flasks for 2 h. Leaflets were subsequently assayed for Chl and amino acids content.

To test the effect of wounding on senescence, samples consisting of four intact leaves (4.5 g) or eight leaf discs (0.3 g) of spinach were enclosed in 0.5-L jars or in 50-mL flasks, respectively. Discs were excised and treated as described previously (16, 17). In one experiment spinach leaves were sprayed prior to their detachment with 100 mL solution of 0.1 mM AVG plus 0.5% (v/v) Tween-20 for elimination of their wound-ethylene production (control leaves were sprayed with water + Tween). The detached leaves were then preincubated for 1 h in petri dishes containing 20 mL of 0.1 mM AVG or H₂O prior to disc excision. Where indicated, AVG (0.1 mM) was added to control leaf discs 4 h after excision and ACC (0.05 mM) was added to AVG-treated discs 2 days after

excision. Both intact leaves and leaf discs were incubated in the dark at 25°C for 6-9 days, and changes in their endogenous ethylene production, respiration rate, ACC, Chl and amino acids levels were assayed daily.

Determination of Ethylene. Ethylene production rates of parsley leaves were monitored daily by enclosing samples of treated intact leaflets (0.5 g) in 50-mL flasks and incubating them at 30°C for 2 h. Ethylene production rates of spinach intact leaves or leaf discs were measured directly in their incubation jars or flasks, respectively, at intervals of 18-20 h. Ethylene concentration in the various vessels was analyzed by withdrawing a 2-mL gas sample and injecting it into a gas chromatograph equipped with an alumina column and a flame ionization detector.

Determination of Respiration Rates. Respiration rates of spinach leaves were determined by measuring the CO₂ accumulated in darkness during a 2-h period. Gas samples of 5 mL were withdrawn from each jar at the indicated periods and CO₂ concentration was determined with a Packard gas chromatograph equipped with a Poropak Q column and a thermal conductivity detector.

Extraction and Determination of Chl, ACC and Amino Acids. Chlorophyll and amino acids were extracted by boiling for 30 min samples of cut leaf segments (0.5 g) enclosed in test tubes containing 10 mL of 80% (v/v) ethanol (7). When necessary an additional 15-min extraction of the leaf segments in 3 mL ethanol was performed, and the ethanolic extracts were combined. Chl was quantitated and expressed as mg Chl/g fresh weight (FW) by transferring 200-μL aliquots of the ethanolic extract into 5 mL of 80% (v/v) acetone and determining the absorbance at 645 and 663 nm according to Arnon's method (3).

The ethanolic extract was then evaporated under vacuum at 55°C, the residues were dissolved in 2 mL of H₂O, and pigments were removed by addition of 0.5 mL chloroform (11). The ACC content in 0.25-ml aliquots of the aqueous solution was determined according to the method of Lizada and Yang (13). Amino acids content was determined in the same aqueous

solution by the ninhydrin method (27), which was adopted to micro quantities suitable for ELISA plates. The reaction was performed with flat-bottom, 96-well, microtiter plates (Immulon 1, Dynatech) in a total volume of 270 μ L/well, consisting of 10- μ L samples aliquots plus the original reaction mixture reduced by ten-fold. A standard methionine curve was included in every plate, and each point was repeated three or four times. After incubating the plates at 95°C for 20 min, the absorbance of the colored reaction product at 560 nm was determined with an automated ELISA Reader (model EL310, BIO TEK). This modified procedure enabled a rapid and accurate quantitation of amino acids in a large number of samples.

Extraction and Determination of Protein. Protein was solubilized from the extracted depigmented leaf segments with 1 N NaOH as described before (9) and estimated by the method of Bradford (4), which was performed with micro quantities on ELISA plates. All treatments and assays were tested in triplicates and the data presented are expressed as means $\pm$ SD.

RESULTS

Effect of Exogenous Ethylene and its Inhibitors on Leaf Senescence. The pattern of endogenous ethylene production in parsley bunches undergoing dark senescence was characterized by the appearance of two rises: a wound-ethylene rise, peaking on the first day, and a subsequent climacteric-like rise, peaking on the 4th day following detachment (Fig. 1A). Unlike the rise of wound-ethylene, the climacteric ethylene rise was paralleled, both in pattern and timing, by a progressive increase in ACC content up to eight-fold (Fig. 1A). The corresponding pattern of Chl loss exhibited two phases, with the first one occurring after the burst of wound-ethylene and the second one appearing in parallel to the climacteric ethylene rise (Fig. 1B).

An effort was made to check whether in addition to wounding, ethylene by itself can enhance the senescence syndrome. For this purpose the effect of ethylene, either exogenously applied or endogenously produced from ACC, was investigated with senescing parsley bunches and detached

leaflets. Gassing parsley bunches with 10 μ L/L ethylene resulted in promotion of Chl (Fig. 1B) and protein (Fig. 2A) degradation as well as in increased accumulation of amino acids (Fig. 2B), which could be observed only from the 4th, 5th or 3rd day (respectively) of ethylene incubation. On the other hand, treatment of detached parsley leaflets with ACC increased ethylene production during the first 5 days (Fig. 3A), and significantly promoted Chl breakdown (Fig. 3B) and accumulation of amino acids (Table I) from the 3rd or 7th day, respectively. It is pertinent to note that while Chl loss in parsley bunches was quite rapid (50% loss during 7 days, Fig. 1B), detached parsley leaflets did not show any significant change in their Chl content during the first 6 days of incubation (Fig. 3B).

If ethylene enhances senescence, it is conceivable that ethylene inhibitors will retard this process. AVG, which is known to inhibit ethylene biosynthesis (26), and Ag⁺, which opposes ethylene action (1, 26), were employed. While AVG inhibited significantly ethylene production of parsley leaflets during 9 days, the Ag⁺ effect was associated with a marked ethylene stimulation during the first 3 days, followed by a gradual decline (Fig. 3A). In spite of these remarkable effects on ethylene production rates of parsley leaflets, neither AVG (Fig. 3B) nor Ag⁺ (data not shown) could alter significantly their Chl content, which remained quite stable during 9 days after detachment (Fig. 3B). Similarly, AVG (Table I) and Ag⁺ (data not shown) did not affect the gradual increase in amino acids content observed in control leaflets.

Effect of Wounding on Leaf Senescence. The effect of excision-induced ethylene production on the rate of leaf senescence was examined by conducting a comparative study between detached intact leaves and excised leaf discs senescing in darkness. Considering the low sensitivity of parsley leaflets to ethylene, spinach leaves were employed for this purpose. Fig. 4A demonstrates that whole detached spinach leaves produced in response to detachment a very moderate ethylene rise, which peaked on the first day following detachment and preceded by one day a moderate respiratory peak. This rise of wound-ethylene production was followed by a climacteric-like ethylene rise,

which lasted for 5 days, and was followed by a second respiration rise peaking on the 8th day (Fig. 4A). Unlike the wound-ethylene rise, the climacteric ethylene rise was accompanied by a corresponding and remarkable accumulation of ACC, starting from the 5th day of incubation (Fig. 4A). A slight increase in amino acids content was observed in intact spinach leaves during the first 2 days after detachment (Fig. 4B), in parallel with their transient rise of wound-ethylene production (Fig. 4A). This moderate increase of amino acids was followed by a second remarkable rise, which initiated on the fifth day (Fig. 4B) and coincided with the climacteric-like rises of ACC, ethylene and respiration. Initiation of Chl loss was observed in parallel to the rise of climacteric-ethylene on the 6th day after detachment (Fig. 4B), well after wound-ethylene had subsided and at least one day after the rise of amino acids (Fig. 4B) had commenced.

The pattern of ethylene production in spinach leaf discs undergoing dark senescence exhibited at least two ethylene rises (Fig. 5A), which differed in magnitude and timing from the peaks observed in intact leaves. Thus, a very sharp burst of wound ethylene, which was 100-fold that of intact leaves, was detected in leaf discs already 2 h following excision (Fig. 5A, inset). Unlike in whole leaves (Fig. 4A), the second ethylene rise, presumably the climacteric-like one, peaked in leaf discs already on the 3rd day after detachment and was 20-fold higher (Fig. 5A). These ethylene rises in leaf discs were accompanied by a transient rise of amino acids as well as by a gradual decline in Chl content, which could be detected already on the first day after excision (Fig. 5B). It seems therefore that excision advanced both wound and climacteric ethylene maxima by 20 h and 6 days, respectively, increased their peaks magnitude by several-fold, and enhanced initiation of Chl loss and amino acids accumulation by 3-4 days.

Role of Endogenous Ethylene Rises in Leaf Senescence. In order to separate the effect of wounding on senescence from the effect of its concomitant burst of wound-ethylene, AVG was employed to eliminate ethylene production following excision. For this purpose spinach leaves were sprayed in the field with AVG prior to their detachment, the detached leaves were then floated for 1 h on an AVG solution prior to

discs excision, and the discs were incubated with AVG immediately after excision. All these treatments resulted in almost complete inhibition of wound-ethylene production and in some reduction of the climacteric-like rise (Fig. 6A). Fig. 6B demonstrates that the AVG-treated discs (wounded tissue without wound-ethylene) retained a significantly higher level of Chl during 5 days as compared with control discs (wounded tissue with wound-ethylene). Alternatively, application of AVG to control discs 4 h after excision - immediately after their burst of wound ethylene has subsided (Fig. 6A) - did not change their rate of Chl breakdown (Fig. 6B). These results suggest that the early burst of wound-ethylene enhances the rate of Chl loss in leaf discs.

In order to check whether the climacteric-ethylene rise, appearing on the 3rd day following excision, has any effect on senescence processes, ACC was applied to AVG-treated discs on the 2nd day of incubation. This treatment increased by five-fold the climacteric-ethylene rise (Fig. 6A). Consequently, a moderate but significant acceleration of both Chl breakdown (Fig. 6B) and amino acids accumulation (Fig. 6C) was observed. It seems therefore that the climacteric-ethylene rise, as well as the wound-ethylene burst, can enhance senescence of leaf discs.

DISCUSSION

Although many senescence phenomena in plants were shown to be ethylene mediated, the role of ethylene in leaf senescence is still controversial (14, 19, 22, 24). An attempt was therefore made to ascertain a role for endogenous ethylene in senescing detached leaves by studying the patterns of their ethylene production rates in relation to changes in various senescence parameters.

The present study demonstrates that intact leaves, leaflets or leaf discs of spinach and parsley exhibited during their dark senescence a basic pattern of ethylene emanation, consisting of two peaks which vary in magnitude and timing (Figs. 1A, 3A, 4A, 5A). The first ethylene rise occurred immediately after detachment or excision, and preceded the metabolic changes characteristic of senescence (Figs. 1, 3, 4, 5, 6). The second, climacteric-like ethylene rise was associated with

senescence and appeared in parallel to (Figs. 3, 4) or well after (Figs. 1, 2B, 5) Chl loss and amino acid accumulation had commenced. This ethylene pattern was found so far to characterize senescing detached dicot leaves and leaf discs incubated for 6-18 days (1, 2, 5, 6, 15, 17), as well as monocot leaves kept for 5-9 days (7, 18). When incubations were shorter, monocot leaves exhibited only the excision-ethylene response (12).

Leaf senescence was estimated by measuring changes in Chl and amino acids content, which are indicators of the two major biochemical changes consistently observed during senescence. In general, proteolysis, which is accompanied by an increase in free amino acids, precedes Chl degradation which results in yellowing (19, 21, 24). Indeed, in the systems of intact parsley and spinach leaves, which exhibit a slow senescence rate, the changes in amino acids content occurred before Chl loss became apparent (Figs. 3B, 4B, Table I). However, in fast-senescing systems such as parsley bunches (Figs. 1, 2B) and spinach leaf discs (Fig. 5B), the changes in the two parameters occurred in parallel.

The process of amino acids' accumulation seems to be more sensitive to ethylene than is Chl breakdown. This conclusion is based on the following observations: (a) Amino acids' accumulation in parsley bunches was promoted significantly by exogenous ethylene already on the 3rd day (Fig. 2B), thereby preceding the appearance of ethylene-enhanced Chl degradation (Fig. 1B) by one day; (b) AVG, which inhibited Chl breakdown in spinach leaf discs (Fig. 6B), did not cause any significant change in their rate of amino acids' accumulation (data not shown), while ACC enhanced this process immediately after its application (Fig. 6C). It seems therefore that AVG-treated leaves contain a residual level of endogenous ethylene which cannot affect Chl loss but can still enhance the process of amino acids' accumulation to the same extent as in control leaves. The promotive effect of ethylene on amino acids' accumulation can be pronounced only when ethylene rises above a threshold level (i.e., after treatment with exogenous ethylene or ACC). Hence, when examining the ethylene effect on senescence rate, the sensitivity to ethylene of the various metabolic processes involved should be considered.

Differences in responsiveness of the tissues to ethylene could be attributed also to variations in their sensitivity to the hormone, a factor which may govern too the ethylene effect in leaves (12, 25). The ability of exogenous ethylene to enhance processes characteristic of leaf senescence has been well documented (1, 7, 17, 25). In the present study, the senescence-associated processes in parsley leaves (slow-senescent system) were promoted by exogenous ethylene (Figs. 1B, 2) or by ACC (Fig. 3B, Table I) only 3 or 7 days after application, whereas in spinach leaf discs (fast-senescent system) the enhancement was obtained immediately after ACC was introduced (Fig. 6B, C). Additionally, inhibitors of ethylene biosynthesis and action, which retarded these senescence-associated metabolic changes in spinach leaf discs (Fig. 6B), had no effect in parsley leaves (Fig. 3B, Table I). Regarding these results, it may be concluded that the relatively slow senescence rate of parsley leaves may be due to their low sensitivity to ethylene, in spite of their relatively high endogenous ethylene production. This conclusion was reinforced by the fact that parsley plants, which were sprayed daily with ACC for 12 days, did not show any significant Chl breakdown in spite of their remarkable ACC-induced ethylene production rates (data not shown). However, it should be noted that the senescence rate of parsley leaves was dependent on the experimental system employed. Thus, parsley bunches kept in water jars (Figs. 1B, 2) senescent at a faster rate than intact parsley leaflets kept on moistened filter paper (Fig. 3B, Table I). This could probably be attributed to loss of nutrients from the whole bunches to the water medium or from older leaves to younger ones within each bunch.

When referring to foliar senescence, one should differentiate between wound-ethylene and climacteric-like ethylene production, which may sometimes be hard to distinguish. Unlike intact spinach leaves (Fig. 4A) and parsley bunches (Fig. 1A), parsley leaflets showed a pattern consisting of one prolonged ethylene rise, which appeared immediately after detachment and lasted for approximately 6 days (Fig. 3A). Since no change in Chl level could be observed during that period (Fig. 3B), this onset of ethylene production was thought to be an excision-related response. The initiation of the late-appearing, climacteric-like

ethylene rise (2) could be detected in this slow-senescing system only on the 9th day of incubation (Fig. 3A). It seems, therefore, that the degree of wounding is expressed in both magnitude and duration of wound-ethylene: While intact leaves - the wounding effect of which is minimal - showed after detachment a moderate ethylene rise lasting for 2-6 days (Figs. 1A, 3A, 4A), leaf discs were characterized by an immediate and sharp burst of ethylene which subsided after a few hours (Figs. 5A, 6A). Similarly, wounding alters the magnitude of the climacteric-ethylene rise as well (Figs. 4A, 5A).

The specific effect of wounding on senescence seems to proceed even without the concomitant burst of wound-ethylene. This conclusion was based on the data showing that the rate of Chl degradation in AVG-treated leaf discs (Fig. 6B) was still much higher than that of untreated intact leaves (Fig. 4B). It seems, therefore, that neither the burst of wound-ethylene production nor the climacteric-like ethylene rise can be regarded as the primary event in the induction of the senescence syndrome. Fig. 6 suggests that both rises play a regulatory role in promoting senescence processes. The induction of leaf senescence may rather be attributed to the wounding effect, which might be followed by: (a) increased ethylene production (14, 21, 22); (b) increased tissue sensitivity to ethylene (12); and/or (c) triggering of other metabolic processes (23, 24). Indeed, recent data obtained with oat leaf segments senescing in darkness, indicate that their endogenous ethylene production can hardly be the main controlling factor of the wounding effect (8). Nevertheless, results depicted in Fig. 6B suggest that the wound-ethylene burst can contribute to the wounding effect a further promotion of senescence. Thus, the rapid senescence of leaf discs is caused by both the wounding effect and the remarkable burst of wound-ethylene appearing 2 h following excision.

In conclusion, the present study supports the notion that ethylene is involved in the regulation of leaf senescence, although it does not play a role in initiation of the process. The results further imply that the ethylene-regulated leaf senescence is governed by (a) ethylene production rates in the tissue; (b) tissue sensitivity to ethylene; (c) sensitivity to ethylene of the various senescence-associated processes; and (d) degree of wounding.

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Table I: Effect of ACC and AVG on the level of amino acids in parsley leaflets during dark senescence. Incubation conditions were as described in Fig. 3. Amino acids content, assayed with samples of ten parsley leaflets, was 7.00 ± 1.4 $\mu\text{mole/g}$ FW at day 0. Means not sharing the same letter are significantly ($P < 0.05$) different from each other according to Duncan's Multiple Range Test.

TREATMENTS	AMINO ACIDS CONTENT				
	DAY 1	DAY 3	DAY 5	DAY 7	DAY 9
	$\mu\text{mole AA/g FW}$				
CONTROL	12.26 a	24.23 a	31.21 a	44.50 b	56.50 b
AVG	9.00 a	23.47 a	30.62 a	44.93 b	54.56 b
ACC	10.07 a	23.88 a	34.34 a	54.56 a	62.23 a

FIGURE 1. Time course of changes in endogenous ethylene and ACC (A) and the effect of exogenous ethylene on Chl degradation (B) in senescing parsley bunches. Samples of parsley bunches were kept in darkness under air or 10 μL/L ethylene in a flow-through system. Ethylene production rates were monitored daily by enclosing 0.7-g leaf samples in 50-ml flasks for 2 h. These samples were subsequently extracted with ethanol for Chl and ACC determinations. The bars represent SD of three replicates.

FIGURE 2. Effect of exogenous ethylene (10 $\mu\text{l/L}$) on changes in protein (A) and amino acids (B) levels during dark senescence of parsley bunches. Details are as described in Fig. 1.

FIGURE 3. Effect of ACC, AVG and Ag^+ on ethylene production rates (A) and Chl loss (B) of parsley leaflets during dark senescence. Samples of ten parsley leaflets were senesced in petri dishes in the presence of 0.1 mM ACC, 0.1 mM AVG or 60 μ M $AgNO_3$ as described in Materials and Methods. Ethylene production was monitored daily by enclosing the leaflets samples in 50-ml flasks for 2 h. Chl was determined subsequently by extracting the same leaflets.

FIGURE 4. Time course of changes in ethylene production, ACC level and respiration rates (A) and in contents of Chl and amino acids (B) during dark senescence of intact spinach leaves. Ethylene and respiration rates of four intact spinach leaves kept in 0.5-L jars, as well as Chl and amino acids levels in 0.5-g samples of these leaves, were monitored daily.

FIGURE 5. Time course of changes in ethylene production (A), and in amino acids and Chl levels (B) during dark senescence of spinach leaf discs. All parameters were assayed daily on samples of eight leaf discs. Ethylene production rates of leaf discs and intact leaves during the first 24 h following detachment are shown in the inset.

FIGURE 6. Effect of modulations in endogenous ethylene production (A) on Chl (B) and amino acids (C) levels of senescing spinach leaf discs. AVG-treated discs were excised from leaves sprayed with 0.1 mM AVG prior to their detachment. Additionally, 0.1 mM AVG was added to control discs 4 h following excision and 0.05 mM ACC was added to AVG-treated discs on the second day after excision. Chl and amino acids were assayed daily as described in Fig. 5.

