ETHYLENE PRODUCTION BY ALFALFA IN RELATION TO APHID FEEDING

By

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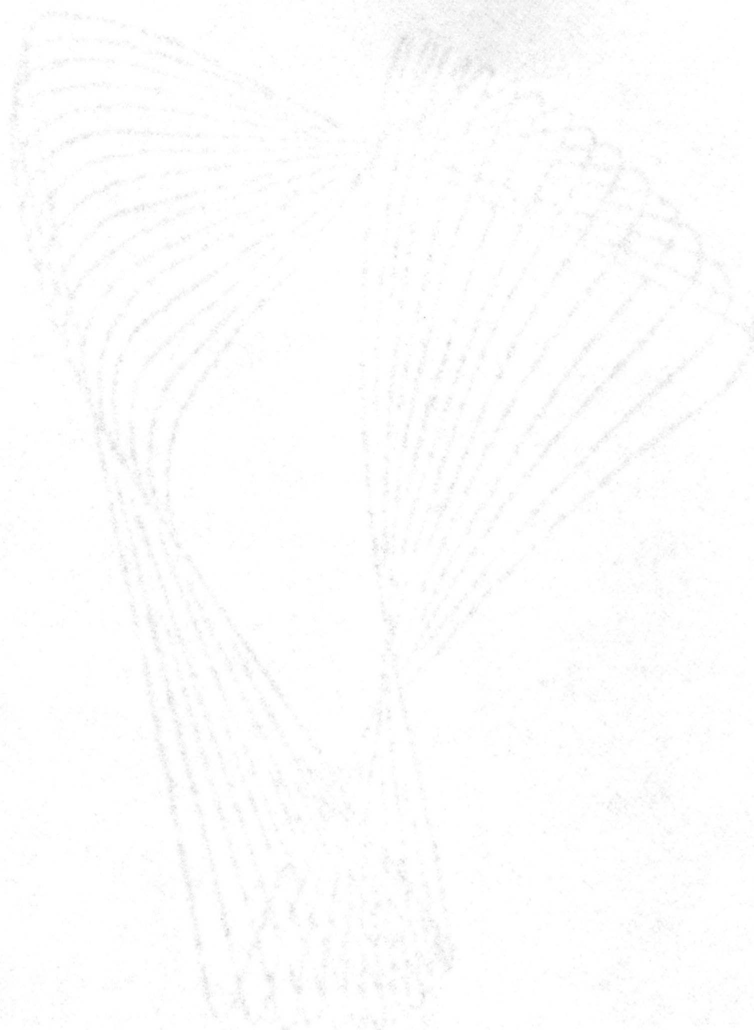

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CHAPTER I

INTRODUCTION

The spotted alfalfa aphid, Therioaphis maculata (Buckton) and the pea aphid, Acyrthosiphon pisum (Harris) are economically important insects in alfalfa production. Alternative control measures for these pests, other than insecticides, can be of ecological and economical advantage (Sorensen et al., 1988). Understanding at the molecular level the relationship between these pests and their host plants permit even more effective control by host plant resistance. Characterizing biochemical markers associated with aspects of this interaction related to feeding of aphids such as ethylene may indicate what biochemical as well as physiological processes are occurring in the relationship between aphids and their hosts.

Ethylene is produced by plants in response to environmental stress and is a marker for plant senescence (Leshem et al., 1986). Ethylene evolution has been demonstrated in conjunction with feeding by the spotted tentiform leafminer, Phyllonorycter blancardella F. (Kappel et al., 1987) and by the cotton fleahopper, Pseudatomoscelis seriatus (Reuter) (Martin et al., 1988). Ethylene production has been shown to be associated with

hypersensitivity reactions (HR) to plant pathogens in resistant plants (Legge & Thompson, 1983). The HR involves production of plant defense chemicals like phytoalexins by induction of key enzymes such as L-Phenylalanineammonia-lyase (EC 4.3.1.5) (PAL). Ethylene is believed to be the compound responsible for the activation of genes mediating production of such enzymes (Ecker & Davis, 1987). Ethylene production by resistant Pinus radiata D.Don in response to attack by Sirex noctilio F. has also been documented (Shain & Hillis, 1972). It is speculated that ethylene may be usedas a marker to distinguish the susceptible and resistant P.radiata.

Lipid peroxidation is another indication of plant stress and senescence. Membranal integrity of cells is compromised by actions of phospholipases, hydrolases, and lipoxygenase (Leshem et al., 1986). Hilderbrand et al. (1986) demonstrated that feeding by the twospotted spider mite, Tetranychus urticae Koch, in soybeans caused an increase in lipid peroxidation and in the activity of lipoxygenase. Products resulting from the action of lipoxygenase upon the phospholipid substrates (lipid peroxides) decompose to give aldehydes and volatile hydrocarbons such as ethane, pentane (Pitkanen et al., 1989; Leshem et al., 1986), and ethylene in combination with 1-aminocyclopropane-1-carboxylic acid (Bousquet & Thimann, 1984; Legge & Thompson, 1983).

The compound 1-aminocyclopropane-1-carboxylic acid

(ACC) is an immediate precursor to ethylene (Adams & Yang,1979). The formation of ACC by ACC synthase is the rate limiting step in ethylene biosynthesis (Bleeker, 1987; Boller et al., 1979).

The overall objectives of this study were to (1.) determine if ethylene is produced by alfalfa in response to aphid feeding and to characterize the production (2.)compare the ethylene production by susceptible, toleant, and resistant clones (3.) describe the biochemical components of ethylene production in response to aphid feeding by investigating ACC, the affects of ethylene inhibitors, and lipid peroxidation. The above objectives reflect the interest in investigating the theory that the response of alfalfa to feeding by aphids is a senescence-like process.

CHAPTER II

LITERATURE REVIEW

Effects of Ethylene on Plants

Since the report by Crocker and Knight (1908), it has been known that small amounts of ethylene have a pronounced influence on all types of plants. As little as 0.1μl/l was shown to cause carnation petals to wither. Gane (1934) provided chemical evidence that plants themselves produced ethylene. Not until 1935 was there a theory implicating ethylene as an important component in plant development. Zimmerman and Wilcoxon (1935) suggested that ethylene may play an important role in the effects caused by indole-3-acetic acid (IAA) such as epinasty. It is now well documented that ethylene is an important part of many processes mediated by auxins such as IAA (Table I) and regulates many important enzymes in plants (Table II).

Ethylene and Senescence

Senescence is defined as the deteriorative process that is a natural cause of death (Leopold, 1961). The first indication that ethylene has a role in foliar senescence was that the incubation of plant material in the presence of ethylene caused loss of chlorophyll (Mack, 1927; Nilsen &

TABLE I

DEVELOPMENTAL PROCESSES WHERE AUXIN-INDUCED ETHYLNENE PRODUCTION IS THOUGHT TO MEDIATE AUXIN ACTION

1. Abscission
2. Apical dominance
3. Branch angle
4. Bud growth
5. Callus, Shoot initiation and growth
6. Epinasty
7. Flowering inhibition
8. Flowering, promotion in bromeliads
9. Flowering, senescence
10. Flowering, sex expression in cucurbits
11. Hypertrophy of hypocotyls
12. Isocoumarin formation in carrots
13. Latex flow, promotion
14. Phenylalanine ammonia lyase
15. Root elongation, inhibition
16. Root initiation
17. Stem elongation, inhibition
18. Swelling, onion leaf bases

Source: Abeles,F.B.."Ethylene and Plant Development: An: Introduction." Ethylene and Plant Development. pp 1-8.

TABLE II

ENZYMES REGULATED BY ETHYLENE

Function / Enzyme
<u>ABSCISSION</u>
Cellulase
Polygalacturonidase
<u>AERENCHYMA</u>
Cellulase
<u>RIPENING</u>
Cellulase
Chlorophyllase
Invertase
Laccase
Malate dehydrogenase
Polygalacturonidase
<u>SENESCENCE</u>
Ribonuclease
<u>STRESS</u>
Beta-1,3-glucanase
Chitinase
Cinnamate-4-hydroxylase
Hydroxycinnamate CoA ligase
Hydroxyproline rich glycoprotein
Phenylalanine ammonia lyase
<u>FUNCTION NOT KNOWN</u>
Ethylene mono-oxygenase
Peroxidase

Source: Abeles,F.B.. "Ethylene and Plant Development: An Introduction." Ethylene and Plant Development. pp 1-8.

Hodges, 1983). The efficacy of the ethylene treatment was most pronounced using excised tissue maintained under continuous light (Gepstein & Thimann, 1981) and may also be dependent upon the maturity of the tissue (Brady et al., 1974). Treatment of excised tissue with ACC also promoted chlorophyll breakdown (Kao & Yang, 1983). Additional evidence that ethylene plays a role in foliar senescence was the demonstration of an ethylene climacteric during the course of the process. A rise in the endogenous levels of ethylene has been observed in both freshly detached senescing leaves (Aharoni et al., 1979) and in mature green leaf tissue excised and induced to senesce after removal from the plant (Aharoni et al., 1979; McGlasson et al., 1975; Roberts & Obsorne, 1981). In the latter instance, the rise in ethylene production can occur within 24 hours after removal and may be related to a wound phenomenon associated with tissue excisions (Even-Chen et al., 1978).

A number of workers have documented the appearance of the ethylene climacteric as an event subsequent to the first visible signs of senescence (Aharoni et al., 1979; Even-Chen et al., 1978). A number of reports correlating the ethylene climacteric with the period of most rapid chlorophyll loss (Gepstein & Thimann, 1981; Aharoni et al., 1979; Ferguson et al., 1983) may implicate it as a regulator of the rate of senescence.

The mode of action of ethylene in plants is not definitively known. However, it is widely accepted that

there is a definite relationship between ethylene production and plant senescence and/or plant stress.