3.3 ROLE OF ETHYLENE IN SENESCENCE OF WATERCRESS LEAVES

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ABSTRACT

The role of ethylene in senescence of watercress, which is one of the most yellowing-susceptible leaves, was investigated by employing inhibitors of ethylene action (CO_2 , Ag^+) and biosynthesis (AVG). Application of 11% CO_2 to watercress bunches in a flow-through system delayed effectively during 4 days all the senescence processes tested, namely chlorophyll degradation, protein hydrolysis and lipid oxidation. On the other hand, application of 0.1 mM AVG or 30 μM Ag^+ to watercress leaves caused retardation of chlorophyll loss during 3 days following detachment, but did not have any effect on protein hydrolysis. Only a combination of AVG + Ag^+ resulted in effective retardation of this senescence process during 2 days after detachment. Similarly, application of exogenous ethylene (10 $\mu\text{L/L}$) to watercress leaves enhanced their chlorophyll degradation without affecting their protein hydrolysis, which was quite rapid even without exogenous ethylene. This suggests that the process of protein hydrolysis is more sensitive to low concentrations of ethylene as compared with chlorophyll degradation, since only the additional Ag^+ -inhibition of the residual ethylene action in AVG-treated leaves was effective in retarding protein hydrolysis. It seems therefore, that the anti-ethylene agents AVG and Ag^+ , varied in their effects on these two senescence processes, whereas CO_2 retarded with similar efficiency all the processes tested. This long-lasting and highly efficient effect of CO_2 on these leaves, which was the most effective retardant among other ethylene inhibitors, may implicate that: (a) the ethylene-receptors in watercress leaves show relatively high affinity to CO_2 , and/or (b) the senescence-retarding activity of CO_2 cannot be solely attributed to its action as an anti-ethylene agent.

Abbreviations: CHL, chlorophyll; AVG, aminoethoxyvinylglycin; MDA, malondialdehyde; TBA, thiobarbituric acid; TCA, three chloro acetic acid.

INTRODUCTION

It has been shown that various detached dicot leaves exhibit under experimental conditions a rapid ethylene burst following excision and a subsequent climacteric-like rise of ethylene production at advanced stages of senescence (1, 8, 9). While the initial ethylene rise is considered as an excision-related response, the second climacteric rise was associated with senescence (1, 8, 2, 15). The role of ethylene in the rapid senescence of watercress leaves was investigated by employing various inhibitors of ethylene synthesis (AVG) and action (CO_2 , Ag^+). The progress of senescence in the detached leaves was estimated by measuring the senescence-associated processes of Chl loss, proteolysis and lipid oxidation.

MATERIALS AND METHODS

Plant Materials and Treatments. Experiments were performed with fully expanded leaves of watercress (Nasturtium officinale R. Br.), which were freshly harvested from the field. Watercress bunches were stored in water-containing flasks and enclosed into 2-L jars fitted with inlet and outlet ports. The jars were sealed and connected to gas cylinders containing either air or 11% CO_2 . A flow-through system was used with a flow rate maintained at 50 mL/min. The jars were incubated for 5 days in the dark at 25°C and changes in ethylene production, Chl, amino acids, protein and total aldehydes content of detached sample leaves were determined daily or in hour intervals.

To test the effect of AVG and Ag^+ on senescence, samples of 6 watercress leaves were floated in petri dishes containing 4.5 mL of either H_2O or 0.1 mM AVG + 0.5% (v/v) Tween-20. Pretreatment of the leaves with silver was done by floating the leaves on 4.5 mL of 30 mM AgNO_3 for 25 min, rinsing them with distilled water and subsequently transferring them to petri dishes containing either water or AVG

solution (0.1 mM). Application of exogenous ethylene was done by enclosing open petri dishes containing leaves floating on water into a sealed 10-L jar, to which 10 $\mu\text{L/L}$ of C_2H_4 was injected. Where indicated, AVG (0.1 mM) was added to control leaves 6 h after excision and C_2H_4 (10 $\mu\text{L/L}$) was added to AVG-treated leaves 20 h after excision. All the petri dishes and the ethylene-containing jar were incubated in the dark at 25°C for 3 days, and changes in their endogenous ethylene production, Chl and amino acids levels were assayed daily.

Determination of Ethylene. Ethylene production rates were monitored daily by enclosing samples of 4 treated leaves (0.5 g) in 50-mL flasks for 1 h at 25°C. Ethylene concentration in the flasks was analyzed by withdrawing a 2-mL gas sample and injecting it into a gas chromatograph equipped with an alumina column and a flame ionization detector.

Extraction and Determination of Chl and Amino Acids. Chlorophyll and amino acids were extracted by boiling for 30 min samples of cut leaf segments (0.5 g) enclosed in test tubes containing 10 mL of 80% (v/v) ethanol (5). When necessary an additional 15-min extraction of the leaf segments in 3 mL ethanol was performed, and the ethanolic extracts were combined. Chl was quantitated and expressed as mg Chl/g fresh weight (FW) by transferring 200- μL aliquots of the ethanolic extract into 5 mL of 80% (v/v) acetone and determining the absorbance at 645 and 663 nm according to Arnon's method (3).

The ethanolic extract was then evaporated under vacuum at 55°C, the residues were dissolved in 2 mL of H_2O , and pigments were removed by addition of 0.5 mL chloroform (8). Amino acids content was determined in the aqueous solution by the ninhydrin method (11), which was adopted to micro quantities suitable for ELISA plates. The reaction was performed with flat-bottom, 96-well, microtiter plates (Immulon 1, Dynatech) in a total volume of 270 $\mu\text{L/well}$, consisting of 10- μL samples aliquots plus the original reaction mixture reduced by ten-fold. A standard methionine curve was included in every plate, and each point was repeated three or four times. After incubating the plates at 95°C for 20 min, the absorbance of the colored reaction product at 560 nm was determined with

an automated ELISA Reader (model EL310, BIO TEK). This modified procedure enabled a rapid and accurate quantitation of amino acids in a large number of samples.

Extraction and Determination of Protein. Protein was solubilized from the extracted depigmented leaf segments with 1 N NaOH as described before (6) and estimated by the method of Bradford (4), which was performed with micro quantities on ELISA plates. All treatments and assays were tested in triplicates and the data presented are expressed as means $\pm$ SD.

Extraction and Determination of TBA reactive compounds. Saturated aldehydes were extracted from 0.5-g tissue by homogenization in 5 mL solution containing 20% TCA + 1.5 mM EDTA. After a 10-min centrifugation at 10,000 rpm, saturated aldehyde were assayed on the supernatant according to the TBA test described by Kosugi and Kikugawa (7), employing slight modifications. Briefly, 1 mL of 0.67% TBA was added to 3-mL aliquots of the supernatant and the solution was incubated at 100°C for 1 h. The solution was then cooled and centrifuged for 10 min at 8000 rpm. The volume of the resultant supernatant was completed to 10 mL with distilled water and the absorbance of the colored reaction product was determined at 455 nm (for total aldehydes) and at 532 nm (for MDA).

All treatments were tested in triplicate and the data presented are expressed as the mean with its standard deviation.

RESULTS AND DISCUSSION

Kinetics of Senescence Processes in Watercress Leaves. Watercress leaves were detached every few hours from bunches kept in jars under a flow-through system of air. Their pattern of ethylene production rates during 24 h after detachment exhibited a sharp burst of wound-ethylene, which initiated rising from the first h following excision, reached its peak 5 h later and gradually subsided (Fig. 1A). Chl content during that period varied significantly, showing a 20% loss between 5-10 h following detachment and then leveling off (Fig. 1A). The senescence-associated process of protein hydrolysis was also evident during the initial 10-h

period, showing a 40% decline in protein content which was accompanied by an increase in the corresponding accumulation of amino acids (Fig. 1B). These results suggest that the senescence processes in watercress leaves are quite rapid and occur within few hours following detachment.

Retardation of Senescence by CO₂. The rate of ethylene production in watercress leaves, which were detached daily from the above described bunches, was determined. The sharp burst of wound-ethylene, peaking 6 h following detachment, declined immediately to a rate of about 3-4 nL/g/h, which remained in this basal level during the next 5 days of incubation (Fig. 2). This remarkable upsurge of wound-ethylene production was accompanied by a rapid Chl degradation in these leaves, which lost 20% of their Chl content during 10 h following detachment (Fig. 1A) and up to 60% within 2 days (Fig. 2, inset). Application of CO₂, which is known as an inhibitor of ethylene action (10), retarded considerably the rapid loss of Chl in these leaves and resulted in retention of 50% of Chl content after 4 days of incubation (Fig. 2, inset). Chl degradation in watercress leaves was accompanied by some other senescence-associated processes such as protein hydrolysis, exhibited by loss of protein content (Fig. 3A) and by the corresponding accumulation of amino acids (Fig. 3B) and lipid oxidation, exhibited by increase in the levels of MDA (Fig. 4A) and total aldehydes (Fig. 4B). Similar to retardation of Chl loss, CO₂ was effective as well in slowing down these two senescence processes. Consequently, after 5 days, CO₂-treated leaves retained about 66% of their protein content (Fig. 3A), and their contents of amino acids (Fig. 3B), MDA (Fig. 4A) and total aldehydes (Fig. 4B) were reduced, respectively, by 58%, 12% and 50% as compared with control leaves.

Since wound-ethylene production is quite high during the initial hours following detachment (Figs. 1, 2), the rapid senescence-associated changes could be greatly affected by this upsurge of wound-ethylene. Therefore, the effectiveness of CO₂ in retarding senescence during the first day after detachment could be attributed to its action as an anti-ethylene factor. However, the long-lasting and highly efficient senescence-retarding effect of CO₂, which was evident also in the subsequent days when ethylene production was relatively low, may

implicate that CO_2 can act in an additional mechanism besides its anti-ethylene action. Alternatively, the possibility that the ethylene-receptors (10) in watercress leaves have a relatively high affinity for CO_2 , cannot be ruled out.

Retardation of Senescence by AVG and Ag^+ . Since watercress leaves underwent a rapid senescence following detachment, presumably due to their high rates of wound-ethylene production, it was of interest to further investigate the effects of ethylene and wounding on the senescence processes occurring in this tissue. For this purpose the ethylene inhibitors AVG and Ag^+ were applied, either individually or simultaneously, at different times after detachment. Incubation was performed by floating the leaves directly on the different solutions for improving uptake. Results depicted in Fig. 5 represent the typical pattern of ethylene production rates under these conditions. The burst of wound-ethylene appeared in control leaves 6 h following detachment (Fig. 5), as in leaves kept under air flow (Figs. 1, 2), but it subsided in a much slower rate during the subsequent days of incubation. A significant but incomplete inhibition of the wound-ethylene burst was obtained in AVG-treated leaves as well as in leaves treated with AVG + Ag^+ (Fig. 5, inset). The inhibition of ethylene production by these treatments was retained during the subsequent 2 days and was relieved on the third day of incubation (Fig. 5). On the other hand, Ag^+ -treated leaves showed a remarkable enhancement of their ethylene production during 2 days of incubation (Fig. 5). It is pertinent to note that the concentration of 30 μM Ag^+ was chosen in this experiment since it was the concentration which gave maximal anti-ethylene activity without causing the leaves any visual damage (data not shown). The results suggest that complete inhibition of ethylene production could not be achieved in watercress leaves treated with AVG, although the inhibition was pronounced in the course of incubation. This might suggest the existence of another pathway leading to ethylene biosynthesis besides ACC synthesis in this system. MACC hydrolysis may represent a possible alternative (see Section 4.2).

The effect of these ethylene inhibitors on senescence processes was investigated. Table I shows that both AVG and Ag^+ , applied either

individually or simultaneously, delayed Chl degradation with similar efficiency during 3 days of incubation. Simultaneous application of these 2 inhibitors 6 h after leaf detachment, when the first burst of wound-ethylene has subsided, resulted in similar retardation of Chl degradation as in leaves initially treated with AVG + Ag⁺. This apparent uneffectiveness of ethylene inhibitors applied either before or after the rise of wound-ethylene, may be attributed to the Ag⁺-enhanced ethylene production observed in the treated tissues. Application of exogenous ethylene to watercress leaves immediately after detachment resulted in significant enhancement of their Chl degradation only on the second day of incubation (Fig. 6). On the other hand, application of exogenous ethylene to AVG-treated leaves 20 h following detachment resulted in an immediate enhancement of Chl loss. Exogenous ethylene was not effective during the first day after detachment probably because of the high saturating levels of endogenous ethylene present in the tissue during that period (Fig. 5). However, on the 2nd day after detachment, when the level of endogenous ethylene was lower and also the tissue became more sensitive to ethylene, the enhancing effect of exogenous ethylene on Chl loss was pronounced. The results suggest that ethylene can enhance Chl degradation at different senescence stages, depending on the saturating ethylene level in the tissue at the time of application.

Unlike Chl degradation, the process of protein hydrolysis, as exhibited by accumulation of amino acids in watercress leaves, was delayed by either AVG or Ag⁺ during the first 6 h following detachment, but no inhibitory effect could be observed in the subsequent days (Table II). No significant promotive effect could be observed also in the presence of exogenous ethylene (data not shown). Only a combination of AVG + Ag⁺, regardless of the time of their application, resulted in significant retardation of this senescence process during 2 days after detachment (Table II). These results imply that only the additional Ag⁺-inhibition of the residual ethylene action in AVG-treated leaves was effective in retarding the increase of amino acids. Considering the fact that complete inhibition of ethylene biosynthesis could not be achieved in these AVG-treated leaves (Fig. 5), it may be concluded that the process of protein hydrolysis is more sensitive to

low concentrations of endogenous ethylene than Chl degradation. This conclusion was reinforced by the fact that exogenous ethylene, which enhanced Chl degradation at certain senescence stages (Fig. 6) did not affect protein hydrolysis, which was quite rapid even in control leaves (Table II). A similar variation in sensitivity to ethylene of these senescence processes was also observed in parsley and spinach leaves, which senesced in a much slower rate (8).

Hence, it seems that unlike CO_2 , which retarded all the senescence processes tested with similar efficiency (Figs. 2-4), AVG and Ag^+ affected the process of Chl loss much more than the process of protein hydrolysis. Since these 2 processes were shown to vary in their sensitivity to ethylene (Tables I, II), the degree of their retardation, obtained by AVG and Ag^+ , is directly related to the efficiency of these 2 inhibitors as anti-ethylene agents (9). Thus, unlike Ag^+ , the remarkable effect of CO_2 in delaying Chl loss in watercress leaves, as compared with its effect in other tissues (1, 2, 5), may result from its inability to induce ethylene production in this tissue (data not shown). Alternatively, CO_2 may act in a different mechanism, which is not necessarily related to its anti-ethylene activity (2).

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Table I: Effect of AVG and Ag⁺ on Chl acids level in watercress leaves during dark senescence.

Incubation conditions were as described in Fig. 5. Chl content, assayed with samples of 4 leaves, was 1.28 ± 0.1 mg/g FW at day 0. Means not sharing the same letter are significantly ($P < 0.05$) different from each other according to the Duncan's Multiple Range Test.

TREATMENTS	CHLOROPHYLL CONTENT			
	6 h	day 1	day 2	day 3
	mg CHL/g FW			
CONTROL	0.93 c	0.69 ab	0.40 c	0.14 bc
AVG	1.29 a	0.90 a	0.45 bc	0.30 a
Ag ⁺	1.04 bc	0.90 a	0.46 bc	0.26 ab
AVG+Ag ⁺	1.20 ab	0.87 a	0.55 ab	0.25 ab
AVG+Ag ⁺ (6h)	-	0.91 a	0.63 a	0.33 a

Table II: Effect of AVG and Ag⁺ on amino acids level in watercress leaves during dark senescence.

Incubation conditions were as described in Fig. 5. Amino acids content, assayed with samples of 4 leaves, was 2.88 ± 0.3 $\mu\text{mole/g}$ FW at day 0. Means not sharing the same letter are significantly ($P < 0.05$) different from each other according to the Duncan's Multiple Range Test.

TREATMENTS	AMINO ACIDS CONTENT		
	6 h	day 1	day 2
	$\mu\text{mole AA/g FW}$		
CONTROL	9.45 a	18.67 bcd	57.40 a
AVG	6.20 b	21.32 b	52.67 ab
Ag ⁺	5.05 c	19.70 bc	50.45 ab
AVG+Ag ⁺	5.48 bc	14.40 d	41.95 cd
AVG+Ag ⁺ (6h)	-	16.43 bcd	35.05 d

FIGURE 1. Time course of changes in ethylene production rates, chlorophyll content (A) and levels of protein and amino acids (B) of senescing detached watercress leaves during 24 hours. Leaves were detached in hours intervals from watercress bunches kept in darkness under an air flow-through system, and enclosed in 50-mL flasks for ethylene determination. The detached leaves were subsequently assayed for Chl, amino acids and protein content. The bars represent SD of 3 replicates.

FIGURE 2. Time course of changes in ethylene production rates and effect of CO₂ on Chl content of detached watercress leaves senescing during 5 days. Watercress bunches were kept in darkness under a flow-through system of either air or 11% CO₂. Ethylene production rates were monitored daily by enclosing samples of detached leaves (0.5 g) in 50-mL flasks for 1 h. These samples were subsequently extracted with ethanol for Chl determination. Chl contents of control and CO₂-treated leaves during 5 days are shown in the inset.

FIGURE 3. Effect of CO₂ on contents of protein (A) and amino acids (B) in watercress leaves during dark senescence. Incubation conditions were as detailed in Fig. 2. Protein and amino acids were assayed daily on the same samples extracted for Chl.

FIGURE 4. Effect of CO₂ on levels of malondialdehyde (MDA) (A) and total aldehydes in watercress leaves during dark senescence. Incubation conditions were as detailed in Fig. 2. MDA and total aldehydes were assayed daily on 0.5-g samples of detached leaves.

FIGURE 5. Effect of AVG and Ag^+ on ethylene production rates in watercress leaves senescing in darkness. Samples of 6 leaves were detached from the field and incubated in darkness at 25°C by floating in closed petri dishes containing either water or AVG (0.1 mM) solution. Pretreatment with Ag^+ was done by floating the leaves for 25 min on 30 μM AgNO_3 solution. Ethylene was monitored at the indicated intervals by enclosing the treated leaves into 50-mL flasks for 1 h. An enlarged scale of ethylene production rates in leaves treated with either AVG or AVG + Ag^+ is shown in the inset.

Figure 6. Effect of exogenous ethylene on Chl content of watercress leaves senescing in darkness. Incubation conditions were as detailed in Fig. 5. Control or AVG-treated leaves were enclosed in a 10-L jar, to which exogenous ethylene (10 μ L/L) was injected. Where indicated, ethylene was introduced to AVG-treated leaves 1 d after detachment. At the indicated intervals, the differently treated leaves were extracted with ethanol for Chl determination.

3.4 THE ROLE OF ETHYLENE IN THE SENESCENCE OF DETACHED BROCCOLI FLORETS

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ABSTRACT

Changes in the rate of endogenous ethylene production, levels of 1-aminocyclopropane-1-carboxylic acid (ACC), its malonyl conjugate (MACC), protein, and the accumulation of amino acids in detached broccoli florets were followed with respect to the senescence as measured by the loss of chlorophyll. A climacteric-like rise in ethylene and respiration, occurred on day 3 coinciding with the onset of chlorophyll degradation. Exposure to exogenous ethylene, at 0.1 ppm and 1.0 ppm, accelerated the onset of senescence, as indicated by chlorophyll loss and amino acid accumulation. Senescence was also hastened by foliar applications of 1.0 mM and 5.0 mM ACC.

Treatments with ethylene biosynthesis inhibitors including the hydroxylamine analog, aminooxyacetic acid (AOA), cobalt and nickel ions, significantly reduced ethylene production and delayed chlorophyll loss. Similarly, continuous exposure to 5% or 15% carbon dioxide, known to block ethylene action, also inhibited ethylene evolution and chlorophyll loss. In contrast, continuous exposure to 1000 μ l/L or 2000 μ l/L norbornadiene (NBD) or foliar applications to 0.5 mM and 1.0 mM silver thiosulfate (STS) increased ethylene production but maintained chlorophyll content. Furthermore, treatment with the protein synthesis inhibitor, cycloheximide, or the metal chelator, α α -dipyridyl, inhibited both chlorophyll degradation and ethylene production at 1.0 mM.

The relative effectiveness of various 0.1 mM hormonal pretreatments in stimulating ethylene production at 24 h was $GA_3 > ABA > IAA > thidiazuron (THD) > BA > control$. Of these BA, THD and GA_3 were effective in delaying chlorophyll loss during senescence. These findings suggest that the sensitivity of the florets to endogenous ethylene produced during senescence affects the subsequent rate of chlorophyll loss.

Senescence is the final stage of development characterized by a series of essentially catabolic processes, including chlorophyll, protein and nucleic acid degradation, which ultimately results in death. Consequently, senescence, like any other developmental phase, is under hormonal regulation. Recent findings by Aharoni and Lieberman (3) on tobacco leaf disks, Gepstein and Thimann (11) on detached oat leaves, and Kao and Yang (14) on excised rice leaves suggest a possible regulatory role for endogenous ethylene in the regulation of senescence.