From what is known about the senescence process, it is assumed that all plant cells produce ethylene at a constant low level. Thus, the ability for ethylene to act as a regulator or as an active participant in physiological processes is dependent upon one or both of the following (1.) a change in sensitivity of the cell to the endogenous levels of ethylene and/or (2.) a response caused by a change in the level of ethylene produced by the tissue (Abeles, 1985).

Biosynthesis of Ethylene

The biosynthesis of ethylene in plants occurs via a relatively simple pathway involving two enzymatic steps. S-adenosyl-L-methionine (SAM) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (S-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) (Bleeker, 1987). The intermediate ACC is subsequently oxidized to ethylene by a poorly understood enzymatic step involving what's commonly referred to as the ethylene forming enzyme (EFE) (Bleeker, 1987). It has been proposed that a pyridoxal phosphate and a Schiff's base intermediate are involved in the conversion of S-adenosylmethionine to ACC (Figure 1). Adam & Mayak (1984) reported on an enzyme in carnation petals that may be EFE, but the results were inconclusive.

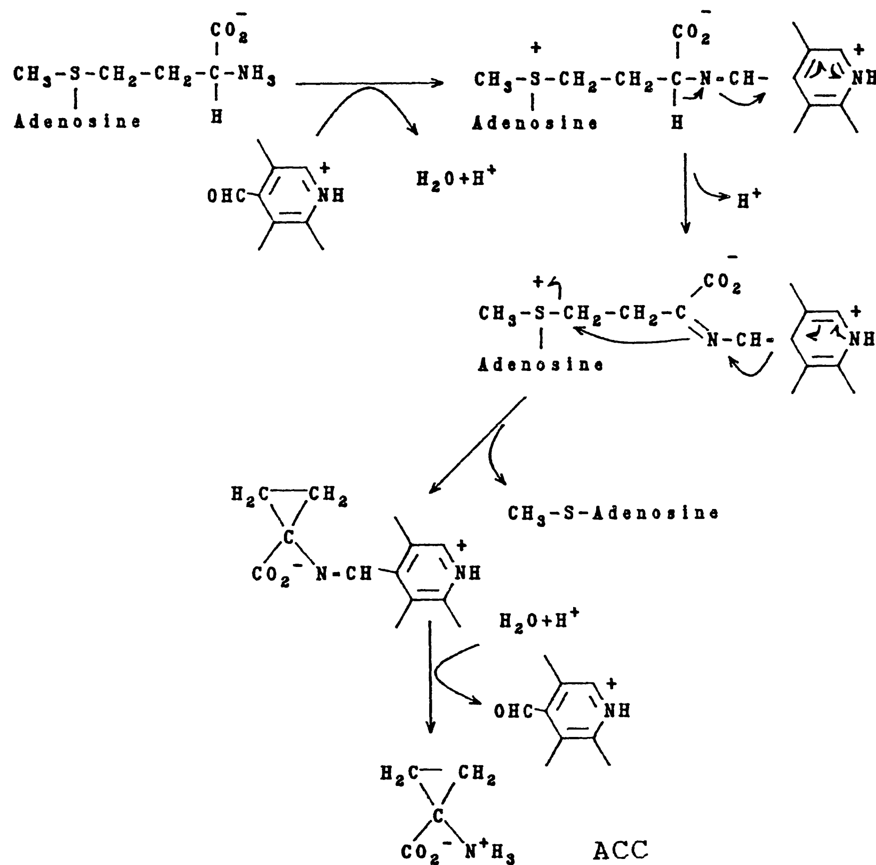


Figure 1. Biosynthetic pathway for ACC production from S-adenosylmethionine catalyzed by ACC synthase. The substituted carboxaldehyde represents enzyme bound pyridoxal phosphate Modified from Smith and Hall, 1984

ACC was first isolated from ripe apples and pears over 30 years ago (Burroughs, 1957). Burroughs (1960) observed that the amount of ACC increased in pears during storage and speculated that this amino acid may be involved in fruit ripening. The importance of ACC in plant physiology was notrealized until the discovery by Adams & Yang (1979) that ethylene was synthesized from ACC (Figure 2). This pathway operates in a number of higher plant tissues.

Cameron et al. (1979) demonstrated that the application of ACC to plant tissues (except for preclimacteric flowers and fruit) caused a marked increase in the production of ethylene. This suggests that the enzyme system converting ACC to ethylene is largely constitutive and that the formation of ACC is the rate-limiting step in this process. The enzyme system that is responsible for the conversion of ACC to ethylene still remains to be completely characterized, but the reaction mechanisms and some of the degradation products have been identified. As noted by Yang (1981) ACC can be oxidized by a hydroxylase to N-hydroxy-ACC which is then fragmented into ethylene and cyanoformic acid. Cyanoformic acid is very labile and breaks down spontaneously to carbon dioxide (derived from the carboxyl group of ACC) and hydrogen cyanide (derived from carbon number one of ACC). Some evidence suggests that the involvement of hydroperoxides, formed by lipid peroxidation are necessary for the formation of a free radical intermediate of ACC, which in turn undergoes a two electron

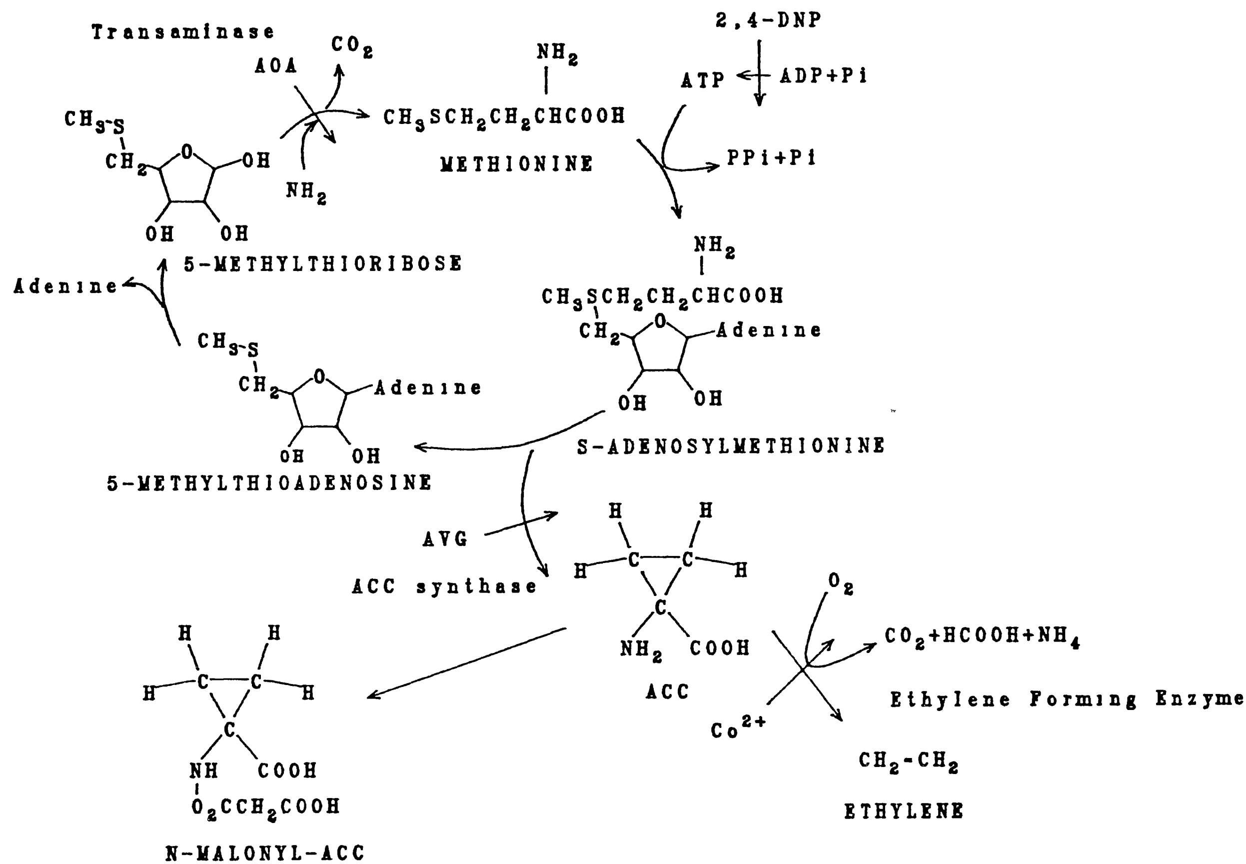


Figure 2 Proposed pathway for the biosynthesis of ethylene from methionine Modified from Adams and Yang, 1979.

oxidation to form ethylene (Figure 3) (Legge & Thompson, 1983).

There is an alternate pathway for the fate of ACC other than the formation of ethylene. Amrhein et al. (1984) discovered in buckwheat seedlings that endogenously supplied ACC was converted to a conjugate that was identified as malonyl-ACC (MACC) (Figure 2). Apelbaum & Yang (1981) had previously observed that the loss of ACC during an incubation period was greater than the quantity of ethylene produced during the same period, suggesting that ACC must have been metabolized by some pathway other than that for ethylene production. Since MACC is a poor producer of ethylene and the conjugation of ACC to MACC is irreversible, it is thought that MACC is a biologically inactive end-product rather than a storage form of ACC in plants (Amrhein et al., 1982).