Evidence in support for a regulatory role for ethylene include the inhibition of senescence by the ethylene biosynthesis inhibitors: cobalt (11, 14) and nickel (14) ions, and the rhizobitoxine analogues, AVG¹ (3, 11, 27) and AOA (14, 28). In detached light-incubated oat (11) and rice (14) leaves, serial concentrations of 1 μ M to 100 μ M Co²⁺ ion decreased ethylene production and inhibited chlorophyll loss. Inhibitors of ethylene action, namely elevated CO₂ concentrations at 1% (11), 5%, 10% and 15% (2), silver ion in the form of AgNO₃ (3) and STS (26, 28), and NBD (23) also delay symptoms of senescence, such as chlorophyll loss, petal in-rolling, and respiration increases.

Early studies by Lieberman and Hardenburg (15) on modified atmospheres suggested that yellowing of broccoli was caused by endogenous ethylene production. Lieberman and Spurr (16) verified the presence of endogenous ethylene during senescence using the 'Triple Response' of pea bioassay. Exposure to exogenous ethylene hastens chlorophyll breakdown in tobacco leaf disks (3). Similarly, applications of exogenous ACC, the immediate ethylene precursor, promoted ethylene production and accelerated senescence of detached oats (11) and rice (14) leaves.

From time course studies, ethylene production has been reported to rise prior to the senescence loss of chlorophyll (19). Gepstein and Thimann (11) found a strong parallel between maximal ethylene evolution and rapid chlorophyll loss in detached oat leaves in light. In contrast, Aharoni et al (4) reported that the climacteric-like rise in ethylene production occurred after the

onset of chlorophyll degradation in leaf blade disks of pinto bean, tobacco and sugar beet.

In the present study, the time course of ethylene production and changes in ACC and MACC levels during the senescence of detached, dark-incubated broccoli florets was investigated to determine the role of ethylene in senescence. Specific objectives of this investigation were to determine the responses to: 1) exogenous ACC and ethylene; 2) decreased ethylene action by Ag^+ , CO_2 , and NBD; 3) reduced ethylene biosynthesis by AOA, Co^{2+} and Ni^{2+} ; 4) the protein synthesis inhibitor, cycloheximide, and the metal chelator, $\alpha\alpha$ -dipyridyl; 5) hormonal pretreatments of ABA, BA, GA_3 , IAA, THD with respect to ethylene production and senescence.

MATERIALS AND METHODS

Plant Materials

Broccoli (*Brassica oleracea* type Italica cv. 'Shogun') grown in Salinas, California, were purchased from a local wholesale distributor. Harvested broccoli heads were immediately stored at 0°C , for a period not exceeding 10 days, in crushed ice. Heads were removed from storage when needed, surface-sterilized by submerging in a 0.5% (v/v) solution of commercial NaOCl for 2 min, and then rinsed several times with distilled water. The florets were removed from the head and allowed to equilibrate for 3 h at 15°C prior to use.

Three sets of eight florets were used for temporal studies of respiration and ethylene production, chlorophyll, ACC, MACC and amino acids content, and protein levels. Each floret, approximately 10 g in weight and 3 cm in diameter, consisted primarily of unopened flower buds and flower stalks. The florets were floated in open petri dishes containing 3 ml of distilled water for 2 h, then sprayed to runoff or dipped in the appropriate treatment solutions containing 1.0 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0 and 0.15% Tween-20, a surfactant, for 30 min, and blotted dry. For hormone

pretreatments, solutions also contained 0.1 mM of the hormone. Thidiazuron stock solution of 1.0 mM was previously dissolved in 2 ml DMSO. Samples were then enclosed in 50-ml Erlenmeyer flasks lined with moistened Whatman filter paper. In the experiments requiring exposure to ethylene, CO₂, norbornadiene, the 50-ml flasks were transferred to 19-L respiration jars with rubber serum cap ports. The respiration jars also contained beakers holding 60-ml of 20% (w/v) KOH to absorb respiratory CO₂. Known concentrations of ethylene, CO₂, and the gas equivalent concentration of NBD were introduced through the serum cap ports. NBD was injected unto a filter paper wick and allowed to vaporize. For ethylene-free atmospheres, a 50-g sachet of Purafil with activated KMnO₄, was placed inside the jar. Ethylene concentrations were checked with a gas chromatograph. The CO₂-free control was obtained by placing a center well containing a filter paper wick moistened with 2-ml 20% KOH. The final concentrations of CO₂ was verified with a Horiba infrared gas analyzer. The open flasks were placed in darkness at a constant temperature of 15°C.

Determination of CO₂ and Ethylene Production

At the indicated times, two 1-ml gas samples were withdrawn from the headspace of sealed 50-ml Erlenmeyer flasks with a hypodermic syringe. The rate of respiration, as CO₂ evolved, was determined with a Horiba infrared CO₂ gas analyzer. Ethylene production was assayed by a gas chromatograph equipped with an alumina column and a flame ionization detector. Both CO₂ and ethylene were allowed to accumulate for 2 h at 15°C prior to sampling, unless otherwise noted. After sampling, flasks were flushed and remained open until the next determination. Rate of CO₂ and ethylene production for zero time was determined by enclosing florets for 30 min after excision.

Determination of Chlorophyll

One gram samples of broccoli florets were homogenized and extracted with a Tekmar^R Ultra-Turrax tissumizer in 5 ml 80% (v/v) acetone containing 10 mM CaCO₃ at 0°C. After centrifugation, the pellet was reextracted as before with cold acetone and the two supernatants were combined. Chlorophyll level was determined spectrophotometrically at 645 and 663 nm and expressed in µg/ml according to the method of Arnon (6).

Determination of ACC and MACC

The acetone extract used for chlorophyll extraction was evaporated *in vacuo* at 50°C, the residue was resuspended in a 0.5-ml chloroform and 2-ml distilled water. The ACC content in the aqueous extract was determined by chemical conversion to ethylene according to the method of Lizada and Yang (17). For quantification of the ACC conjugate the aqueous extract was hydrolyzed in 2N HCl at 100°C for 3 h, followed by neutralization with NaOH, and the resulting solution was assayed for ACC content. The difference in ACC content before and after hydrolysis was taken to as the amount of MACC (13).

Determination of Amino Acids

A 0.01-ml aliquot, from the clear, aqueous supernatant of the chloroform:water solution, was made to 1 ml with distilled water and its amino acid content was determined spectrophotometrically at 570 nm according to the procedure of Yemm and Cocking (31).

Determination of Protein

Two gram samples of florets were homogenized in 10 ml solution of 0.1 M Tris, pH 6, and 0.2 M NaCl at 0°C. The homogenate was filtered through two layers of Miracloth and centrifuged at 10,000 g for 12 min. Five ml of Bradford reagent (9) were added to a 0.01-ml aliquot and the absorbance at 595

nm was determined after 5 min.

Three replicates were used for CO₂ and ethylene studies. Chlorophyll determinations are an average of two to three replicates. Experiments were repeated at least three times, and similar results were obtained. The data reported herein were from three representative experiments.

RESULTS

Changes in Ethylene Production, Respiration, ACC, MACC, Chlorophyll, Amino Acid and Protein Contents During Senescence.

The senescence of detached broccoli florets held in darkness was characterized by the loss of chlorophyll and protein concomitant with an increase in free amino acids. Preliminary experiments showed florets detached from an intact head senesced in a manner and time sequence similar to heads held under similar conditions. However, respiration and ethylene production rates of detached florets are slightly higher than whole heads [data not shown] in agreement with other findings (5). Figure 1 presents the time courses of changes in ethylene production, respiration rate, ACC and MACC contents with respect to senescence, as measured by chlorophyll, protein and amino acid levels. Ethylene production steadily rose during senescence, reaching an eight-fold increase over the initial level at 51 h, and subsequently declined [Figure 1a]. The rise in ethylene coincided with the increase in respiration, suggesting a climacteric-like response in detached florets during senescence. A climacteric-like rise during senescence has been reported for several other vegetative tissues including leaf segments of tomato (19), leaf disks of sugar beet, tobacco and pinto beans (4).

An increase in ACC levels occurred concomitantly with ethylene production with a maximum peak of 1.77 nmol/g occurring at 51 h before declining to the basal levels [Figure 1b]. MACC content remained constant during the initial 24 h and then increasing more than three-folds over the

next 4 d. Chlorophyll levels decreased throughout senescence in a linear fashion although the first visible symptoms of 'yellowing' occurred on day 2, at which time ethylene production is at a maximum [refer to Figure 1c]. From Figure 1d, protein levels declined sharply throughout senescence; approximately 57% of the total protein content was lost after 5 d in darkness. Concomitant with proteolysis was the 5-fold rise in free amino acids at the end of the time course. The accumulation of amino acids may play some role in senescence since serine, cysteine and glycine have been suggested to promote protease activity (22).

Effect of Exogenous Ethylene and ACC on Chlorophyll Content

Exposing florets continuously to 1.0 ppm and 10 ppm ethylene markedly accelerated senescence as measured by chlorophyll loss, with greater loss occurring at the higher concentration. Figure 2 shows that the pattern of chlorophyll loss under ethylene was similar to ethylene-free control except the rate was hastened. When the detached florets were sprayed with 1.0 and 5.0 mM ACC, the immediate ethylene precursor, the senescence rate as determined by chlorophyll loss, was accelerated. Ethylene production was stimulated 4-fold and 16-fold respectively after 24 h over the control for the same time period [refer to Figure 3]. Ethylene production in ACC-treated florets continued to increase for the 24 h following treatment, before declining.

Effect of Ethylene Biosynthesis Inhibitors on Chlorophyll and Amino Acid Content

In order to determine whether ethylene serves as the trigger for senescence (11) or acts as a regulator in senescence (28), detached florets were sprayed with serial concentrations of AOA, which inhibits ACC synthesis from SAM (14). In addition, two other ethylene production inhibitors which block the conversion of ACC to ethylene, Co^{2+} and Ni^{2+} , were also applied at serial concentrations as foliar sprays. All three inhibitors decreased ethylene production and chlorophyll loss [Figure 4]. However, AOA was more effective in reducing ethylene production at each concentration; at 100 mM foliar spray

AOA inhibited 78% of ethylene production at 36 h as compared to 38% and 70% for Co^{2+} and Ni^{2+} at the same concentrations, respectively. Since inhibition of chlorophyll degradation and ethylene production is linearly related to the concentrations of inhibitors, this suggests that the mode of application, as foliar sprays, does not result in saturable levels of the inhibitors to sites of synthesis.

In contrast to previous studies by Kao and Yang (14) and Woodson et al (28), the concentrations of AOA used in this investigation were substantially (10-fold) higher. However, the differences partly reflect the bulk and size of the broccoli tissue and the short duration of one-time applications of the ethylene biosynthesis inhibitors.

Effects of Ethylene Action Inhibitors on Ethylene Production and Chlorophyll Content

Elevated CO_2 concentrations (2, 3), Ag^+ ion (2, 8, 26) and NBD (23) have been reported to oppose the physiological effects and action of ethylene on growth and senescence. Table 1 shows the effectiveness of these ethylene action inhibitors on ethylene production and chlorophyll content. CO_2 , at both 5% and 15%, inhibited chlorophyll loss by 58% and 80% respectively after 3 d in darkness. Ethylene production rates of florets under continuous exposure were not significantly different than those held in air. Ag^+ , as a foliar STS spray, at 0.5 mM and 1.0 mM, markedly enhanced ethylene production and was less effective in retarding chlorophyll loss. Ethylene production of Ag^+ treated florets at both concentrations were 10-fold higher than those sprayed with the MES buffer control. At 0.5 mM STS, the chlorophyll content was similar to that of the MES-buffer spray control, with the florets visibly yellowed after 72 h. The higher concentration at 1.0 mM STS retained 41% of the initial chlorophyll as compared to 31% for the MES buffer control. In comparison to elevated CO_2 concentrations and Ag^+ foliar sprays, continuous exposure to NBD was most effective in retaining chlorophyll content. Exposure to 1000 $\mu\text{l/l}$ and 2000 $\mu\text{l/l}$ NBD retarded

chlorophyll loss by 88% and 71%, respectively, after 3 d in darkness. Similar to Ag^+ ion, NBD promoted ethylene production although the change in magnitude was less than that of Ag^+ , on the order of 2.5- to 5-fold increase over the control. Figure 5 shows that the NBD effect on ethylene action can be reversed with the addition of exogenous ethylene.

Effect of Cycloheximide and Dipyridyl on Ethylene Production, Chlorophyll and Amino Acid Content

Table 2 presents the effectiveness of cycloheximide (CHI), a protein synthesis inhibitor, and α, α -dipyridyl (DPI), a metal chelator, in inhibiting senescence and ethylene production. CHI and DPI inhibited chlorophyll loss at higher concentrations with CHI being more effective in maintaining chlorophyll content. In addition, CHI also inhibited slightly amino acid accumulation as compared to the MES-buffer control and DPI. With the exception of 0.1 mM cycloheximide, both CHI and DPI inhibited ethylene production. After 60 h, ethylene production of detached florets treated with either 1.0 mM CHI or DPI was 38% or 69% that of the control.

Effect of Hormonal Pretreatments on Ethylene Production and Chlorophyll Content

From Table 3 the relative effectiveness of several 0.1 mM plant growth regulator foliar sprays in stimulating ethylene production 24 h after application was $\text{GA} > \text{ABA} > \text{IAA} > \text{THD} > \text{BA}$. Ethylene production rates of pretreated tissues were slightly higher or equivalent to the MES buffer control during the initial 24 h period. During this period, the rates of ethylene production by the florets pretreated with 0.1 mM GA_3 or ABA were twice that of the MES buffer control.

Following the accelerated rise in ethylene production, rates of ethylene evolution by GA_3 -treated tissue remained constant whereas ABA-treated tissue declined in the succeeding 24 h. Ethylene production by florets

pretreated with either ABA or BA at 48 h showed a 42% decline from production levels at 24 h. Florets pretreated with IAA and THD displayed an initial increase in ethylene evolution compared to the buffer control at 24 h which then declined at 48 h.

Pretreatments with BA and THD were most effective in inhibiting chlorophyll loss by 96% and 92% respectively. Florets pretreated with BA and THD remained visibly green at 60 h comparable to control held at 0°C for the same duration. At this time, visible yellowing occurred most significantly in buffer control followed by IAA and ABA. Gibberellic acid was partially effective in delaying chlorophyll loss. Although no visible yellowing occurred, GA₃-treated florets were lighter green than BA, THD-treated and cold stored florets.

DISCUSSION

From the results presented herein, ethylene is a regulator of senescence in detached broccoli florets. Evidence for a regulatory role for ethylene include: 1) the kinetics of endogenous ethylene production during senescence indicates a causal relationship, 2) the reduction of ethylene biosynthesis and production delays senescence, as measured by chlorophyll loss, 3) the exposure to exogenous ethylene or ACC, the immediate ethylene precursor, accelerates senescence, 4) the presence of NBD, an antagonist of ethylene action, delays senescence, and 5) pretreatments with senescence retardants such as CHI, DPI, and specific hormones, like BA and THD, reduce ethylene production.

During the senescence of detached florets, the extent to which endogenous ethylene regulates this process is observed in Fig. 1a. Concomitant with the increase in ethylene evolution is the rise in CO₂ level with maximal production rates for both occurring at 52 h during the period of rapid chlorophyll degradation. The rise and then decline in ethylene

production and respiration rates in detached florets resembles the climacteric characteristic of fruit tissues at the onset of ripening (1). Previous findings by McGlasson et al (19) on tomato leaf segments and Gepstein and Thimann (11) on oat leaf segments suggest that the ethylene evolved during this 'climacteric' regulates senescence in leaf tissues as in fruit tissues.

In senescing florets, botanically immature floral tissue, the changes in ethylene production, ACC and MACC levels closely parallel those of ripening fruit (1) and senescing leaves (4, 19). Concomitant with the rise in ethylene evolution was the increase in ACC levels similar to those observed in senescing carnations (30) and hibiscus (28) flowers. In freshly detached florets at equilibrium with experimental temperature at 15°C, the initial levels of ethylene production (Fig. 1a), ACC and MACC content (Fig. 1b) were low. During senescence both ethylene production and ACC content increased significantly and then declined simultaneously indicating that ACC was required in ethylene biosynthesis during senescence as in apple fruit tissue (1).

However, as Kao and Yang (14) noted the rise in ethylene during senescence studies employing detached tissues may indeed be a wound or stress related response. They concluded from their study with rice leaf segments which were transversely cut and floated on water, that the ethylene evolved was an excision response since the area of wounding was small and water stress was unlikely given the slight increase in fresh weight. Similarly, in the present study, the 'climacteric' ethylene at 52 h in detached broccoli florets is also an excision rather than a wound response. The cut surface was less than 3 cm in diameter and the slight increase in fresh weight during sampling of the florets suggests that wounding or stress is unlikely.

Further evidence for the regulatory role of ethylene during senescence was obtained with exposures to exogenous ethylene (Fig. 2) and ACC (Fig. 3). Increasing concentrations of either ethylene or ACC significantly accelerated senescence in agreement with detached hibiscus flowers (28) and excised rice leaf segments (14). Similar antisenesence effects were observed with antagonists of ethylene action including NBD, CO₂ and STS in order of

effectiveness. NBD was most effective in preventing chlorophyll loss (Fig. 5 and Table 1) at 1000 $\mu\text{l/L}$. In the presence of saturating levels of 0.1 or 1.0 ppm ethylene, senescence was accelerated (Fig. 5). The senescence-retarding effect of NBD was completely nullified by continuous exposure to saturating levels of ethylene as characterized by chlorophyll loss (Fig. 5) which suggests that NBD acts solely on ethylene regulated senescence rather than other biological processes. The enhancement of ethylene production and delay of senescence by NBD seem contradictory although similar results have been reported by Sisler et al (23) on ethylene production and abscission in citrus leaf explants.

The ineffectiveness of STS in delaying ethylene regulated senescence suggests that Ag^+ was toxic. The rates of ethylene evolved increased with the Ag^+ concentrations. Similar findings of Ag^+ toxicity, at concentrations above 50 mg/l, were noted by Aharoni and Lieberman (3) in excised tobacco leaf discs as increased ethylene production and respiration rates, yellowing and blackening of the cut surfaces. The anti-senescence response in florets stored under CO_2 is in agreement with findings by Beyer (8) that CO_2 is a weak inhibitor of ethylene action, given the low rates of ethylene production by florets of less than 2 nl/g fresh weight.

From Table 2, increasing concentrations of CHI and DPI decreased ethylene production and prevented senescence as measured by chlorophyll loss. CHI was more effective than DPI in preventing amino acid accumulation given the nature and function of the respective compounds as protein synthesis inhibitor and chelator (25). CHI may have prevented the synthesis of an individual or group of hydrolytic enzymes which in turn maintained cellular integrity and function.

From the aforementioned findings, it appears that the inhibition of ethylene synthesis and action prevents ethylene-regulated senescence. However, this attractive simplification is complicated by the hormonal pretreatment study in Table 3 which shows that THD, BA and GA_3 prevent senescence while ABA and IAA promotes it. From the data presented in Table 3, the maintenance of chlorophyll by THD and BA may be attributed to

the suppression of ethylene production at 48 h. However, the initial ethylene production levels at 24 h of both THD- and BA- treated tissues were greater than the control. In addition, ethylene production rates of IAA- and THD-treated tissues were similar although their effectiveness in preventing chlorophyll loss were markedly different. Furthermore, GA₃ minimalized chlorophyll degradation despite its enhancement of ethylene production. These seemingly contradictory findings are consistent with several previous studies including those of McKeon et al (20), Yip and Yang (32), Wright (29) and Yoshii and Imaseki (33).

McKeon et al (20) found differential rates of ethylene production in excised stressed and nonstressed wheat leaves pretreated with one of several hormones. Ethylene production by BA-treated, nonstressed leaves were slightly higher than the respective controls and resulted in little ACC accumulation prior to excision. In contrast, they found that BA-treatments to water-stressed wheat leaves significantly stimulated ethylene production through enhanced ACC synthesis. In the present study, the florets were not water-stressed as indicated by the increase in fresh weights. Consequently, BA slightly enhanced ethylene production during the initial 24 h. However, as Fuchs and Lieberman (10) and Aharoni et al (2) noted ethylene production in response to BA varies depending on the age and the corresponding sensitivity of the tissue. Thus at 48 h, BA was effective in lowering ethylene production and in retaining chlorophyll. Pretreatments of detached florets with THD produced similar results with an initial stimulation of ethylene production at 36 h followed by a subsequent decline at 60 h. THD was also effective in preventing chlorophyll degradation. The abilities of both THD and BA in delaying senescence is consistent with the work by Yip and Yang (32) which showed THD mimics BA in three distinct physiological systems. Thus, whether the retention of chlorophyll is directly related to decreased ethylene production, by THD and BA pretreatments is unknown since the latter also acts on the maintenance of protein synthesis (21) and suppression of RNase (7).