Another mechanism for the production of ethylene by plants is through the formation and decomposition of lipid peroxides formed from polyunsaturated fatty acids such as linoleic acid. Membranal phospholipids are acted upon by phospholipase A_2, phospholipase B, lysophospholipase, and lipolytic acyl hydrolase to form free polyunsaturated fatty acids. These in turn are acted upon by the enzyme lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) to form conjugated 9- and 13-hydroperoxy fatty acids (Galliard, 1979). Verhagen et al. (1978) indicated that hydroperoxy fatty acids are further degraded by

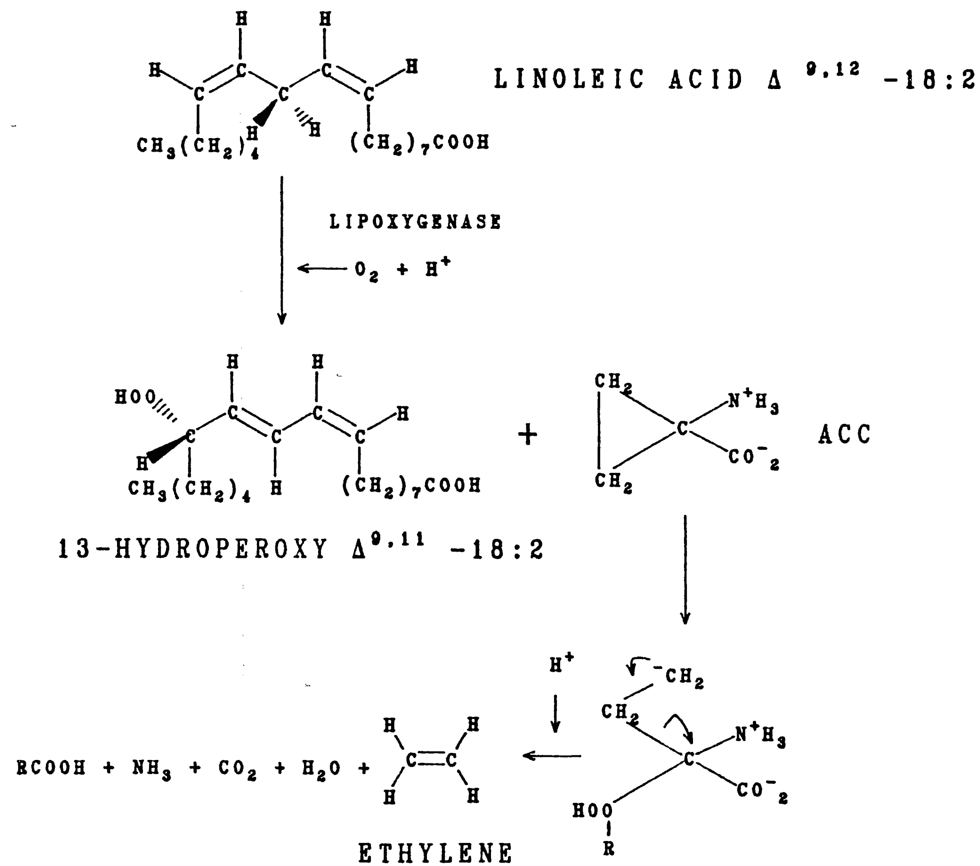


Figure 3. Proposed mechanism for the involvement of lipid peroxides in ethylene biosynthesis

lipoxygenase to yield volatile hydrocarbons such as pentane, ethane and ethylene.

Peroxidation is described as the process (which includes autoxidation) that produces peroxides and their degradation products, which includes products such as malondialdehyde (MDA), ethane, pentane, and ethylene. MDA is used as a measure of lipid peroxidation in many systems (Dhindsa et al., 1980).

The enzyme lipoxygenase plays an important role in the generation of lipid peroxides. This enzyme catalyzes the oxidation of unsaturated fatty acids containing a 1,4-cis, cis-pentadiene system (Vliegenthart & Veldink, 1982). Of particular consequence is the oxygenation of linoleic and linolenic acids to hydroperoxy derivatives. This process is detrimental to the proper function of membranes in plants, causing the loss of fluidity of membranes (membrane lipids become more crystalline) at physiological temperatures (Pauls & Thompson, 1980). Moreover, this contributes to cell deterioration and causes membranes to become "leaky". This process in turn contributes to the loss of chlorophyll and photosynthate in senescing tissues (Kar, 1986).

Ethylene Production and Insect Feeding

Ethylene production by plants in relation to insect feeding has been demonstrated by apple leaves in response to feeding by the spotted tentiform leafminer (Kappel et al., 1987) and by cotton in response to cotton fleahopper (Duffey

& Powell, 1979; Martin et al., 1988; Burden et al., 1989). It was noted that when fed upon, apple leaves produced significantly more ethylene than those without feeding. Duffey & Powell (1979) postulated that the ethylene produced by cotton in response to fleahopper feeding resulted from inoculation of the plant with a plant pathogen by the fleahopper. However, more recently Martin et al. (1988) have suggested that salivary enzymes of the fleahopper are responsible for the effect. One of these enzymes, polygalacturonase, caused an increased level of ethylene production in comparison to controls. This was due to necrosis caused by degradation of the middle lammelae. Burden et al. (1989) demonstrated that the fleahopper itself contains indole-3-acetic acid (IAA), an auxin known to meditate ethylene activity and 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor to ethylene. When injected into plants, whole body homogenates of fleahopper nymphs and adults caused an increase in ethylene evolution compared to controls.

CHAPTER III

MATERIALS AND METHODS

Insect and Plant Rearing

Colonies of spotted alfalfa aphids (SAA) were reared under greenhouse conditions at 25 ± 7°C with a minimum of 16 hr. photophase. Pea aphids (PA) were maintained in an environmental cabinet at 25 ± 2°C with a photoperiod of 14:10 (L:D). Insects were reared on alfalfa bouquets placed in funnel cages.

Plants used were spotted alfalfa aphid resistant, tolerant, and susceptible selections of alfalfa (*Medicago sativa* L.) cultivar OK-08, an Oklahoma Common that were previously described by Jimenez et al. (1988). In the remainder of this thesis, these plants will be referred to as susceptible, tolerant, and resistant. Plants were grown in an environmental chamber at 25 ± 1°C, relative humidity of 60%, and a photoperiod of 16:8 (L:D).

Aged Aphids and Plant Material

Aphids of three and seven days of age (plus or minus one day) were obtained by placing virginopara of SAA and PA on trifoliolates and allowing the aphids to produce nymphs. The reproductives were removed 24 hrs. later and the nymphs

were allowed to feed and develop for three or seven days. Newly excised trifoliolates were placed in the cages as supplement food over seven days.

Leaves of known age were obtained by marking newly emerging trifoliolates from the plant terminal with enamel paint at the base of the petiole. Leaves were then excised on a particular day as needed.

Ethylene Assays

Three SAA or PA (as treatments) or zero aphids (as uninfested controls) were placed on susceptible, resistant, or tolerant trifoliolate explants. One aphid was placed on each of the three leaflets. These explants were placed in 12 X 75mm culture tubes (Sargent-Welch Co.), with the cut ends of petioles inserted in 1ml of 1% tissue culture gradeagar (Gibco, Lab.) made with 0.1% Hoagland's solution (Hoagland & Arnon, 1938), or by placing the cut end of each petiole into liquid 0.1% Hoagland's solution contained in a 500μl microcentrifuge tube, which was then placed in the culture tube. Sample tubes were capped with rubber serum caps four hours prior to sampling. Nymphs that had been produced by the SAA or the PA, were removed daily prior to capping.

Sampling was done by removing 1ml from the headspace of the sample tube and injecting onto a 1/8" X 5'stainless steel column packed with activated alumina (60/80 mesh). The column was installed in a Hewlett-Packard Model 5840 Gas

Chromatograph with an oven temperature of 90°C isothermal, injector temperature of 100°C, and Flame Ionization Detector at 150°C, with Helium as a carrier gas at a rate of 30 ml/min. The volume of ethylene produced was determined by comparing the peak height of sample peaks with a standard curve generated using different concentrations of an ethylene standard (Neogen,Inc.) The rate of ethylene (C_2H_4) production was expressed as follows:

nl C_2H_4 / wt. of tissue (gm) / time (hrs)

where

1 ppm = 1nl/ml

nl C_2H_4 = nl/ml C_2H_4 x vol. of sample tube

Inhibition of Ethylene Biosynthesis

Inhibition of ethylene production by aminoethoxy-vinylglycine (AVG) and aminooxyacetic acid (A0A) was measured at various inhibitor concentrations. The inhibitor solutions were placed into 500μl microcentrifuge tubes which were placed inside 12 X 75mm culture tubes. Cut ends of petioles of explants were then placed in the tubes to allow for tissue uptake of the inhibitor. Explants were exposed to the inhibitors for seven days. The headspace of each tube was sampled daily four hours after capping and the amount of ethylene produced in that time period was quantified as described earlier. Inhibition was expressed as a percentage of ethylene produced by uninhibited controls. The use of these inhibitors had no appearent affect on

an aphid longevitiy or fecundity.

Determination of Lipid Peroxidation

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA, a product of lipid peroxidation) content determined by a reaction with thiobarbituric acid (TBA) (Dhindsa et al.,1980). Trifoliolates were homogenized in 500μl 0.1% trichloroacetic acid (TCA) then centrifuged for 20 minutes in a Microfuge E (Beckman Instruments). One hundred microliters of supernatant were decanted and combined with 400μl of 20% TCA containing 0.5% TBA. This mixture was heated for 30 min. at 95°C, cooled in an ice bath and centrifuged for 20 min. The supernatant was decanted and absorbance then read with a Beckman DU-65 specrtrophotometer at 532nm and the value for non-specific absorbance at 600nm was subtracted. The MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}.