Although its slight promotive effect on ethylene production was similar to that of THD and BA, IAA promoted senescence in marked contrast to the effect of THD and BA. Wulster et al (30) demonstrated the senescence rate of carnation flowers was determined by an increase in duration and amount of ethylene production. From the present results, IAA was more active in comparison with either BA and THD in stimulating ethylene production at equal concentrations. This effectiveness of IAA in stimulating ethylene production is consistent with previous findings (4) by acting on ACC synthesis (33).

Although GA₃ was the most effective hormone in promoting ethylene production of those used, it paradoxically prevented chlorophyll loss. The promotive effect of GA₃ has been reported previously in both turgid and wilted wheat leaves (29) and 2-day old etiolated pea seedlings (10). Thimann (25) found that GA₃ prevented senescence in terms of chlorophyll and α -amino nitrogen content in detached *Tropaeolum* leaves.

In contrast, ABA at 48 h suppressed ethylene production but hastened senescence, similar to previous findings by Kao and Yang (14) in detached rice segments. However, the enhanced ethylene production at 24 h may have been accelerated by pretreatment with ABA since such high levels would normally occur at 48 h as in the control. Mayak and Dilley (18) reported a similar finding in carnation flowers in which ABA hastened senescence which was preceded by a stimulation of accelerated ethylene production.

Despite the often-times paradoxical findings between ethylene production after hormonal pretreatments and senescence rate, it may not be necessary to correlate the two in terms of magnitude and time. Instead, attention should be diverted to the age and sensitivity of the tissue to ethylene, which is the most significant factor in the regulation of senescence. Consequently, the feedback mechanism proposed by Aharoni et al (2), Beyer (8), Gepstein and Thimann (11) may be irrelevant. Similarly, the conclusion by Aharoni and Lieberman (3) that the concentration "internal endogenous ethylene ... plays a considerable role in the regulation of leaf senescence" may not be as critical as

the sensitivity of the tissue to ethylene. In addition to the sensitivity of the tissue, other factors to consider include the age of the tissue (10) and the plant species from which the tissue was derived (12), both of which have been shown to affect the sensitivity of the tissue. Gertman and Fuchs (12) showed that the effects of ABA, IAA and GA on ethylene production by pea seedlings were directly opposite those of orange peel plugs.

In determining a role for ethylene in senescence, consideration should also be paid to its interaction within the existing "hormonal pool" of the tissue. The choice of parameters in determining senescence is equally important; the use of chlorophyll retention, one of the characteristic tests for cytokinin activity, may have confused the relationship between ethylene evolution and senescence rate. Consequently, further work is required to elucidate whether ethylene production rates need to parallel ethylene action in the regulation of senescence. Furthermore, the role of tissue sensitivity to ethylene alone or in combination with others in the "hormonal pool" must be examined in greater detail before assigning ethylene as the dominant regulator of senescence.

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Table 1: Effects of NBD, Ag⁺, CO₂ treatment on ethylene production and chlorophyll content of detached broccoli florets held in darkness. Ethylene production and chlorophyll content were determined at the end of 72 h incubation in darkness. Rate of ethylene production was determined after incubation for 2 h periods. Continuous exposure to NBD or CO₂ was as described in Material and Methods. Ag⁺ was applied as a foliar 0.5 mM or 1.0 mM spray until runoff. The initial chlorophyll content was 0.83 mg/(g fresh weight).

Treatment	Ethylene Production	Chlorophyll Content	
		<u>mg/g</u>	<u>% inhibition of loss</u>
	<u>nl/g FW-h</u>		
Control	1.22	0.29	0
NBD (1000 µl/l)	2.01	0.76	88
NBD (2000 µl/l)	6.22	0.67	71
STS (0.5 mM)	13.63	0.26	0
STS (1.0 mM)	17.74	0.38	17
CO ₂ (5%)	1.31	0.61	58
CO ₂ (15%)	1.07	0.72	80

Table 2: Effects of CHI and DPI on ethylene production, chlorophyll and amino acid content of detached broccoli florets held in darkness. Ethylene production rates were determined after 60 h in darkness and represent means of two replicates. Chlorophyll and amino acid content were determined after 108 h in darkness. The initial chlorophyll content was 0.56 mg/(g fresh weight).

Treatment	Ethylene Production	Chlorophyll Content		Amino Acid Content
	<u>nl/g FW-h</u>	<u>mg/g</u>	<u>% inhibition of loss</u>	<u>μmol/g</u>
Control	3.12 ± 0.11	0.24 ± 0.02	0	51.5 ± 3.5
Dipyridyl - 0.1 mM	2.35 ± 0.24	0.34 ± 0.07	32	66.2 ± 5.1
Dipyridyl - 1.0 mM	2.14 ± 0.47	0.42 ± 0.01	56	61.5 ± 1.9
Cycloheximide - 0.1 mM	3.39 ± 0.25	0.36 ± 0.01	37	48.0 ± 0.1
Cycloheximide - 1.0 mM	1.19 ± 0.02	0.50 ± 0.05	81	43.8 ± 1.4

Table 3: Effect of hormonal pretreatments on ethylene production and chlorophyll content of detached florets held in darkness. Ethylene production determinations were made at 24 h and 48 h. Chlorophyll content was determined at 60 h. Ethylene production and chlorophyll content are the average of three replicates + SE. The initial chlorophyll content was 0.56 mg/(g fresh weight).

Treatment	Ethylene Production		Chlorophyll Content	
	<u>nl/g FW-h</u>		<u>mg/g</u>	<u>%inhibition of loss</u>
	<u>24 h</u>	<u>48 h</u>		<u>60 h</u>
control	1.21	2.34	0.15	0
ABA	2.35	1.54	0.19	9.5
BA	1.41	0.82	0.52	90
GA	2.43	2.15	0.42	66
IAA	1.70	1.40	0.16	2.7
THD	1.60	1.34	0.49	84
control (0° C)	0.06	0.09	0.53	93

Figure 1: Changes in ethylene production and respiration rates (1A), ACC-MACC levels (1B), chlorophyll content (1C), amino acid and protein contents (1D) of detached broccoli florets during senescence in darkness. Arrow refers to first signs of visible yellowing. Means of at least two replicates. Vertical bars represent SE.

Figure 2: Effect of continuous exposure to exogenous ethylene at 1 ppm and 10 ppm on chlorophyll loss of detached broccoli florets. Chlorophyll content was determined at the times indicated after the start of the ethylene treatment. Arrows indicate the first visible signs of yellowing. Means of three replicates. SE were within 10%.

Figure 3: Effect of exogenous ACC on chlorophyll loss (3A) and ethylene production (3B) of detached broccoli florets. Chlorophyll determinations were as described for Figure 2. Arrows indicate the first visible signs of yellowing. Rates of ethylene production are means of three replicates. SE were within 10%.

Figure 4: Effect of serial concentrations of applied AOA, CoCl_2 and NiCl_2 on ethylene production and retention of chlorophyll content after 36 h incubation in darkness.

Figure 5: Effect of continuous exposure to NBD, ethylene, and combinations of both on chlorophyll content. Continuous exposure to NBD and ethylene were as described in Materials and Methods.

4. PATTERNS OF ETHYLENE PRODUCTION DURING SENESCENCE OF VARIOUS LEAVES AND HERBS

4.1 WOUND-ETHYLENE PRODUCTION AND SENESCENCE PARAMETERS IN WATERCRESS LEAVES SENESCING ON THE PLANT

Shimon Meir, Sonia Philosoph-Hadas and Nehemia Aharoni

Watercress leaves were found to undergo a relatively rapid senescence upon detachment, which was followed by a remarkable burst of wound-ethylene (see sections 3.1 and 3.3). In this study various parameters of ethylene biosynthesis and senescence processes were examined in leaves detached from the plant at different senescence stages.

Materials and Methods. Leaves at different stages of senescence, determined on a chlorophyll (Chl) content basis and their location on the stem, were detached from watercress plants grown in the field. Accordingly, four different stages were defined: A (100% Chl), B (60% Chl), C (40% Chl) and D (5% Chl). Samples of 6 detached leaves from each stage were placed on petri dishes containing a filter paper moistened with distilled water. The petri dishes were closed and incubated for 24 h in darkness at 25°C. Ethylene production was monitored at hour intervals by enclosing the leaf samples in 50-ml flasks for 1 h. These samples were subsequently extracted in 80% ethanol and assayed for contents of Chl, ACC, MACC, amino acids and protein. Estimation of malondialdehyde (MDA) and total aldehydes contents was performed with new leaf samples detached at the same intervals. Extraction and assays procedures of the various parameters tested were performed as detailed in sections 3.2 and 3.3 (3). MACC was extracted and assayed according to Jiao et al (2).

Results and Discussion. Ethylene production rates of watercress leaves detached at various senescence stages and incubated for 24 h are depicted in Fig. 1. All leaves showed the characteristic ethylene pattern consisting of a wound-ethylene rise peaking between 1-4 h following detachment. While the high wound-ethylene rise peaked in young leaves (A,B) 4 h following detachment, in older leaves (C,D) this rise

was lower and it peaked earlier. The ethylene production rates during the 24-h incubation period were found to be age-dependent, with the youngest leaves (A) showing the highest rate. The data show that the lower capacity of aged watercress leaves to produce ethylene during the first 24 h following detachment, may be related to their advanced senescence stage. The high ethylene production rates in young leaves (A) were accompanied by high corresponding levels of ACC and MACC, which declined with senescence (Fig. 2). However, at all senescence stages (except of stage D) levels of ACC and MACC increased with time of incubation. While ACC levels did not vary significantly during senescence, large differences in MACC levels of leaves at the various senescence stages could be observed. Thus, young leaves contained higher levels of MACC as compared to older ones. This may be attributed to a higher malonylation capacity in young leaves, as well as to transportation or leakage of MACC from the aged leaves.

The changes in 3 senescence-associated processes occurring at various senescence stages were examined. As defined, Chl content of the leaves declined with age as well as with increasing incubation time after detachment (Fig. 3A). However, the degree of Chl loss during 24 h following detachment was constant (about 25%) at all the senescence stages. Similarly, a constant and remarkable 4-fold increase of amino acids could be observed 24 h following detachment at all senescence stages (Fig. 3B). However, while protein content decreased moderately (by 16%) with senescence on the plant, the content of amino acids decreased by 77% (Fig. 4A). This age-dependent decrease in amino acids during senescence in vivo could be attributed to their translocation outside the leaf when it is still attached to the plant. On the other hand, the accumulation of amino acid in detached leaves during 24-h of incubation could be ascribed to their enhanced senescence.

The process of lipid oxidation, as exhibited by accumulation of total aldehydes and MDA, was also pronounced in leaves senesced on the plant. Results depicted in Fig. 4B demonstrate that while the content of total aldehydes increased moderately with age, MDA did not change significantly. This implies that MDA may further react with other compounds in the membrane and therefore may not represent an end product

of lipid oxidation (1).

The results suggest that leaf detachment imposes some variations in the pattern and rate of the senescence-associated processes examined. Thus, unlike in attached leaves, all the senescence-associated processes were enhanced in a fixed rate by detachment, and amino acids content increased during senescence of detached leaves rather than declined.

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FIGURE 1. Patterns of ethylene production rates during 24 h-incubation of watercress leaves detached at different senescence stages. The senescence stages of 4 types of detached leaves were defined on the basis of their initial % of Chl: A (100%), B (60%), C (40%) and D (5%). Samples of 6 leaves from each senescence stage were incubated at 25°C in darkness and their ethylene production rates were monitored at the indicated hour intervals. Bars represent SD of 3 replicates.

FIGURE 2. Changes in levels of ACC and MACC in watercress leaves detached at different senescence stages and incubated for 24 h. Incubation conditions were as detailed in Fig. 1. Leaf samples from each stage were extracted in ethanol and their levels of ACC and MACC were determined immediately after detachment (0 h) and 24 h later.

FIGURE 3. Changes in levels of Chl (A) and amino acids (B) in watercress leaves detached at different senescence stages and incubated for 24 h. Incubation conditions were as detailed in Fig. 1. The same ethanolic extract of leaves from each stage (Fig. 2) was used for determination of Chl and amino acids contents immediately after detachment (0 h) and 24 h later.

FIGURE 4. Patterns of protein hydrolysis (A) and lipid oxidation (B) in watercress leaves detached at different senescence stages. Four types of leaves, defined in Fig. 1, were detached and extracted either with ethanol (for determination of amino acids and protein), or with TCA (for determination of MDA and total aldehydes). The senescence stages A-D are represented by % chl.

4.2 CHANGES IN ETHYLENE BIOSYNTHESIS DURING SENESCENCE OF SPINACH AND PARSLEY LEAVES

Shimon Meir, Sonia Philosoph-Hadas, Eynat Pesis and Nehemia Aharoni

It has been shown that various detached dicot leaves exhibit under experimental conditions a rapid ethylene burst following excision and a subsequent climacteric-like rise of ethylene production at advanced stages of senescence (1). The magnitude of these ethylene peaks varied in leaves senescing at different rates (6). An attempt was made to explain these variations in detached senescing leaves of spinach and parsley by studying their patterns of ethylene production in relation to changes in the levels of ACC and MACC, which regulate ethylene biosynthesis (9).

Materials and Methods. Experiments were performed with mature, green, fully expanded leaves of spinach (*Spinacia oleracea* L.) and parsley (*Petroselinum crispum* Mill.), which were freshly harvested from the field. Leaves were rinsed with distilled water and surface-sterilized with 0.5% sodium hypochlorite as described previously (1, 5, 6). Samples consisting of four intact leaves (4.5 g) or eight leaf discs (0.3 g) of spinach were enclosed in 0.5-L jars or in 50-ml flasks, respectively. Discs were excised and treated as described previously (5, 6). Both intact leaves and leaf discs were incubated in the dark at 25°C for 6-9 days, and changes in their endogenous ethylene production, ACC and MACC levels were assayed daily. Parsley bunches were randomly distributed in groups of 12 stems and placed in 100-ml flasks containing 50 ml of distilled water plus 50 µg/ml chloramphenicol. The flasks were then enclosed into 2-L jars, which were sealed and connected through fitted inlet and outlet ports, to gas cylinders containing either air or 10 µL/L ethylene. A flow-through system was used with a flow rate maintained at 50 mL/min. The parsley bunches in the jars were incubated for 7 days in the dark at 30°C and changes in their endogenous ethylene production, ACC and MACC levels were determined daily with samples of ten excised leaflets. To avoid possible contamination during the incubation period the stems bottom were cut and the solution in the flasks was renewed every second day.

Ethylene production rates of spinach intact leaves or leaf discs were measured directly in their incubation jars or flasks, respectively, at hourly and daily intervals. Ethylene production rates of parsley leaves were monitored daily by enclosing samples of treated bunches (0.7-0.9 g) in 50-ml flasks and incubating them at 30°C for 2 h. Ethylene concentration in the various vessels was analyzed by withdrawing a 2-ml gas sample and injecting it into a gas chromatograph equipped with an alumina column and a flame ionization detector. For determination of ACC and MACC, samples of the various leafy systems (0.5 g) were cut into small pieces and boiled in 10 mL of 80% ethanol for 20 min. All subsequent handling of the extracts was performed as described previously (3). ACC content in 0.25-ml aliquots of the aqueous solution was determined according to the method of Lizada and Yang (4). Quantification of MACC in the extract was carried out by hydrolyzing a 0.2-ml aliquot of the aqueous solution in 2 N HCl at 100° for 3 h as described previously (2, 3). The difference in ACC content after and before HCl-hydrolysis was taken as the amount of MACC in the extract (2).

Results and Discussion. A comparative study of the ethylene biosynthesis pathway was conducted between detached, whole leaves and excised leaf discs of spinach senescing in darkness. Whole detached spinach leaves produced in response to detachment a very moderate ethylene rise, which peaked on the first day following detachment (Fig. 1A). This rise of wound-ethylene production was followed by a second, climacteric-like rise, which lasted for the next 5 days. The pattern of ethylene production in spinach leaf discs undergoing dark senescence exhibited at least two ethylene rises (Fig. 1B) which differed in magnitude and timing from the peaks observed in intact leaves. Thus, a very sharp burst of wound ethylene, which was 100-fold that of intact leaves, was detected in leaf discs already 6 h following excision (Fig. 1B). Unlike in intact leaves, the climacteric-like ethylene rise peaked in leaf discs already on the 3rd day after excision and was 20-folds higher (Fig. 1B).

The climacteric-like rise of ethylene was related to the progressive

changes of ACC levels (Fig. 2) and senescence parameters (see Section 3.2). ACC began to accumulate in whole spinach leaves 2 days after the climacteric-like rise of ethylene has begun, reaching at the 9th day a remarkable 24-fold rise (Fig. 2). On the other hand, MACC levels, which were higher than those of ACC during the initial 3 days following detachment, decreased slightly later on, before the increase in ACC content has commenced (Fig. 2). These patterns imply that following detachment ACC is predominantly metabolized to MACC and ethylene, while in advanced stages of senescence MACC may be further metabolized (3, 7). A similar pattern consisting of a remarkable late ACC rise was also observed in senescing oat leaves (7).

The sequence of changes in ACC and MACC levels of spinach leaf discs (Fig. 3) was similar to that of whole leaves. However, during the first 2 days following excision ACC was converted rapidly to ethylene and MACC, thereby resulting in relatively low ACC and remarkably high MACC levels (Fig. 3). In the subsequent days MACC subsided during senescence, while ACC showed a moderate increase (Fig. 3). The remarkable MACC peak preceded by one day the climacteric ethylene peak of leaf discs, which appeared on the 3rd day following excision (Fig. 1B). It should be noted that the decrease of MACC level in spinach leaf discs was very sharp (Fig. 3), whereas that of intact leaves was quite moderate (Fig. 2). It seems therefore that excision advanced both wound and climacteric ethylene maxima by 20 h and 6 days, respectively, and increased their peaks magnitude, as well as MACC levels, by several folds. Inasmuch as part of the MACC in spinach leaf discs may be metabolized to ACC and ethylene (3), a molar calculation indicates that MACC should be metabolized to another compound as well. When comparing whole leaves to leaf discs it seems that the climacteric ethylene rise in whole leaves (Fig. 1A) results from increased ACC synthesis (Fig. 2), whereas the first climacteric-ethylene peak on the 3rd day (Fig. 1B) may result from hydrolysis of MACC to ACC (Fig. 3).

The effect of exogenously applied ethylene on ethylene production and contents of ACC and MACC was investigated in senescing parsley leaves whose wounding effect was minimal. The pattern of endogenous ethylene production in parsley bunches undergoing dark senescence was

characterized by the appearance of two rises: a wound-ethylene rise, peaking on the first day, and a subsequent climacteric-like rise, peaking on the 4th day following detachment (Fig. 4). Unlike the rise of wound-ethylene, the climacteric ethylene rise was accompanied by a progressive increase in ACC content up to eight-fold, peaking on the 4th day (Fig. 5A). On the other hand, MACC level increased sharply during the first day following detachment and continued to increase gradually in the subsequent days (Fig. 5B). Gassing parsley bunches with 10 μ L/L ethylene resulted in suppression of their endogenous ethylene production (Fig. 4), which was accompanied by reduced levels of both ACC (Fig. 5A) and MACC (Fig. 5B). These reduced ACC and MACC levels could explain the remarkable autoinhibitory effect of ethylene in this tissue (5, 8).

While the ACC peak in parsley leaves (Fig. 5A) paralleled that of ethylene (Fig. 4), MACC accumulated during their senescence and unlike in spinach leaves, no metabolism of MACC was apparent (Fig. 5B). Consequently, ACC levels and ethylene production rates subsided in the advanced stages of senescence, since increased ACC malonylation reduced the level of free ACC available for ethylene formation. The degree of ACC malonylation in the tissues tested may explain the differences obtained in the magnitude of their climacteric-ethylene rises. Thus, the climacteric-ethylene rise in parsley leaves, which showed a high malonylation capacity, was 2-fold lower than their wound-ethylene rise (Fig. 4). On the other hand, the climacteric-ethylene peak of spinach intact leaves, whose MACC levels remained relatively low during senescence (Fig. 3), was 2-fold higher than their wound-ethylene rise (Fig. 1A).

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FIGURE 1. Time course of changes in ethylene production rates during dark senescence of detached whole leaves (A) and leaf discs (B) of spinach. Ethylene production rates of 4 detached spinach leaves (4.5 g) kept in 0.5-L jars, or of 8 leaf discs (0.3 g) enclosed in 50-mL flasks, were monitored daily. The bars represent SD of three replicates.