Assay for ACC

ACC concentration in alfalfa tissue was measured as a function of the amount of ethylene produced from isolated ACC. ACC was isolated from 0.2 gms of tissue homogenized in 4ml of methanol:water:chloroform at a 15:5:3 v/v/v ratio. This homogenate was shaken for 24 hrs. at 4°C. One milliliter each of chloroform and water were added to the homogenate, this mixture was then centrifuged for 10 minutes. The aqueous phase was separated, lyophilized, then

resuspended in 40ml of 25% acetonitrile. This solution was then applied to a 500 mg strong cation exchange column (SCX) (Supelco, Inc.). The SCX column was then washed 2X with 750μl of acetonitrile, followed by 2ml of 25% acetonitrile containing 5% ammonia, which was then collected in a 12mm X 75mm culture tube. This tube contained all the amino acids extracted from in the tissue, including ACC, which is the only amino acid which reacts with the reaction mixture forming ethylene. This mixture was 100μl of 100μM $HgCl_2$ and 100μl of 2:1 v/v solution of 5% NaOCl (Clorox bleach) and saturated NaOH (Concepcion et al., 1979). The $HgCl_2$ solution was injected into the culture tube (sealed with a rubber serum stopper) first, shaken, then the NaOCl/NaOH solution was added. Ethylene was determined as described above.

Statistical Analysis

The ANOVA procedure and F tests were used to test forsignificant differences in means and Scheffe's method was used to make multiple comparisons.

CHAPTER IV

RESULTS AND DISCUSSION

Ethylene Production

The standard curve for the analysis of ethylene was linear in the range from 0.1 ppm to 1.0 ppm of ethylene (Figure 4). The amount of ethylene in samples was determined from this type standard curve.

In an initial study to determine if ethylene was produced in response to SAA feeding, trifoliolates from aphid infested and uninfested plants were compared. Trifoliolates were removed from plants and placed in capped tubes for four hours and then ethylene was measured. Aphid-infested plants produced significantly more ethylene than controls (Figure 5).

Since ethylene may be produced by excised trifoliolates in response to being removed from the plant (wounding), the production of ethylene was next monitored over a period of several days. In this experiment trifoliolates were removed from uninfested plants and each was infested with three aphids. Ethylene was then determined each day for seven days. The production of ethylene by uninfested leaves was highest 24 hours postinfestation and then decreased to low levels (Figure 6). Ethylene production by infested leaves