FIGURE 2. Time course of changes in ACC and MACC contents during dark senescence of detached spinach leaves. ACC and MACC were assayed daily with 0.5-g samples of leaf blade tissue.

FIGURE 3. Time course of changes in ACC and MACC contents during dark senescence of spinach leaf discs. ACC and MACC were assayed daily with samples of 8 leaf discs (0.3 g).

FIGURE 4. Effect of exogenous ethylene on the time course of ethylene production in senescing parsley bunches. Samples of parsley bunches were kept in darkness under air or 10 μ L/L ethylene in a flow-through system. Ethylene production rates were monitored daily by enclosing 0.7-g samples into 50-mL flasks for 2 h.

FIGURE 5. Effect of exogenous ethylene (10 μ L/L) on contents of ACC (A) and MACC (B) in senescing parsley bunches. Incubation conditions were as described in Fig. 4. ACC and MACC levels were assayed with 0.5-g leaf samples detached from the parsley bunches.

4.3. ETHYLENE PRODUCTION AND SENESCENCE OF IPOMEA LEAVES

Ma. Concepcion C. Lizada and S.F. Yang

Ipomea convulvulus was examined as a potential system for characterizing the relationship between ethylene and senescence in detached leaves.

Materials and Methods

Seeds were washed with 1% (v/v) NaOCl, rinsed with and allowed to imbibe in distilled water overnight before sowing in vermiculite saturated with a quarter strength of nutrient solution (1). Seedlings were grown in continuous light (82 $\mu\text{E}/\text{m}^2\text{-sec}$) and watered with 1/10 strength of nutrient solution. The first leaves from 11-day old seedlings were excised and incubated at the indicated temperatures in the dark in 13 x 100 mm tubes containing 0.1 ml water, either as whole leaves or as 2-cm sub-apical segments.

Results and discussion: At 30°C intact leaves of *I. convulvulus* showed two patterns of ethylene production. Although all leaves showed an early peak occurring 24 h after excision, about half of the leaves had an average peak rate of 2.8 nl/g-h, while the other half had an average peak rate of < 1 nl/g-h. Regardless of the peak rate, yellowing became evident 80 h after excision and 46 h before the onset of a second rise in ethylene production. The latter occurred when the leaves showed severe yellowing. Sub-apical segments exhibited an earlier peak in ethylene production which occurred 10 h after excision. As with whole leaves, yellowing became evident 80 h after excision. The onset of the climacteric rise, in all cases, occurred before the onset of the second rise in ethylene production. Thus, it appears that the second increase in ethylene production might not be involved in the onset of senescence.

As might be expected, lowering the temperature to 20 or 25°C delayed the onset of yellowing by 46 and 66 h, respectively. No second rise in ethylene production was observed at either temperatures, even when the leaves were almost fully yellow. At 20°C, incubation in the light increased the peak rate of ethylene production (2.2 nl/g-h) 20 h after excision) relative to incubation in the dark (0.7 nl/g-h), but delayed the onset of yellowing by 3 days.

Work with *I. convulvulus* leaves was discontinued due to high variability observed in leaf morphology.

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5. EFFECTS OF PLANT HORMONES ON ETHYLENE PRODUCTION AND ACTION IN SENESCING TISSUES

5.1 QUANTITATION OF ABSCISIC ACID IN RICE LEAVES BY ELISA USING A MONOCLONAL ANTIBODY

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ABSTRACT

Immunological methodology was developed for convenient quantitative analysis of abscisic acid (ABA) levels in leaf extracts. Monoclonal antibodies (mAb) were generated against (+)-ABA conjugated to bovine serum albumin (BSA) through its carboxyl group (C₁). The mAb reacted preferentially with the (+)-ABA enantiomer, and equally well with both free and esterified ABA. Cross-reactivity with several ABA-related compounds was negligible. All reactions were tested with an indirect enzyme-linked immunosorbent assay (ELISA), employing horseradish peroxidase. Linearity was obtained between 3 and 1000 pmol of (+)-ABA. The ABA-mAb was further used to quantitate pmol concentrations of (+)-ABA in partially purified rice leaf extracts. Such ELISA quantitation compared favorably with other presently used techniques for ABA determination, in regard to their detection limits, cost and time consumption. This suggests that the combination of specific mAb with a sensitive ELISA technique is quite promising for quantitation of ABA.

ABBREVIATIONS: BHT, 2,6-di-tert-butyl-4-methylphenol; DPA, 4'-dihydro-
phaseic acid; BSA, bovine serum albumin; ELISA, enzyme- linked
immunosorbent assay; GLC, gas liquid chromatography; HRP, horse-
radish peroxidase; IgG, immunoglobulin; mAb, monoclonal antibody; PA,
phaseic acid; RIA, radioimmunoassay.

INTRODUCTION

Research in the physiological roles of ABA in leaves (1, 4, 18) has been slow due to difficulties in measuring small amounts of endogenous ABA. The most common methods for ABA quantification are by HPLC-UV followed by GLC. These techniques require rigorous cleanup procedures to ensure elimination of contaminants that may cochromatograph with ABA (13). Recent advances in immunological techniques (3, 5, 22) have provided attractive alternatives to the traditional assays of ABA, which minimize the need for purification and allow rapid analysis of many samples (2, 6, 10, 12, 19, 21). Hence, we have developed a mAb for ABA and employed a very sensitive ELISA system (17). Unlike previous ELISA quantitations of ABA, which coupled the hormone with alkaline phosphatase (2, 21), this report describes an indirect ELISA method that provides substantial amplification of the response by employing a second antibody, goat anti-mouse, coupled to the enzyme HRP (17). An application of this method has been recently published (16).

MATERIALS AND METHODS

Chemicals. All chemicals, unless otherwise specified, were obtained from Sigma or Gibco and were of the highest purity available. Affinity purified goat anti-mouse IgG conjugated to HRP was from Bio-Rad, Richmond CA, the Diazald methylation reagent was from Aldrich and the Horse serum was from J.R. Scientific, CA. Phaseic acid (PA) and dihydrophaseic acid (DPA) were generous gifts from Dr. M. Brenner, University of Minnesota, St. Paul, MN. The ABA glucose ester was a generous gift from Dr. T. Davenport, University of Florida, Homestead, FL. The ABA methyl esters were prepared by methylation of ABA with ethereal diazomethane prepared from Diazald.

Plant Material and Incubation Conditions. Rice (Oryza sativa L. Taichung Native 1) seedlings were grown in vermiculite for 9-10 d in a growth chamber at 30°C, 65% RH and 18 h-light period (light intensity of 540 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, provided by a mixture of cool-white and Gro-lux tubes). For experiments using detached leaves, samples of 10 rice leaf segments (the apical 5 cm of the third leaves), excised from 9-days old

seedlings and weighing about 0.1 g, were floated in covered petri dishes containing 10 ml test solution. Incubation was carried out in the growth chamber, under the same conditions and light regime used for plant growth. At the indicated periods, ABA was extracted from samples of 10 detached leaf segments. Extraction of ABA from attached leaves was carried out parallelly with 10 segments freshly harvested at the indicated periods, beginning with 9-day old seedlings.

Extraction and Partial Purification of ABA. The methods for extraction and purification of ABA were slightly modified from those described by Hubick and Reid (8). Briefly, samples of 10 rice leaf segments (0.1 g) were homogenized for 1 min in 10 ml extraction medium (methanol: ethyl acetate: acetic acid, 50:50:1, v/v) containing BHT (20 mg/L) as an antioxidant. Cellular debris were filtered through Whatman No.1 filter paper and the filtrates were dried in vacuo at 35°C. The residues were dissolved in 1 ml methylene chloride and loaded (with a glass syringe) onto Sep-pak silica cartridges (Waters Associates, MA), prewashed with 5 ml methylene chloride. The cartridges were then washed with a series of organic solvents with increasing polarity, for removal of contaminating compounds and elution of ABA: 5% (v/v) ether, 5% ethyl acetate, 5% acetone and 4%, 10%, and 20% methanol in methylene chloride. ABA fractions, eluted from the Sep-pak with 10-20% methanol in methylene chloride, were bulked, evaporated to dryness and dissolved in 1 ml of 0.5 M phosphate buffer (pH 8.0) for ABA analysis by ELISA.

Separation of free ABA from its conjugates was performed according to methods described previously (6, 8). The ABA extracts, dissolved in phosphate buffer after the Sep-pak elution, were acidified to pH 2.5 with 6 N HCl and partitioned (3 times) against equal volumes of ethyl acetate. The resultant organic fractions containing free ABA were evaporated to dryness and dissolved in 0.5 ml phosphate buffer (pH 8.5) for ABA analysis by ELISA. The corresponding aqueous fractions containing ABA-conjugates were hydrolyzed (pH 11.0, 60°C, 1 h), acidified and treated as described above for free ABA.

Synthesis of ABA-BSA Conjugate and Immunization. ($\pm$)-ABA (Sigma, cis-trans isomers, 99+%) was coupled through C₁ to BSA (Sigma, 98-99%

albumin, FA and globulin free) by using 1-ethyl-3 (3-dimethyl-aminopropyl)-carbodiimide-HCl (EDC), as described by Weiler (20). ($\pm$)-ABA (66 mg), dissolved in 1.5 ml solution of water-dimethyl-formamide (1:2), was added dropwise to an icecold solution of 125 mg BSA in 5 ml H₂O, while keeping the pH >8.1 with 1 N NaOH. EDC (105 mg) was then added in 4 portions over a period of 2 h and the solution was stirred under N₂ at 4°C in the dark overnight. The conjugate (ABA-BSA) was dialyzed for 4 d against water and stored at -18°C. Three male Bulb/c mice were injected intra-mascular (i.m.) with 100 μ g/animal of ABA-BSA conjugate emulsified in 0.2 ml of complete Freund's adjuvant, and boosted after 16 and 32 d with further 100 μ g antigen per animal. Sera were collected 10 d after each boost and screened by ELISA for the presence of antibodies reactive with the ABA-BSA conjugate.

Cell Fusion and Cloning. The mouse giving the antiserum with the highest titer was finally boosted 4 d prior to fusion with 50 μ g of ABA-BSA conjugate in saline solution inoculated into the peritoneum, followed by a second boost of 50 μ g ABA-BSA conjugate, inoculated i.v. into the tail vain after 24 h. Fusion was performed 3 d later according to Galfre and Milstein (3), as modified recently by Hadas and Theilen (5). The mouse spleen cells were fused with the NSO/1 myeloma cell line (3) using 41% polyethylene glycol (MW 1450, Kodak).

After fusion cells were distributed into 20 microtiter plates (Costar) at a concentration of 5×10^4 spleen cells/well and cultured in 0.1 ml Dulbecco's Modified Eagle medium (DMEM, high glucose, Gibco), supplemented with the following: 20% horse serum (HS, J.R. Scientific, CA), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.1 hypoxanthine (H), 16 μ M thymidine, 50 U/ml penicillin and 50 μ g/ml streptomycin. Cultures were fed with 0.4 μ M aminopterin (A) 24 h after fusion and with additional 0.1 ml/well of DMEM + 20% HS + HAT medium on day 6. Aliquots of the supernatants were removed on days 11-14 after fusion and were tested by ELISA for the presence of ABA specific mAb. Following this initial screening, 25 μ l-aliquots from the positive (A₄₉₂ > 0.1) wells were transferred into new plates and fed with 225 μ l/well of DMEM + 15% HS + HT medium. A second detailed screening for characterization of the mAb was performed after 3 consecutive transfers.

The desired wells producing mAb against ABA were then cloned by limiting dilution (3) and the resulting clones were frozen. The number of clones in each well was estimated from the percentage of wells that contained growing hybrid colonies (5).

ELISA Procedure. Immunoassay was performed according to the methods described before (17). Flat-bottom, 96-well, microtiter plates (Immulon 1, Dynatech) were precoated overnight at 4°C with ABA-C₁-BSA conjugate (100 ng/well) in 100 μ l of coating buffer (0.1 M Na₂CO₃, pH 9.6). The coating solution was then decanted and the coated wells were blocked for 1 h at 25°C with 100 μ l of 5% non-fat dry milk dissolved in 50 mM Tris-HCl pH 8.0. Excess liquid was drained and the wells were rinsed (3 times) with distilled H₂O.

For the assay, 50 μ l of (+)-ABA standard (in serial dilutions) or sample was first added to each well, followed by 50 μ l of the anti-ABA mAb diluted 8-fold in ELISA buffer (0.15 M NaCl, 1 mM EDTA, 50 mM Tris, 0.05% tween-20 and 1 mg/ml BSA, pH 7.4). After 2 h incubation at 37°C, wells were rinsed 4 times with water and 100 μ l of diluted (3000-fold in ELISA buffer) tracer (affinity purified goat-anti-mouse IgG conjugate to HRP) was added. The plates were incubated at 37°C for additional 1 h and then rinsed 5 times with water. Bound tracer activity was determined by adding 100 μ l/well of HRP substrate (2 mg/ml O-phenylenediamine in 50 mM citric acid pH 5.0 and 0.008% H₂O₂). The colored reaction product was allowed to develop at 25°C for 2 min and reaction progress was stopped by addition of 50 μ l/well of 4N HCl. Absorbance at 492 nm against 405 nm as background was determined with a dual wavelength automated microplate ELISA Reader (model EL310, BIO TEK). Under the assay conditions described A₄₉₂ = 1.0 was obtained for maximum binding, and unspecific binding (determined with excess ABA) was automatically subtracted. To account for plate-to-plate variability, a standard ABA curve was included in every plate. Each point was repeated 3-4 times.

RESULTS

Characterization of ABA-mAb and Immunoassay. From an initial collection of cell lines that produced mAb specific for the ABA-BSA conjugate, one clone that produced a mAb of high affinity and specificity for (+)-ABA, was selected. This mAb was further used to quantitate pmol concentrations of (+)-ABA in rice leaf extracts by enzyme immunoassay. A cross-reactivity study of the mAb obtained revealed its high degree of selectivity for the naturally occurring (+)-ABA, whereas the (-)-ABA enantiomer was not immunoreactive (Table I). The ABA catabolites PA and DPA were only slightly reactive (300 and 1000-fold, respectively, less reactive). On the other hand, both ABA-methyl ester, which may occur as an extraction artefact (20), and ABA-glucosyl ester, the predominant ABA conjugate in plants (18), were reactive. The results of Table I show that the mAb formed in response to (±)-ABA conjugated to BSA through its carboxyl group (C₁) distinguished sharply between the 2 ABA enantiomers by reacting preferentially with the (+)-ABA form, but it reacted equally well with both free and esterified ABA, as described previously for polyclonal Ab (20, 21). Therefore, a separation between the free and conjugated forms of ABA in the extracts was required prior to ABA analysis.

Fig. 1 shows typical standard curves for (±)-ABA, (+)-ABA and ABA-glucosyl ester in the sigmoid and linearized plots. The linearity of the latter, between a range of 3-1000 pmol (+)-ABA or 3-200 pmol (±)-ABA, shows that reaction equilibrium has been reached within the incubation time. Our mAb-ELISA system is 10-fold less sensitive than the systems with polyclonal Ab reported previously (6, 19, 20, 21), but 3-fold more sensitive than the mAb-RIA system described by Mertens et al (12). This discrepancy may be probably attributed to the use of mAb produced against (+)-ABA coupled to BSA through C₁, in conjunction with the naturally occurring (+)-ABA as a standard (20).

To detect any potentially interfering compounds present in the plant extracts, serial dilution curves of different extracts were made and a constant amount of (+)-ABA was added to the extract as an internal standard. The curves obtained in both cases showed parallelism with the

standard curve (Fig. 2). These results indicate the absence of interfering materials cross reacting with the mAb and having different affinity constants as compared with ABA.

Estimation of Endogenous ABA Levels in Excised Rice Leaves. The ABA-mAb was further employed for rapid determination of ABA content in partially purified leaf extracts. Fig. 3 describes the quantitative changes of endogenous free ABA content and its conjugated form in both detached and attached leaves during 28 h from zero time (9-days old seedlings), as determined by immunoassay. A sharp 2-fold rise in free ABA content of attached leaves, followed by a 2-fold decrease was observed during the first 8 h from zero time (Fig. 3A). On the other hand, free ABA levels in detached leaves showed a moderate decrease followed by a gradual increase during that period of incubation (Fig. 3A). A detailed examination of the changes in ABA content reveals that the reduction in free ABA level of detached leaves during the first h following excision was accompanied by a concomitant significant rise in the content of conjugated ABA (Fig. 3B). Subsequently, both free and conjugated ABA declined parallelly during the next 3 h (Figs. 3A,B). The moderate increase of free ABA observed between 4-8 h after excision (Fig 3A) was accompanied by a sharp rise in the level of conjugated ABA, which levelled off later on (Fig. 3B). Similarly, free and conjugated ABA levels of attached leaves raised parallelly during the first 4 h from zero time, and changed in opposite manners during the subsequent 4-8 h (Fig. 3A,B).

The age-dependent changes in endogenous free ABA level during long-term senescence of detached and attached leaves were also examined during 6 days. The initial fluctuations in free ABA content observed during the first 24 h (Fig. 3A) and probably derived from water stress (14), were not plotted. Fig. 4 demonstrates that free ABA content remained in attached leaves at a basal level, which did not change during 6 days. Senescing detached leaves, however, showed a gradual increase in free ABA level which peaked on the 3rd day after detachment.

DISCUSSION

Methodology was developed for convenient quantitative analysis of ABA concentrations in leaf extracts at different stages. This included development of an ABA-mAb (3, 5), together with an enzyme-linked immunoassay (17) which provides substantial amplification of the response. To our knowledge, this combination of mAb with ELISA has been applied only recently for ABA quantitation (16), and all other studies dealing with immunoassays for ABA used polyclonal Ab (2, 6, 10, 20, 21) or mAb coupled with RIA (12) rather than with ELISA. Employing these immunological techniques minimized the need for prepurification of the ABA extracts, so that a large number of samples could be processed with little procedural effort, and correspondingly little sample loss.

In the present study, the mAb was raised against (+)-ABA and standartidized with (+)-ABA, which is the naturally occuring form (18). Although this variable enantiomers combination was not recommended due to loss of assay sensitivity and precision (20), our data (Table I, Figs. 1, 2) show high specificity and selectivity of the ABA-mAb as compared with previous studies (2, 6, 10, 19, 20, 21). This most likely results from the use of mAb rather than polyclonal sera, which contain also low affinity Ab that are probably responsible for non-specific cross reactions. When polyclonal Ab are employed for ABA determination, an additional purification by HPLC is required (11). Hence, the mAb approach, though more time consuming, is most advantageous since it allows the preparation of unlimited amounts of pure Ab with well defined characteristics (3, 22).

The ABA levels in rice leaf extracts determined by the present ELISA method (Fig. 3) are higher than those quantitated by RIA in other leaves (19, 20), but are comparable with the values obtained by ELISA for citrus leaves, where the values of 5.3 and 25.1 nmol/g, for free and conjugated ABA, respectively, were reported (6). The differences between the two immunoassays may be ascribed to the higher sensitivity of ELISA as compared with RIA (22, 17). ELISA quantitation of ABA showed also good correlation with ABA determination by GLC (10) and compared favorably with all presently used techniques for ABA determination (1,

4, 9, 14, 15) in regard to their detection limits, cost and time consumption (2, 22). Therefore, the combination of specific mAb with a sensitive ELISA technique used in the present study seems promising.

Published data (15, 20) report that the concentrations of conjugated ABA may match or even far exceed the concentration of free ABA. Results depicted in Fig. 3 confirm these observations, showing that conjugated ABA levels were about 4-fold higher than those of free ABA. The findings showing that during the first 24 h free ABA levels in attached leaves were significantly higher than those of detached leaves (Fig. 3A) may be ascribed either to fluctuations in their water potential status (14, 18) or to a stress imposed on attached leaves upon their experimental excision. However, during senescence of rice leaves, endogenous ABA levels of detached leaves were much higher than those of attached ones (Fig. 4). While the initial rise of ABA (Fig 3A) may be related to changes in the water potential of attached leaves caused by the experimental conditions, the second ABA increase which appeared only in detached leaves (Fig. 4), seems to be a senescence-related response. Indeed, detached rice leaves senesced much faster than attached ones and their senescence was further enhanced by exogenous ABA (see Section 5.2). These data may further implicate ABA as a senescence-enhancing hormone (18).

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Table I. Specificity of mAb raised against (±)-ABA. Increasing concentrations of ABA-related compounds were tested under standard assay conditions for their ability to displace 50% of the tracer. Data are expressed as % cross reaction on molar basis, relative to that of (+)-ABA. Percent cross reaction = 100 X/Y, where X = moles of (+)-ABA required to displace 50% of the tracer and Y = moles of cross-reactant required to produce the same effect.

Compound	Cross Reaction
	(%)
(+) - ABA	100
(±) - ABA	46
Methyl (±) - ABA	100
Methyl (+) - ABA	92
Phaseic acid	0.3
Dihydrophaseic acid	<0.1
(±) - ABA - glucose ester	12

Figure 1. Typical standard curves for ELISA quantitation of ABA enantiomers and their glucose-esters. B/B_0 is the relative binding (%) of tracer in the presence (B) as compared to the absence (B_0) of respective standards. Dilution of ABA-mAb was 1:16. Bars represent mean $\pm$ SD of standard triplicates in $n = 15$ assays (except for ABA-GE, $n = 2$ assays). Inset; linearized logit/log plot of the data, where $\text{logit } B/B_0 = \ln[(B/B_0)/(100 - B/B_0)]$. (±)-ABA-GE = (±)-ABA-glucose ester.

Figure 2. Parallelism of extract dilution curves for rice extracts and standard (+)-ABA curve. Different rice extracts were assayed at various dilutions with and without exogenous ABA. Where indicated, 10 pmole of (+)-ABA per mg fresh weight was added to the extract. Extract (1) = detached rice leaves; Extract (2) = attached rice leaves.

Figure 3. Effect of excision on levels of free (A) and conjugated (B) ABA in attached and detached rice leaves. ABA was extracted at the indicated periods and separated to its free and conjugated forms, which were further quantitated by ELISA. Bars represent 2 SE of triplicates in n = 3 assays.

Figure 4. Time course of changes in free ABA content during senescence of attached and detached rice leaves. Details are as described in Fig. 3.

5.2 EFFECT OF ABA ON ETHYLENE PRODUCTION IN RELATION TO EXCISION AND SENESCENCE OF RICE LEAVES

Sonia philosoph-Hadas, Nehemia Aharoni and Shang Fa Yang

ABA and ethylene are known to regulate leaf senescence, when both hormones induce symptoms typically associated with the natural aging process (1, 3, 8, 10, 11, 14, 16, 18). However, the precise interaction and sequential relationship between these two growth regulators in controlling senescence remained unclear. Evidence that ABA promoted ethylene production in leaf explants (3, 14), led to the suggestion that ABA may accelerate senescence by stimulating ethylene production (11, 12, 14). On the other hand it was found that ABA treatment, which promoted senescence, substantially suppressed ethylene production in leaves (8, 12, 13, 16, 19), thus suggesting that ABA may control ethylene production during senescence via a feedback mechanism (10). The basis for this discrepancy could be related to various factors such as levels of endogenous hormones, leaf age and particularly whether leaves are attached or detached. In general, an accelerated senescence was observed in detached leaves (5), which were shown to undergo a rapid increase in their ethylene production within minutes or few hours following excision (1, 6, 15). This rapid surge of ethylene production was thought to be an excision-related response which may result either from a rapid induction of the ethylene system (6, 15) or from a possible release of an ethylene inhibitor (20). Because of its ability to suppress ethylene production in young monocot leaves (8, 12, 16, 17, 19), ABA might be a logical candidate for such an internal inhibitory factor in these leaves. In the present study we have examined the effect of ABA on parameters of ethylene biosynthesis pathway during senescence of attached and detached rice leaves. Our data indicate that ABA promotes senescence of rice leaves but inhibits their ethylene production rates through suppression of ACC conversion to ethylene.

Material and Methods. Rice (*Oryza sativa* L. Taichung Native 1) seedlings were grown in vermiculite for 9-10 d in a growth chamber at 30°C, 65% RH and 18 h light period (light intensity of 540 $\mu\text{E.m}^{-2}.\text{s}^{-1}$, provided by a mixture of cool-white and Gro-lux tubes). For experiments using

detached leaves, samples of 10 rice leaf segments (the apical 5 cm of the third leaves), excised from 9-days old seedlings and weighing about 0.1 g, were floated in covered petri dishes containing 10 ml test solution. Incubation was carried out in the growth chamber, under the same conditions and light regime used for plant growth. Where indicated, 0.1 mM of either ABA, benzyl adenine (BA) or methyl jasmonate (MJ) were included in the medium. Methyl (+)-jasmonate was a generous gift from Dr. E. Demole of Firmenich SA, Geneva, Switzerland. At the indicated periods, samples were placed into 14-ml test tubes containing 0.5 ml of deionized H₂O to prevent dehydration, sealed with rubber serum caps after flushing with air, and ethylene accumulated in darkness at 30°C during 1 h was measured. Ethylene measurements in attached leaves were carried out parallelly with segments freshly harvested at the indicated periods, beginning with 9-day old seedlings. Samples of 10 leaf segments were placed immediately after excision in the 14-ml test tubes for the 1-h ethylene measurement, or processed for further analyses.

Ethylene (a 1-mL sample) was withdrawn from each 14-ml test tube with a hypodermic syringe after 1 h incubation, and determined by a gas chromatograph equipped with an activated alumina column and a flame ionization detector. The leaf samples, containing 10 detached or attached rice leaves, were subsequently cut into small pieces, extracted in 10 mL of 80% ethanol for 20 min and assayed for determination of Chl (2), ACC (9) and MACC (7) contents. Extraction and assays procedures of the various parameters tested were performed as detailed in Sections 3.2 and 4.2.

Results and Discussion. Ethylene production rates of detached rice leaf segments floating on petri dishes and of freshly detached segments (representing attached leaves) during short periods (1 h), were compared. Since a rise in ethylene production by excised leaf segments did not occur after 2 h (6, 19), measurements of ethylene production by leaf segments within 1 h of excision were assumed to represent the ethylene production rates in attached leaves (6). It is to be noted that both detached and attached leaves were exposed to the same regime of light / dark cycle and temperature. Results depicted in Fig. 1 show that the rise of ethylene production in detached rice leaves became

detectable 2 h after excision, and reached a peak between 8-10 h from the onset of detachment, which lasted up to 24 h. Subsequently, the ethylene production rates declined moderately, but remained substantially above the initial value for 48 h. Ethylene emanation in attached leaves remained relatively constant at basal levels. The initial surge (4-10 fold) of ethylene production following excision could be the consequence of a wounding response (1, 6, 15). The speed of this ethylene response suggests that the increase in the ethylene biosynthesis parameters might be due either to a rapid induction of synthesis *de novo* or due to an inhibitor release. Thus, attached leaves can be viewed as being capable of synthesizing ethylene at high rates, but this synthesis is suppressed by some unknown inhibitor that excision is able to relieve, thereby resulting in a surge of ethylene production (20). Additionally, a rapid increase in ethylene may occur also via release of ethylene precursors from compartmentation within the tissue, or other inducing factors (elicitors). The findings showing that additional cutting of detached leaves into small pieces did not change significantly their ethylene production rates (Fig. 1) may be interpreted according to both possibilities: Either the excision-induced effect is so great that it masks any other effect derived from the subsequent cuttings, or the relieve of a potential ethylene inhibitor is obtained already by the initial detachment of the leaves, and is therefore unaffected by the additional cuttings.

Several plant growth regulators, applied exogenously to detached leaf segments, were assayed for their effect on the excision-induced ethylene system. Fig. 2 demonstrates that unlike BA, which caused a slight increase of the excision-induced ethylene production, application of exogenous ABA or MJ to detached leaf segments immediately after excision, reduced markedly their ethylene production rates to levels produced by attached leaves. A similar slightly stimulatory effect of BA and an inhibitory effect of ABA on ethylene production rates of detached leaves have been reported previously with wheat (12) and rice (8).

Since external application of ABA reduced markedly the onset of ethylene production in detached leaves (Fig. 2), we tried to further check how ABA affects parameters of ethylene biosynthesis pathway during

24 h following leaf excision. Results depicted in Fig. 3A demonstrate that the rapid increase in ethylene production rates of detached leaves during the initial 24 h-interval was accompanied by a concomitant increase in their ACC (Fig. 3B) and MACC (Fig. 3C) levels, which could be observed, respectively, from the 2nd or 8th h of incubation. As expected, ACC and MACC levels of attached leaves remained fairly constant and relatively lower throughout this 24-h period (Fig. 3). It should be noted that during the initial 2 or 4 h following excision, no significant differences could be observed in ACC and MACC levels of detached and attached leaves. Nevertheless, after 24 h of incubation, ACC and MACC levels of detached leaves were 7-fold and a 4-fold higher, respectively, than the corresponding levels of attached leaves (Fig. 3B, C). The results suggest that the similarity between attached and detached leaves during the first hours may result from a stress effect imposed on both leaves due to experimental conditions. Results depicted in Figs. 3B,C demonstrate that in detached leaves the excision-induced increase in ACC content did not coincide closely with the increase in MACC levels, which were pronounced only 6 h later. Application of ABA to these leaves, which reduced their ethylene production rates to those of attached leaves (Fig. 3A), increased markedly both their ACC (Fig. 3B) and MACC (Fig. 3C) levels. It seems therefore that ABA application to detached rice leaves enhanced both their ACC synthesis and malonylation. It may therefore be concluded that the ABA-inhibited ethylene production of detached rice leaves may stem from inhibition of ACC conversion to ethylene.

Since our short-term kinetics (24 h following excision) showed that detachment imposed immediate changes on the endogenous rates of ethylene production (Figs. 1, 2), it was pertinent to check the age-dependent changes of ethylene and its precursor during senescence of detached and attached leaves. For this purpose the time course of chlorophyll loss (Fig. 4A), ethylene production rates (Fig. 4B) and ACC content (Fig. 4C) were followed simultaneously in senescing detached and attached rice leaves during 5 days in the light. The pattern of endogenous ethylene during senescence of detached rice leaves is characterized by the appearance of a sharp rise in ethylene production occurring immediately after excision, followed by a gradual decrease in ethylene, (Fig. 4B).

On the other hand, attached leaves exhibited basal rates of ethylene production during senescence (Figs. 4B). Similar to previous reports (1) our data clearly demonstrate that leaf detachment hastens their senescence, as evidenced by the complete loss of Chl observed in detached leaves after 5 days. The curve of Chl degradation in detached leaves was characterized by a 20% decrease during the first 8 h, followed by a rapid decrease during the subsequent 4 days. On the other hand Chl loss in attached leaves, which proceeded during the initial 8 h in a similar rate to that of detached leaves, levelled off in the subsequent days. Consequently, 5-days old attached leaves retained about 70% of their Chl content (Fig. 4A). The sharp rise of ethylene production observed in detached leaves (Fig. 4B) coincided with their Chl loss during the initial 8 h, but preceded the subsequent phase of Chl degradation which lasted for 4 days (Fig. 4A). Attached leaves exhibited only a slight ethylene rise during the corresponding first day. It may be therefore concluded that the excision-induced ethylene rise of detached leaves could account for their second and rapid phase of Chl degradation occurring from the first day following excision. Thus, it is conceivable that among other factors excision may hasten senescence of detached rice leaves also via increased ethylene production.

It should be noted that in spite of its inhibitory effect on ethylene production, exogenous ABA stimulated Chl loss (data not shown), and endogenous ABA levels were found to increase with advanced senescence (See Section 5.1). A seemingly contradictory observation of ABA promoting senescence but inhibiting ethylene production was reported previously in rice leaves by Kao and Yang (8), who have suggested that ABA may increase tissue sensitivity to ethylene. The regulatory significance of this ABA inhibitory role is best evaluated when considering the indirect mode of action of ABA in inducing leaf senescence versus the more direct senescence response elicited by ethylene (1, 3, 11, 14). Since ABA was found to inhibit ethylene production in some monocot leaves (8, 12, 16, 17, 19), one is bound to conclude that it is advantageous for the plant to limit the high amounts of ethylene produced in response to either water stress (12, 19) or excision (Fig. 3), thereby retarding its ethylene-enhanced senescence. A

similar conclusion was reported recently for ABA affecting oat leaves in darkness (16, 18).

The origin of the ethylene rise associated with detachment is presently unknown. Most likely it occurs via stimulation of the ACC pathway (Figs. 3, 4). However, our results do not support yet conclusively any of the possible modes of action by which this rapid stimulation may occur.

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FIGURE 1. Effect of excision and cutting on ethylene production rates of rice leaves. Samples of ten rice leaf segments, freshly excised (attached), preincubated (detached) or preincubated and further cut into pieces (detached-cut), were enclosed in 14-mL test tubes at the indicated intervals. The test tubes were incubated in darkness at 30°C and ethylene production rates were monitored after 1-h of incubation. The bars represent SD of 3 replicates.

FIGURE 2. Effect of exogenous plant hormones on excision-induced ethylene production in detached rice leaves. Samples of ten rice leaf segments were excised and incubated in the light with 0.1 mM of BA, ABA or MJ, and their ethylene production rates were determined at various intervals following excision. Parallely, ethylene production of ten attached leaves, freshly harvested at the indicated periods, was determined immediately after their excision.

FIGURE 3. Effect of excision and ABA on ethylene production rates (A), ACC (B) and MACC (C) contents of rice leaves. Samples of ten rice leaf segments were incubated in the light in the presence or absence of 0.1 mM ABA and their ethylene production rates were monitored as described in Fig. 1. The segments were subsequently extracted for determination of their ACC and MACC levels.

FIGURE 4. Time course of changes in chlorophyll content (A), ethylene production rates (B) and ACC levels (C) of attached and detached rice leaves during senescence in the light. Ethylene was determined as described in Fig. 1. The leaf segments were then extracted with ethanol for quantitation of Chl and ACC.

5.3 EFFECTS OF IAA, GA₃ AND BA ON ETHYLENE PRODUCTION AND SENESCENCE OF SPINACH LEAF DISCS

Sonia Philosoph-Hadas, Eynat Pesis and Nehemia Aharoni

It is well documented in various leaves that indoleacetic acid (IAA) and benzyl adenine (BA) enhanced ethylene production rates (1, 3, 7, 8, 9), while gibberellic acid (GA₃) did not affect (3, 4, 7) or caused only a moderate stimulation of ethylene (1). Nevertheless, application of these hormones resulted in many cases in retardation of leaf senescence (2, 3, 5). The effects of these juvenile plant hormones on various factors of the ethylene biosynthesis pathway and on senescence processes in spinach leaf discs were therefore examined.

Materials and Methods. Experiments were performed with fully expanded leaves of spinach (*Spinacia oleracea* L.), which were freshly harvested from the field. Whole detached leaves were dipped for 30 sec in a NaOCl (0.5%) solution for sterilization and subsequently washed with distilled water. Samples of 8 discs, 1 cm in diameter, were excised from the sterilized leaves and incubated on a filter paper in 50-mL flasks containing 1.8 mL of water. Where indicated, 0.1 mM of either IAA, GA₃, or BA solutions were included. The sealed flasks were incubated in darkness at 25°C, and ethylene production and respiration rates, as well as contents of ACC, MACC, Chl and amino acids were assayed daily.

Ethylene and CO₂ were assayed by withdrawing, respectively, 1-mL and 5-mL gas samples from the flasks and determining the concentration by gas chromatographs (see Section 3.2). The disc samples were subsequently extracted with 10 mL of 80% ethanol and the extract was processed for determination of Chl, ACC, MACC and amino acids as detailed in Sections 3.2 and 4.2.

Results and Discussion. A) Effect of IAA. Ethylene production rates of IAA-treated spinach leaf discs were stimulated markedly on the first day of incubation, but subsequently decreased to control levels (Fig. 1A). It seems that IAA treatment resulted in enhancement of wound-ethylene production and in advancement of the climacteric-ethylene rise by one

day. On the other hand, IAA decreased respiration rates during 6 days of incubation, but it did not change the time of appearance of the respiratory climacteric rise (Fig. 1B). Fig. 2A demonstrates that IAA, which did not affect significantly ACC levels during the first 3 days of incubation, prevented ACC accumulation during the subsequent days. On the other hand, MACC levels were markedly inhibited in IAA-treated leaves throughout the incubation (Fig. 2B). This suggests that in the presence of IAA most of the ACC produced was converted to ethylene rather than to MACC. Despite the stimulation of ethylene production by IAA, this hormone inhibited amino acid accumulation (Fig. 3A) and loss of Chl (Fig. 3B). It should be noted that this inhibition of both amino acids accumulation and Chl loss by IAA was pronounced, respectively, only from the third or second day of incubation (Fig. 3). The results indicate that IAA delays significantly senescence processes in spinach leaf discs.

B) Effect of GA₃. Application of GA₃ to spinach leaf discs enhanced markedly their ethylene production rates on the first day and from the fourth day of incubation (Fig. 4A). A GA-enhanced ethylene production was reported so far only in the system of bean leaves (1), whereas in some other leaves no GA stimulatory effect has been found (3, 4, 7). In GA₃-treated leaf discs the enhancement of respiration was completely inhibited during the initial 5 days of incubation (Fig. 4B). Respiration in control discs peaked on the second day, whereas that of GA₃-treated ones was delayed by 5 days. The pattern of changes in ACC content during incubation was quite different from that of ethylene production. Hence, ACC content of control discs on the second day of incubation (Fig. 5A) was relatively low, since most of it was converted either to ethylene (Fig. 4A) or to MACC (Fig. 5B). During the last days of incubation, the decline in ethylene production rates (Fig. 4A) was accompanied by a gradual accumulation of ACC (Fig. 5A). This ACC accumulation could be ascribed mainly to a reduced EFE activity in senescing leaves, but may also partially result from the sharp decline in MACC levels observed during this period (Fig. 5B). These data may implicate that in spinach leaf discs MACC might be hydrolyzed to ACC during advanced stages of senescence (6). GA₃ reduced MACC content (Fig. 5B) and increased ACC levels (Fig. 5A) during the first 3 days of incubation. This may account for the GA₃-stimulated ethylene production rates observed during the

first day of incubation (Fig. 4A). Nevertheless, GA₃ retarded senescence of spinach leaf discs, as evidenced by their reduced levels of amino acids (Fig. 6A) and their higher chlorophyll content (Fig. 6B). Additionally, the rapid phase of chlorophyll loss, which commenced in control discs already after the 3rd day of incubation, was delayed in GA₃-treated discs by 2 days (Fig. 6B). It seems therefore that GA₃, like IAA, retards senescence processes in leaves (2, 3, 5) in spite of its stimulatory effect on ethylene biosynthesis pathway.

C) Effect of BA. Likewise to the previous two juvenile hormones, BA stimulated ethylene production rates during the initial day of incubation, delayed the peak of ethylene by 3 days (Fig. 7A) and inhibited respiration rates during 6 days of incubation (Fig. 7B). Similar to GA₃, BA caused a moderate increase in ACC content on the second day of incubation which was followed by a significant inhibition during the subsequent days (Fig. 8A). This was accompanied by a significant reduction of MACC levels in BA-treated discs during the first 3 days of incubation (Fig. 8B). BA retarded senescence of spinach leaf discs as exhibited by reduced accumulation of amino acid content (Fig. 9A). However, the BA-retarding effect was not pronounced significantly on chlorophyll content (Fig. 9B). This could be attributed to the relatively high BA concentration (10^{-4} M) employed in this experiment, since efficient retardation of senescence was obtained with much lower concentrations of cytokinins (2, 3, 5).

D) Conclusions. The retarding effect of the juvenile plant hormones on senescence of spinach leaf discs was evidenced by inhibition of some characteristic events in the process, namely delay of protein hydrolysis, chlorophyll loss and the appearance of the climacteric-like rises in respiration and ethylene. The hormones delayed senescence in spite of their stimulatory effect on ethylene biosynthesis pathway during the first incubation day, which was exhibited by enhanced rates of wound-ethylene production, increased ACC levels and inhibition of MACC formation. Since ethylene was reported to enhance leaf senescence (see Section 3.1), it can be concluded that these plant hormones may be capable of antagonizing ethylene action, thereby preventing its senescence-enhancing effect. In the present study, application of the hormones at high concentrations (10^{-4} M) resulted in a remarkable stimulation of ethylene production rates (Figs. 1A, 4A, 7A), and

therefore only a moderate retardation of chlorophyll loss was achieved (Figs. 3B, 6B, 9B). Efficient retardation of leaf senescence was reported in other systems with much lower concentrations of these hormones (2, 3, 5). Accordingly, IAA was most efficient at 10^{-6} M, whereas GA₃ and kinetin were most efficient at 10^{-4} M in retarding chlorophyll loss of tobacco (3) and lettuce (2) leaves. It seems therefore, that the hormones are more efficient in retarding senescence when applied at low concentrations, which do not stimulate markedly ethylene production. A possible commercial application of this hormonal treatments to delay senescence of fresh herbs is described in Section 5.6.

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FIGURE 1. Effect of IAA on rates of ethylene production (A) and respiration (B) in senescing spinach leaf discs. Samples of 8 spinach leaf discs were incubated in 50-mL flasks at 25°C in the dark, with or without 0.1 mM IAA. Ethylene and CO₂ emanation in the flask were monitored daily.

FIGURE 2. Effect of IAA on levels of ACC (A) and MACC (B) in senescing spinach leaf discs. ACC and MACC were determined on the ethanolic extracts of the leaf discs treated as in Fig. 1.

FIGURE 3. Effect of IAA on levels of amino acids (A) and chlorophyll (B) in senescing spinach leaf discs. Contents of amino acids and chlorophyll were determined on the ethanolic extracts of the leaf discs treated as described in Fig. 1.

FIGURE 4. Effect of GA₃ on rates of ethylene production (A) and respiration (B) in senescing spinach leaf discs. Samples of 8 leaf discs were incubated with or without 0.1 mM GA₃ as detailed in Fig. 1.

FIGURE 5. Effect of GA₃ on levels of ACC (A) and MACC (B) in senescing spinach leaf discs.

FIGURE 6. Effect of GA₃ on levels of amino acids (A) and chlorophyll (B) in senescing spinach leaf discs.

FIGURE 7. Effect of BA on rates of ethylene production (A) and respiration (B) in senescing spinach leaf discs. Samples of 8 leaf discs were incubated with or without 0.1 mM BA as detailed in Fig. 1.

FIGURE 8. Effect of BA on levels of ACC (A) and MACC (B) in senescing spinach leaf discs.

FIGURE 9. Effect of BA on levels of amino acids (A) and chlorophyll (B) in senescing spinach leaf discs.

5.4 HORMONES-ENHANCED ETHYLENE PRODUCTION IN LEAVES

HORMONE-ENHANCED ETHYLENE PRODUCTION IN LEAVES

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ABSTRACT

The effects of the phytohormones gibberellin (GA₃), cytokinin (BA) and abscisic acid (ABA) on ethylene biosynthesis were studied in a model system of spinach leaf discs. All three hormones were found to accelerate ethylene production. The GA₃ and BA-enhanced ethylene production during 2 days of incubation resulted mainly from increased ACC synthesis and its conversion to ethylene, as well as from suppression of ACC malonylation. On the other hand, application of ABA, although causing enhancement of both ACC synthesis and ethylene formation from ACC, resulted in a remarkable stimulation of ACC malonylation. The hormones effects were found both on endogenous and on ACC-dependent ethylene production. Further investigation of the stimulatory effect of ABA on EFE activity revealed that it is CO₂-mediated. The significance of these hormonal effects on ethylene production will be discussed.

Abbreviations: ABA = abscisic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; AS = ACC synthase; BA = benzyl adenine; EFE = ethylene-forming enzyme; GA = gibberellic acid; IAA = indole acetic acid; MACC = 1-(malonylamino)cyclopropane-1-carboxylic acid; MT = malonyl transferase; SAM = S-adenosylmethionine.

INTRODUCTION

The pathway of ethylene biosynthesis was originally established by Adams and Yang (1979) as proceeding from methionine via SAM and ACC. In addition to serving as a precursor for ethylene, ACC can also be widely metabolized to its malonyl conjugate, MACC (Yang and Hoffman, 1984). The effect of plant hormones on ethylene biosynthesis has already been investigated. Evidence was reported that auxins, cytokinins, GA and ABA (Abeles and Rubinstein, 1964; Lieberman, 1979; McKeon and Yang, 1987) can influence ethylene production in vegetative tissues, mainly on the step converting SAM to ACC (Yang and Hoffman, 1984). However, recent findings (Suttle, 1986) suggest that cytokinins can also stimulate the step converting ACC to ethylene by enhancing EFE activity. The conjugation of ACC to MACC by MT, which was suggested as the third regulatory step in the sequence of ethylene biosynthesis (Yang and Hoffman, 1984), was also affected by IAA and kinetin (Amrhein et al., 1982;

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Kionka and Amrhein, 1984).

The present work describes the effects of GA₃, BA and ABA on the three regulatory steps of ethylene biosynthesis in spinach leaf discs.

MATERIALS AND METHODS

Experiments were performed with fully expanded leaves of spinach (*Spinacia oleracea* L.) which were freshly harvested from the field. Whole detached leaves were incubated immediately after excision in the dark at 25°C under 95% RH for 24 h prior to the experiment. The preincubated intact leaves were then dipped for 30 sec in a solution containing 10⁻³ M of the specified hormone + 0.5% Triton. Discs, 1 cm in diameter, were excised from the treated leaves and incubated on a filter paper in 50-ml flasks containing 1.8 ml of water. Where indicated, 10⁻⁴ M of hormones (IAA, GA₃, BA, ABA) was included. Ethylene and CO₂ evolved in the flasks were absorbed by 0.25 M Hg(ClO₄)₂ and 10% KOH solutions, respectively, as detailed previously (Meir et al., 1985). The flasks were sealed and when necessary 15% CO₂ or 10 µl/L ethylene was injected, as described before (Philosoph-Hadas et al., 1986). The sealed flasks were incubated in the dark at 25°C, and ethylene production, ACC and MACC content as well as *in vivo* EFE and MT activities were assayed periodically.

Ethylene was assayed as described by Meir et al. (1985). ACC was extracted as described by Jiao et al. (1986) and determined according to the method of Lizada and Yang (1979). The ACC extract was passed through an ion exchange resin column of Dowex-50 (H⁺ form) for separation of MACC (Liu et al., 1983). Quantification of MACC in the Dowex effluent was performed according to Jiao et al. (1986). *In vivo* EFE and MT activities were determined by measuring the capabilities of the leaf discs to produce ethylene or MACC, respectively, in the presence of a saturating ACC concentration. ACC was applied by floating the discs for 2 h on either 0.5 mM or 3 mM ACC.

RESULTS

Application of the plant hormones GA₃ and BA to spinach leaf discs resulted in an increase in ethylene production during 6 days of incubation, while IAA had almost no effect (Fig. 1). Since the hormone-stimulating effect was more pronounced in the first 2 days, further experiments were concentrated on this initial period. Additionally, the effect of ABA on ethylene biosynthesis was investigated, since its physiological effects in the plant tissue are antagonistic to BA and GA₃ effects (Goldthwaite, 1987). Table 1 shows that BA and ABA stimulated total ACC produced by the tissue during 48 h, while the GA₃-stimulatory effect was observed only between 24 and 48 h. The effect of these three hormones on ethylene formation was similar (Table 2). Additionally, GA₃ and BA reduced ACC malonylation, while ABA treatment resulted

FIGURE 1. Effect of hormone (10^{-4} M) on the time course of ethylene production rates in spinach leaf discs.

TABLE 1. Effect of hormone (10^{-4} M) treatment on total ACC biosynthesis in spinach leaf discs. Total ACC formed in the system was calculated according to the sum of nmol ethylene + nmol ACC + nmol MACC produced at the indicated time.

TIME	TREATMENT		
	GA ₃	BA	ABA
h	total ACC (% of control)		
0-24	80	174	187
24-48	471	560	663

TABLE 2. Effect of hormone (10^{-4} M) treatment on ethylene production in spinach leaf discs.

TIME	TREATMENT		
	GA ₃	BA	ABA
h	ethylene (% of control)		
0-24	81	192	142
24-48	172	424	355

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in increased MACC formation (Table 3). As a consequence of increased consumption of ACC for both ethylene and MACC formation, ABA-treated discs showed very low ACC levels (data not shown). A similar effect of these three hormones on the steps of ethylene biosynthesis was obtained also with intact spinach leaves (not shown).

TABLE 3. Effect of hormone (10^{-4} M) treatment on MACC formation in spinach leaf discs.

TIME	TREATMENT		
	GA ₃	BA	ABA
h	MACC (% of control)		
0-24	10	9	163
24-48	42	17	900

In order to study further the hormones effects on the conversion of ACC to ethylene and to MACC, discs were fed with exogenous 3 mM or 0.5 mM ACC, respectively. These ACC concentrations were found to be the saturating concentrations for the *in vivo* reactions of the enzymes involved (EFE and MT). Results depicted in Table 4 show that EFE activity was stimulated in GA₃-, BA- and ABA-treated discs, with ABA being the most effective. On the other hand, GA₃ and BA inhibited MT activity, while ABA stimulated it (Table 4).

TABLE 4. Effect of hormone (10^{-4} M) treatment on the capabilities of spinach leaf discs to convert exogenous ACC to ethylene and to MACC. Control activities were 3.0 ± 0.1 nmol C₂H₄/gFW and 24.5 ± 2 nmol MACC/gFW.

TREATMENT	ACC---->C ₂ H ₄	ACC---->MACC
activity (% of control)		
GA ₃	172	74
BA	232	76
ABA	350	150

The effect of ABA on EFE activity, which was the most pronounced one, was further studied. Since both CO₂ (Kao and Yang, 1982; Philosoph-Hadas et al., 1986) and ethylene (Chalutz et al., 1984; Philosoph-Hadas et al., 1986) have been reported to enhance EFE activity, we checked the possibility that the ABA stimulatory effect on this step is

mediated through these two gases. Accordingly, ACC-dependent ethylene production was monitored in ABA-treated discs in the presence of 15% CO₂ or 10 μ l/L ethylene. Figures 2A and B demonstrate that both CO₂ and ABA could, each individually, promote EFE activity, while simultaneous application of the two did not cause any further increase as compared with the effect of CO₂ alone. On the other hand, ABA could promote EFE activity also in ethylene-treated discs (Fig. 2B). These data indicate that the ABA-promoting effect, which cannot be attributed to the ethylene effect, may be CO₂-mediated.

FIGURE 2. Effect of ABA (10⁻⁴M) and CO₂ (15%) on ACC-dependent ethylene production in spinach leaf discs not treated (A) or pretreated (B) with ethylene (10 μ l/L).

Discussion

The stimulating effect of cytokinins on ethylene production in vegetative tissues has already been established (Abeles and Rubinstein, 1964; Lieberman, 1979; McKeon and Yang, 1987). In our system, consisting of spinach leaf discs, BA enhanced ethylene biosynthesis as expected (Fig. 1, Table 2). Treatment with ABA was very effective in spinach leaf discs, causing increased ethylene production, especially on the second day of incubation (Table 2). So far, the reported effects of ABA on ethylene biosynthesis in vegetative tissues were inconsistent, showing suppression (Gertman and Fuchs, 1972; Kao and Yang, 1983), enhancement (Aharoni, 1975; Sagee et al., 1980), or no effect (McKeon et al., 1982; Yoshii and Imaseki, 1981) on ethylene production rates. Additionally, ABA was found to repress IAA or wilting-induced ethylene production in leaves (McKeon and Yang, 1987).

GA₃ enhanced markedly ethylene production in spinach leaf discs (Fig. 1, Table 2) as well as in intact leaves of spinach, dill and chives (data not shown). In many studies GA₃ was found to be inactive in stimulating ethylene production (Aharoni et al., 1979; Fuchs and Lieberman, 1968; Gertman and Fuchs, 1972; Lieberman, 1979). The bean leaf was the only system, to our knowledge, that showed GA-enhanced ethylene production (Abeles and Rubinstein, 1964).

The hormone-enhanced ethylene production in spinach leaves resulted from enhancement of ACC formation (Table 1) and enhancement of conversion of ACC to ethylene (Table 4). Increased total ACC biosynthesis (Table 1), which resulted probably from the hormones effect on AS, was closely correlated with the hormone-enhanced ethylene production (Table 2), suggesting this step to be a rate-limiting one in this tissue, as proposed by Yang et al. (1980). This hormone-stimulated ACC synthesis is in agreement with other reports, which suggest that the hormones auxin, cytokinin and ABA regulate ethylene production in vegetative tissues at the level of ACC synthesis (McKeon and Yang, 1987; Yoshii and Imaseki, 1981). Nevertheless, our data indicate that the hormones enhance also the step of ACC conversion to ethylene (Table 4), with GA₃ being as effective as the other hormones. A similar enhancing effect of cytokinin on EFE activity has been reported recently (Suttle, 1986). It is therefore suggested that, although ACC synthesis is the rate-limiting step in our system, the enhancing effect of the hormones on the two other steps of the ethylene pathway can contribute to their overall stimulatory effect on ethylene biosynthesis. These effects may be more prominent in tissues where the ACC is not the limiting factor.

Besides their effect on ACC formation and on ACC conversion to ethylene, the hormones also affected ACC malonylation (Table 3). While BA and GA₃ inhibited MACC formation, ABA enhanced this step, in parallel to its stimulating effect on ACC formation. The fact that the levels of extractable ACC in ABA-treated discs were very low (data not shown), indicates that ABA-induced malonylation resulted from both increased AS and MT activities. These conclusions were further confirmed by showing the GA₃- and BA-inhibitory effects and the ABA-stimulating effect on the *in vivo* MT activity in the presence of exogenous ACC (Table 4).

Since the effect of ABA on EFE activity was quite remarkable (Table 4), we investigated its nature further. Based on the fact that ABA did not cause any additional stimulation of EFE activity in the presence of CO₂ (Fig. 2), it is suggested that the ABA effect on EFE activity is CO₂-mediated.

The results suggest that the stimulatory effect of GA₃ and BA on ethylene biosynthesis resulted from increased availability of free ACC, through both enhancement of ACC formation and suppression of ACC malonylation. Since these hormones are known as senescence-retardants (Goldthwaite, 1987), it is conceivable that GA₃ and BA, like CO₂ and Ag⁺ (Yang and Hoffman, 1984), block ethylene action

although stimulating its synthesis. On the other hand, ABA, which is known as a potent accelerator of leaf senescence (Goldthwaite, 1987), was found to stimulate all three steps of the ethylene biosynthesis pathway. It seems therefore that the two senescence hormones, ethylene and ABA, act in the same direction.

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6. EFFECT OF STRESS-INDUCED ETHYLENE PRODUCTION ON SENESCENCE AND STORABILITY OF FRESH HERBS

6.1 EVALUATION OF TEMPERATURE-STRESS DAMAGES IN BASIL LEAVES

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Basil is a plant of tropical origin which is susceptible to chilling. Exposure of basil leaves to low temperatures (below 12°C) during storage and transport resulted in development of chilling injury, which is visually expressed by browning of the leaves followed by their decay. These temperature-stress damages, causing a great commercial loss of the crop, are of economic significance since basil is one of the main fresh herbs exported from Israel. Therefore, early detection of chilling injury may be of great importance for evaluation of optimal storage conditions as well as for selection of cultivars which are more resistant to low temperatures.

Cell membranes are considered as likely sites of primary effects of chilling as exhibited by phase transition (11), increased membrane permeability (5, 7, 17) and changes in membrane composition (15, 16). Additionally, many metabolic changes have been recorded during prolonged chilling of various chilling-susceptible plants. Prominent among these are: (A) Changes involving oxidative reactions, expressed in chilling-promoted generation of free radicals, leading to lipid peroxidation (15, 16); (B) Chilling-induced ethylene production (10, 13, 14), which was accompanied by increased levels of ACC (1, 12) and MACC (12). In several tissues it was found that prolonged low-temperature storage caused inhibition of ethylene emanation due to inhibition of the ethylene forming enzyme (EFE) activity (2, 3, 11). Thus, increased levels of ethylene and ACC and inhibition of EFE as response to chilling, may be regarded as indicators of the plant susceptibility to chilling injury (1, 10). These various metabolic responses of chilling-susceptible species to low temperatures were examined in basil leaves, for evaluating biochemical changes that may precede the appearance of visual symptoms.

Materials and Methods. Experiments were conducted with mature leaves and bunches of basil (*Ocimum basilicum*) freshly harvested from the greenhouse or the field. The bunches (100 g each) were packed in 1-kg cartons, lined with a perforated polyethylene liner, and incubated for 6-7 days in cold-storage rooms at various temperatures ranging from 4 to 12°C. The chilling-induced damages observed after cold storage of basil leaves were estimated visually according to a chilling injury index ranging from 1 (= none) to 5 (= severe). Parallely, various parameters of ethylene biosynthesis and senescence were determined daily, either at the storage temperatures or after a 5-hours warming period at 25°C, with samples of 4-5 leaves excised from the stored bunches.

Determination of ethylene biosynthesis parameters: Basil bunches or detached leaves were stored for 6 days at 6°C (chilled) or at 12°C (control) into 2-L or 0.5-L jars, respectively, containing a moistened filter paper and sealed with paraffin. Every day samples of 7 leaves or 2-3 stems were enclosed inside other 0.5-L or 2-L jars, respectively, which were sealed and incubated for 5 h either at the storage temperatures or at 25°C (warming) for ethylene accumulation. Ethylene production rates were determined by a gas chromatograph with 2-mL gas samples withdrawn from the sealed jars. ACC was extracted by homogenizing samples of 1-g basil leaf tissue in 10 mL of 80% ethanol. All further processing of ACC was as described previously (9). MACC content was quantitated after hydrolysis of the extract as detailed by Hoffman et al (4). In vivo activity of EFE was determined by measuring the capability of basil leaves to produce ethylene in the presence of a saturating concentration of ACC. Thus, leaves were floated on a 0.1 mM ACC solution for 3 h with a gentle shaking, blotted dry and then enclosed for 2 additional h into 0.5-L jars, for measuring their ACC-dependent ethylene production (9).

Determination of ion leakage: The changes in membrane permeability of basil leaves, excised from bunches stored at various temperatures (6, 8, 10 and 12°C), were estimated daily by measuring the degree of ion leakage from leaf discs into the water medium (8). Thus, samples of 7 basil leaf discs (8 mm in diameter), excised from the stored leaves, were floated on a 30-mL water solution kept in 50-mL flasks. The sealed

flasks were incubated for 3.5 h with a gentle shaking at 30°C, and the electric conductivity of the solution was determined at the end of incubation by a Microprocessor conductivity meter model LF-535. For determination of total leakage from the cells, the conductivity of the solution in the incubation flasks was monitored after a 20-min heating in an autoclave. This value was taken as 100% of leakage from the leaf discs.

Determination of lipid oxidation: The level of total aldehydes, which are products of lipid oxidation, was determined daily in basil leaves stored at 4, 8 and 12°C. Aldehydes were extracted from 1-g leaves with 20% TCA and assayed with the thiobarbituric acid (TBA) test described by Kosugi and Kikugawa (6), as detailed in Section 3.3.

Results and Discussion. When basil bunches were stored continuously at 6-10°C, visual symptoms of chilling injury could be observed only after 4 days, while very slight leaf browning was detected after a 6-day storage at 12°C (Fig. 1). Transferring the bunches on the 5th day of storage to shelf-life (12°C) for 24 h, resulted in increased differences between the symptoms observed to occur at the various temperatures. It seems that 12°C is the optimal temperature for basil storage, whereas at 6°C the most severe chilling damages occurred. Therefore, control leaves were kept at 12°C throughout the experiments. Fig. 2 demonstrates a significant acceleration of ion leakage in basil bunches stored at 6°C as compared with the other storage temperatures. The remarkable accelerated ion leakage at 6°C was pronounced already from the 4th day of storage, parallely with the appearance of leaf browning (Fig. 1). On the other hand, no increase in ion leakage could be detected in basil bunches stored at 8 or 10°C for 5 days until they had been transferred to shelf-life at 12°C. It seems therefore that ion leakage may indicate the degree of chilling damage developed, but it does not precede the appearance of the visual browning symptoms.

When examining the effect of low-temperature storage on the process of lipid oxidation, as exhibited by accumulation of aldehydes, a different pattern was observed. Storage of basil bunches at both 4°C and 12°C, which caused, respectively, severe or very slight chilling injury

(Fig. 1), resulted in increased accumulation of aldehydes (Fig. 3).

However, bunches exposed to a moderate chilling damage at 8°C retained a constant and lower level of aldehydes during 7 days of storage.

Significant differences between aldehydes levels following storage at 4 and 8°C were pronounced only after 5 days of storage (Fig. 3), parallelly to the changes in visual symptoms (Fig. 1) and ion leakage (Fig. 2). A possible explanation for this discrepancy may be attributed to retardation of senescence processes in bunches stored at 8°C, as exhibited by their low aldehydes level in spite of their moderate chilling injury. Hence, the increased aldehydes accumulation observed in bunches stored at 4°C resulted from a chilling injury effect, while in control leaves stored at 12°C the increase resulted from enhanced senescence. It may be concluded therefore, that accumulation of aldehydes is not a unique symptom of chilling injury during prolonged incubation of this tissue.

Since a chilling-induced ethylene production has been reported to occur in various tissues (3, 10, 13, 14), the changes in ethylene emanation caused upon exposure of basil bunches to low temperatures were examined. Previous reports showed that the increase in ethylene parameters is more pronounced when assayed after a warming period rather than at the storage temperature (14). Fig. 4A demonstrates that a chilling-induced ethylene production, assayed after warming, could be detected in basil bunches on the 5th day of storage at 6°C. This significant ethylene rise was accompanied by a corresponding gradual increase in ACC content, initiating on the same day (Fig. 4B). Control leaves stored at 12°C showed a typical climacteric-like ACC rise, peaking on the 5th day of storage (Fig. 4B). It seems that the significant chilling-induced changes in both ethylene and ACC occurred after 4-5 days of storage, in parallel to the appearance of the visual (Fig. 1) and biochemical (Figs. 3, 2) symptoms of chilling injury. Therefore, parameters of ethylene biosynthesis pathway were further examined on the 5th day of storage. Remarkable rises in ethylene production rates (Fig. 5A), as well as in contents of ACC (Fig. 5B) and MACC (Fig. 5C), which peaked after 4.5-5.5 hours of warming, were detected in basil bunches assayed on the 5th day of storage. All these parameters were significantly higher in leaves stored at 6°C than in

control leaves stored at 12°C, suggesting a chilling-induced change. The increase in MACC level observed after 4.5 h of warming (Fig. 5C) could account for the commencement of decrease in ACC (Fig. 5B) and ethylene (Fig. 5A) observed at that time.

When examining the ethylene parameters on the whole basil bunch, some discrepancy may occur due to variations in the chilling susceptibility of young and old leaves. Further experiments were therefore carried out with old, basal leaves, which were found to be most sensitive to chilling injury (data not shown). The general pattern of changes in ACC content of these leaves (Fig. 6) was similar to that observed in whole bunches (Fig. 4B). However, unlike in bunches, significant differences in ACC content between old leaves stored at 6 and 12°C were already detected on the third day of incubation (Fig. 6A, inset). Moreover, when ACC levels were assayed after a warming period, a remarkable difference could be detected even earlier, on the second day of storage (Fig. 6B, inset). These results suggest that the chilling-induced rise of ACC preceded in old leaves all the visual and biochemical parameters tested. The pattern of ethylene production rates in old basil leaves (Fig. 7A) was similar to that observed in whole bunches (Fig. 4A), exhibiting a significant chilling-induced ethylene rise from the 4th day of storage. No corresponding increase in EFE activity could be observed in old leaves stored at 6°C and assayed without warming, since EFE activity decreased gradually during 6 days of storage at both 6 and 12°C (Fig. 7B). However, when ethylene and EFE were assayed in old leaves after a warming period, the chilling-induced ethylene rise could be detected on the third day of storage at 6°C (Fig. 8A), while the chilling-reduced EFE activity was observed already from the first day (Fig. 8B). Additionally, control leaves stored at 12°C showed a climacteric-like rise in EFE activity, peaking on the 5th day of storage (Fig. 8B). It should be noted that EFE activity after one day of storage at 6°C, assayed either before (Fig. 7B) or after (Fig. 8B) warming, was reduced by about 50% as compared with leaves stored at 12°C. Since EFE is a membraneous enzyme, this immediate decline in its activity might indicate a rapid response to low temperatures occurring in membranes of chilling-susceptible species. The results suggest that low-temperature stresses in basil, which is a chilling-susceptible plant, are exhibited

initially by reduced EFE activity and increased levels of ACC. These parameters of ethylene biosynthesis pathway precede by several days the appearance of visual and other biochemical symptoms of chilling injury in this crop.

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FIGURE 1. Time course of changes in the visual symptoms of chilling injury occurring in basil bunches stored at various temperatures. Basil bunches (100 g each) were packed in 1-kg polyethylene-lined cartons and stored for 6 days at the indicated temperatures. The chilling-induced damages were estimated according to a chilling injury index ranging from 1 (= none) to 5 (= severe). Bars indicate SE of 3 cartons.

FIGURE 2. Effect of storage temperature on degree of ion leakage from basil leaf discs. Samples of 7 basil leaf discs were excised daily from bunches stored as described in Fig. 1, and were floated on 30-mL water solution for 3.5 h at 30°C. The conductivity of this solution was measured before and after a 20-min heating in an autoclave. Results are expressed as % of ion leakage, when the conductivity after autoclavization represents 100% leakage.

FIGURE 3. Effect of storage temperature on content of total aldehydes in basil leaves. Aldehydes were extracted from 1-g basil leaves detached daily from bunches stored as described in Fig. 1, and their content was assayed with the TBA test.

FIGURE 4. Effect of cold storage on ethylene production rates (A) and ACC content (B) of basil bunches. Samples of 8-10 leaves, detached daily from bunches stored as described in Fig. 1, were enclosed in 0.5-L jars. Ethylene was determined in the jars after a 5-h warming period at 25°C, while ACC was extracted from the leaves immediately after storage.

FIGURE 5. Changes in ethylene production rates (A) and contents of ACC (B) and MACC (C) during a warming period of basil leaves. Leaves were detached on the 5th day from bunches stored as described in Fig. 1. Ethylene production rates and levels of ACC and MACC of the detached leaves were assayed in hourly intervals during incubation at 25°C.

FIGURE 6. Changes in ACC content of old basil leaves incubated in 0.5-L jars for 6 days at 6°C or 12°C, and assayed daily either at the storage temperatures (A) or after a 5-h warming period at 25°C (B). Insets, changes in ACC content during the first 3 storage days.

FIGURE 7. Effect of low temperature on ethylene production rates (A) and EFE activity (B) of old basil leaves assayed without warming. Old basil leaves, stored as described in Fig. 6, were assayed for their ethylene production rates and EFE activity immediately after storage.

FIGURE 8. Effect of low temperature on ethylene production rates (A) and EFE activity (B) of old basil leaves assayed after warming. Details are as described in Fig. 7, except that assays were performed after a 5-h warming period at 25°C.

7. EVALUATION OF PRACTICAL METHODS, BASED ON THE USE OF ANTI-ETHYLENE AGENTS, FOR REGULATING SENESCENCE OF EDIBLE PRODUCTS

7.1 MODIFIED ATMOSPHERES IN FILM PACKAGES DELAY SENESCENCE AND DECAY OF FRESH HERBS

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Abstract

The effect of modified atmospheres (MA) produced inside film packages, on the keeping quality of yellowing-susceptible fresh herbs, was examined. The experiments were conducted under simulated conditions of air transport from Israel to Europe, as well as with an actual shipment, during which temperature fluctuations were between 4 and 15°C. Under these conditions, bulk packaging in perforated polyethylene (P.PE)-lined cartons reduced water loss markedly, but were not effective in retarding yellowing and decay of watercress, chives, chervil, sorrel, coriander, dill or parsley. Packaging of these herbs in non-perforated PE (NP.PE)-lined cartons resulted in a remarkable retardation of both yellowing and decay. The beneficial effect of the MA produced in the NP.PE liner, in delaying senescence, could be improved further by a combination with vacuum precooling. The best results for watercress, chives, chervil and sorrel, which are the most yellowing-susceptible herbs, were obtained by packaging them in sealed PE (S.PE)-lined cartons. However, sealed film packaging is applicable only if the temperature during transit and storage is well controlled.

Retardation of yellowing and decay in our experiments was attributed mainly to the accumulation of respiratory CO_2 within the package, rather than to the depletion of oxygen. Thus, chlorophyll degradation of the herbs was delayed effectively when 5% CO_2 in air was applied in a flow-through system. Addition of 10 ppm ethylene to this flow system negated the CO_2 effect partially or completely, depending on the herb species. This implies that the senescence-retarding effect of elevated CO_2 concentrations in green herbs can be attributed mainly to the ability of CO_2 to antagonize ethylene action.

1. Introduction

Twenty two species of fresh herbs are exported from Israel to Europe, mainly during the winter season. The herbs are packed in bulk (1-or 3-kg cartons) and transported by air to the overseas markets.

Hruschka and Wang (1979) have shown that watercress, parsley and mint, packed in P.PE bags, kept well for several weeks at 0°C. However, when green herbs, which are vulnerable to yellowing and decay, are transported by air and then exposed to warm temperatures during marketing, packaging in NP.PE liners (non-sealed) was found to be more effective in delaying leaf senescence. This was attributed to the elevation of CO₂ concentration (to 1-5%) within the PE liners (Aharoni et al., 1988).

Application of MA is the most effective method other than low temperatures, to retard senescence of harvested leafy vegetables (Lipton, 1975). Various aspects of MA during transport and storage of green vegetables have been reviewed in the last decade (Isenberg, 1979; Brecht, 1980; Kader, 1986). Chlorophyll loss is retarded about equally well by low O₂ (less than 5%) and by elevated CO₂ (generally more than 5%). The combination of these two gases is slightly more effective than either one alone (Lipton and Mackey, 1987). Although this prominent effect of MA, evident on many leafy vegetables, is well known, only few reports are available on MA in green herbs (Apeland, 1971; Nsengimana and Bangerth, 1981).

The use of MA produced in film package during transit and storage could be harmful if the temperatures can not be well controlled. This may result in an improper balance between CO₂ and O₂ which may lead to anaerobic respiration (Hardenburg, 1971). Thus, the produce packed in sealed films may develop severe off-odors when held too long at non-optimal temperatures. Prevention of anaerobic respiration during a short transport under uncontrolled temperatures could be achieved by rapid removal of field heat of the produce packed in film packages. Vacuum cooling is the most efficient method of precooling leafy products and therefore it might be used in combination with non-sealed film packaging (Aharoni and Ben-Yehoshua, 1973).

In this work we examined the use of different methods of film packaging, with or without vacuum precooling, as to their suitability for yellowing-susceptible herbs. The results were obtained in the laboratory in transit-simulated conditions as well as in an experimental air shipment to England.

2. Materials and methods

Green herbs were grown on various farms in Israel during the winter season. Coriander, dill, parsley and watercress were grown outdoors, and chives, chervil and sorrel were grown in unheated greenhouses. The fresh herbs were packed in bulk or in small bunches (~100g each), in corrugated cartons (herb weight = 1 or 3 kg)

lined with 0.03 mm-thick P.PE or NP.PE. The upper edges of the liner were folded over inside the carton. In order to enhance formation of MA, some of the NP.PE liners were tightly sealed with rubber bands.

Vacuum precooling of either P.PE or NP.PE (unsealed) was performed 6-8 after harvest, in a commercial installation (Voskamp and Vrijland, Holland). In preliminary experiments conducted with dill, coriander, chives, chervil, watercress and mint, it was found that fresh herbs are most suitable for vacuum precooling due to their high ratio of surface area to volume. Our experiments revealed that the effect of film package on the cooling rate was negligible. The herbs, either vacuum precooled or untreated, were transferred to a cold-storage room and kept under simulated conditions of air shipment to Europe. The holding temperatures are given in the Results section. In one experiment, with vacuum-precooled herbs, the products were airfreighted to England. At the end of the transit and storage, the keeping quality of the herbs was estimated visually. Yellowing and decay indexes were determined as follows: 1 = none; 2 = slight; 3 = moderate; 4 = severe; and 5 = very severe. Each determination consisted of 4-6 cartons (replicates) from which 6-8 bunches per carton were sampled and scored separately.

3. Results and discussion

Packaging of fresh herbs in PE-lined cartons reduced markedly water loss and prevented completely wilting of any of the herbs stored for 5 days at 6°C plus 2 days at 12°C (Fig. 1). Packaging in a sealed PE (S.PE)-lined carton resulted in the maximal reduction of both yellowing and decay. Measuring the changes of the respiratory gases inside the packages of chives (1-kg cartons) revealed that the accumulation of CO₂ (5.7%) rather than the decrease in O₂ (12%) could be the reason for retardation of senescence (Fig. 1).

In watercress packed in 3-kg cartons, the 4-5% CO₂ and 15.5-15.8% O₂ formed in the NP.PE liners was insufficient to retard yellowing (Fig. 2, Table 1). This was achieved by elevation of CO₂ to 7-10% and reduction of O₂ to 4-6%, following sealing of the liners with rubber bands. In this case, it seems that both reduced O₂ and elevated CO₂ could contribute to maintenance of the high quality of watercress. However, this sealing caused a slight alcoholic smell in the S.PE packages, which dissipated completely upon aeration. When watercress was packed in S.PE-lined cartons of 1 kg instead of 3 kg, the same rate of yellowing retardation was achieved (Table 1), without the development of off-odors. A better balance between CO₂/O₂ concentrations inside the package was achieved by the

faster cooling rate of the smaller packages as well as by the higher ratio between film surface and produce weight. The results suggest that in order to retard efficiently the yellowing of watercress, without development of off-odors during holding conditions as described (Fig. 2, Table 1), the CO_2 level should be above 7% and the O_2 level should not drop below 5%.

A relatively high efficiency of the S.PE liner in maintaining the quality of chives and watercress, was found also with chervil, sorrel, coriander, dill and parsley (not shown). However, severe off-odors developed when the produce packed in the sealed NP.PE liner was held during the first 24 h at 18°C (not shown). Therefore, we tried to improve the keeping quality of the herbs packed in NP.PE-lined cartons by rapid removal of field heat, achieved by vacuum precooling. This experiment was conducted in an actual air shipment to London, England, where some fresh herbs, packed in 3-kg cartons were tested.

The temperature of the herbs was reduced in the vacuum cooler from 15-17°C to 1-2°C within 25 min. regardless of the type of liner. The herbs were stored for 36 h in a cold-storage room (6-8°C) and then airfreighted to England. Upon arrival, the temperature of the precooled and non-precooled herbs was 9-10°C and 13-14°C, respectively. Examination of their quality was done after an additional 5 days' storage, at 7.0-8.0°C. Very good results were achieved with coriander, which was vulnerable to both yellowing and decay (Fig. 3). These developments were retarded completely by packaging the herb in a NP.PE liner followed by vacuum precooling.

In this experiment and in some others, vacuum precooling improved the keeping quality of all the other yellowing susceptible herbs packed in NP.PE liners. However, in all cases, vacuum precooling by itself (without MA) was not sufficient to retard yellowing and decay. In some herbs, packed in P.PE liners and vacuum precooled, a slight wilting was noticed. This was not found in vacuum precooled herbs packed in NP.PE liners.

Ethylene has long been known to enhance leaf senescence (Burg, 1968, Lieberman, 1979) and to reduce the market quality of floral and immature-fruit vegetables and ornamental foliage (Kader, 1985). In many plant tissues CO_2 was found to antagonize the senescence enhancing effects of ethylene (Burg and Burg, 1967; Abeles, 1972; Aharoni et al. 1979; Lieberman, 1979).

In order to examine whether the senescence-retarding effect of CO_2 is related to its blocking effect on ethylene action, CO_2 (5% in air) was applied in a flow-through system. In both dill (Fig. 4) and chives

(Fig. 5), 5% CO₂ retarded chlorophyll loss markedly. Application of 10µl/l ethylene to this flow system negated completely the CO₂ effect in dill but had no effect in chives. It should be noted that in chives, exogenously applied ethylene did not enhance chlorophyll loss at all. These results may indicate that CO₂, besides retarding senescence as an antiethylene agent, may act also independently in other ways.

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Table 1 - Effect of weight of watercress, packed in a polyethylene-lined carton, on CO₂/O₂ concentrations and yellowing index. Cartons were held for 6 days at 6°C followed by 2 days at 12°C.

Packed- carton weight (kg)	Type of package	Gas concentrations(%) at 12°C		Yellowing index*
		CO ₂	O ₂	
1	non-sealed	0.5-3.0	18.1-20.5	3.7
	sealed	7.4-8.9	8.3-12.0	1.6
3	non-sealed	4.0-5.0	15.5-15.8	3.2
	sealed	7.0-10.0	4.0-6.0	1.6

* From 1 = none to 5 = very severe yellowing.

Figure 1 - Effect of the package type on weight loss, decay and yellowing of chives stored for 5 days at 6°C plus 2 days at 12°C.

Figure 2 - Effect of MA produced in PE-lined cartons of watercress on the yellowing indexes.

Figure 3 - Effect of vacuum precooling on the yellowing and decay of stored coriander packed in PE-lined cartons. Coriander was packed in 3-kg cartons and airfreighted to England. The quality was checked 7 days after harvest.

Figures 4 and 5 - Effect of exogenously applied CO₂ and ethylene on chlorophyll loss in dill and chives. Gases were applied in a flow-through system.

D. DESCRIPTION OF COOPERATION

The Israeli and the American teams have continued in this study the close cooperation already established in their previous BARD project. Contact was maintained by correspondence, as well as by exchange and meetings of personnel. All the scientists participating in the project have met at the Gordon Conference on Post-harvest Physiology in July 1986, just after the initiation of the project. Mutual plans for the future experiments were outlined during discussions at this meeting. Dr. Philosoph-Hadas from the Israeli group was a guest scientist in Dr. Yang's laboratory and began there the research on this project. She has continued her work on this study in Dr. Aharoni's laboratory in Israel since September 1986. Dr. Meir has joined the Israeli group in September 1987. Dr. Lizada from the Phillipines has participated in the project during her sabbatical leave in Dr. Yang's laboratory from 1987 to 1989. Dr. Aharoni visited the American team in May 1987 for comparison of results and discussions concerning the plans of the future research.

Part of the results are included in students theses as follows:

- (A) Section 3.4 is included in the M.Sc. thesis of T. Ching, done in the laboratory of Dr. SF. Yang.
- (B) Section 6.1 is included in the M.Sc. thesis of A. Reuveni, done in the laboratory of Dr. N. Aharoni.
- (C) Section 5.3 is included in a high-school final project of E. Pesis, done in the laboratory of Dr. N. Aharoni.

E. EVALUATION OF RESEARCH ACHIEVEMENTS

In the present study we have examined the effects of ethylene on senescence processes and keeping quality of leafy vegetables and fresh herbs as related to interaction with plant growth regulators (IAA, BA, kinetin, thidiazuron, GA₃, ABA), inhibitors of ethylene synthesis and action (AVG, AOA, Co²⁺, Ni²⁺, CO₂, Ag⁺, STS, NBD) and inhibitors of photorespiration (MSX, INH). Evaluation of leaf senescence was improved and broadened by developing and applying various parameters for a simultaneous quantitation of the different senescence-associated

processes (loss of chlorophyll and protein, increase in ion leakage, amino acid content and products of lipid oxidation). Additionally, a monoclonal antibody against ABA was developed and combined with a sensitive ELISA technique for quantitation of ABA in leaves.

The present research work has expanded and deepened our understanding of the control of ethylene biosynthesis and its role in deteriorative processes of perishable leafy commodities. Unlike in fruit ripening, the role of ethylene in leaf senescence has not been clearly demonstrated. Since excision often induces ethylene production, the response of foliar tissues to exogenous ethylene has been complicated by the possible effects of excision. Attempts were therefore made to distinguish between the effects of ethylene and excision on senescence of detached leaves. While ethylene was found to be the enhancer of leaf senescence, excision was suggested as one of the factors initiating the process. Some additional new and original findings regarding the control of ethylene biosynthesis were established in the course of this study as follows:

(A) CO_2 was found to exert its stimulatory effect on the conversion of ACC to ethylene by enhancing not only the activity but also the synthesis of EFE in tobacco leaf discs.

(B) The sucrose-enhanced ethylene production was found to be derived from a sucrose-stimulated activity of an enzyme hydrolyzing IAA-amine conjugates.

(C) Unlike in other leafy tissues, we could find in spinach leaves a GA_3 -enhanced ethylene production, which resulted mainly from increased ACC synthesis and its conversion to ethylene as well as from suppression of ACC malonylation.

(D) In spinach leaves (dicot) ABA exerted a stimulatory effect on EFE activity which was CO_2 -mediated, whereas in rice leaves (monocot) ABA inhibited ethylene production via suppression of ACC conversion to ethylene.

(E) Indications for a senescence-dependent metabolism of MACC were found in spinach leaves.

(F) The CO_2 -inhibited photorespiration in rice leaves was found to be responsible partially for the senescence-retarding effect of CO_2 in the light.

(G) An early chilling-induced rise in ACC content and reduction in EFE

activity were found in basil leaves. This early detection of chilling injury is applied for evaluation of optimal storage conditions as well as for selection of basil cultivars which are more resistant to low temperatures.

Based on our findings regarding the senescence-enhancing effect of ethylene in edible products, it was suggested to block ethylene action by naturally-occurring compounds. Practical implications of this method with leafy vegetables and fresh herbs resulted in a combined treatment of plant hormones and CO₂. This was found as an effective and applicable method for increasing shelf-life and improving the keeping quality of various perishable, commercially-important commodities.

F. LIST OF PUBLICATIONS

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