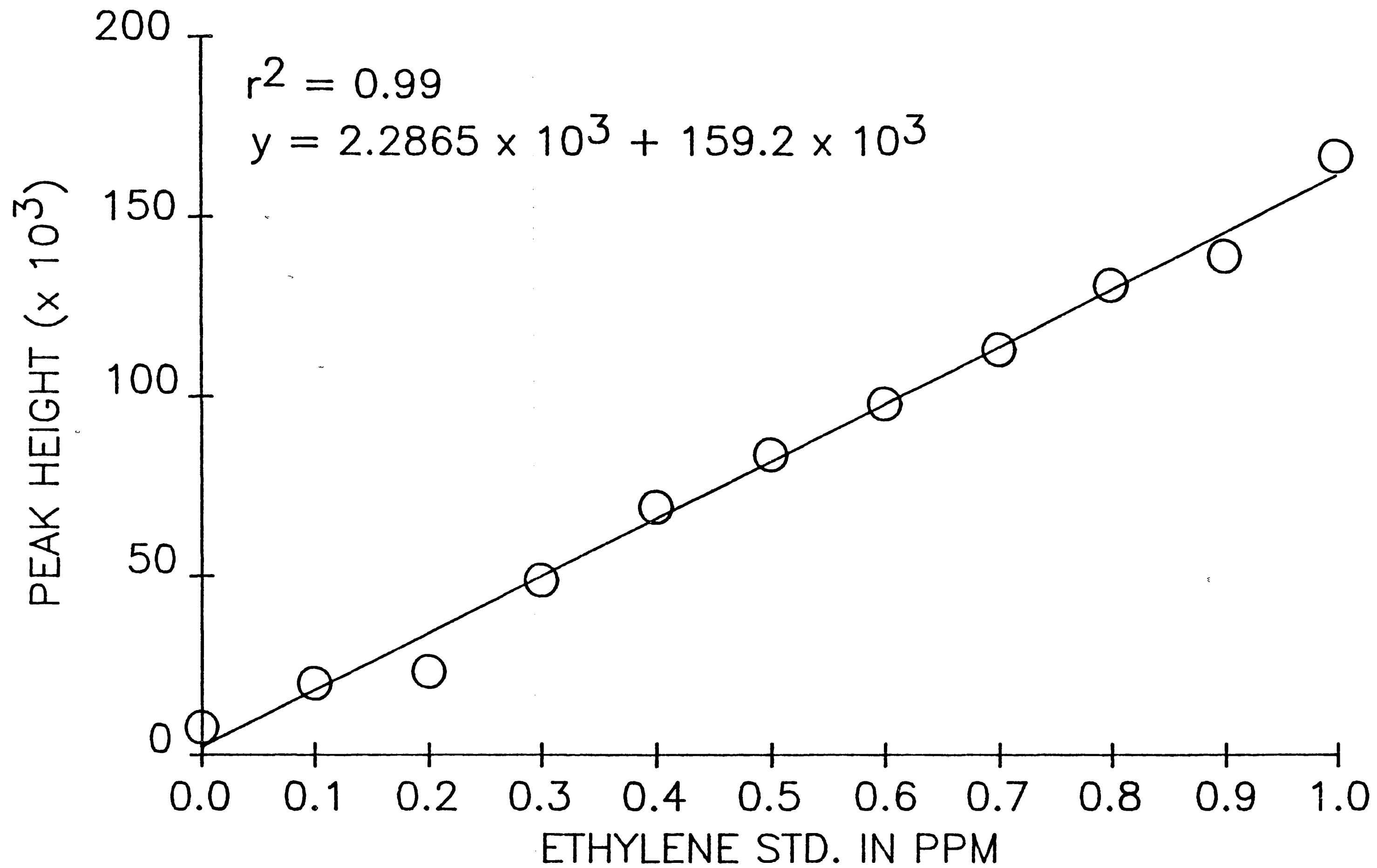


Figure 4. Ethylene standard: Peak height as a function of concentration.

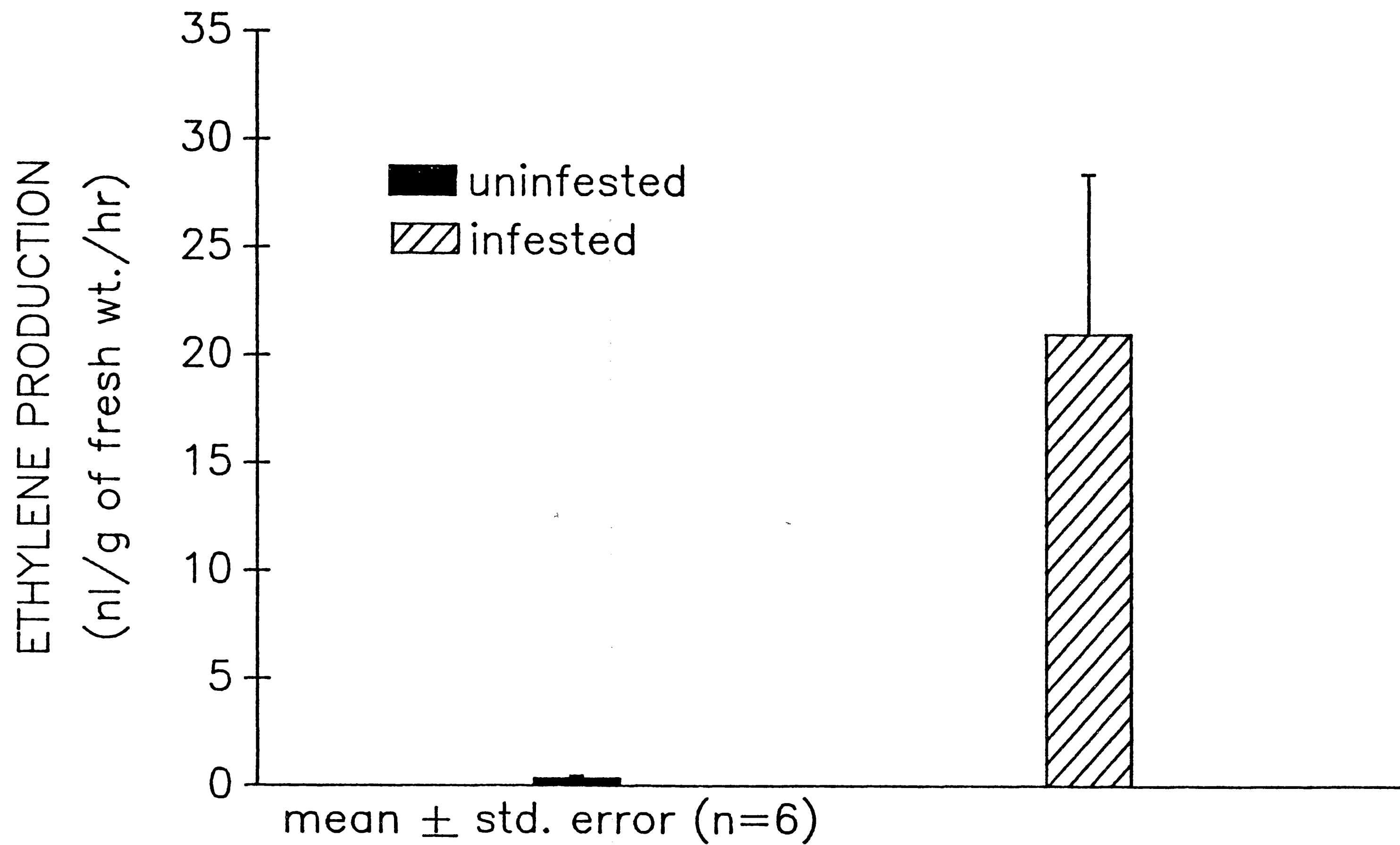


Figure 5. Ethylene production by newly excised leaf tissue. Trifoliolates that were either uninfested or infested with SAA removed from plants and ethylene measured after four hours.

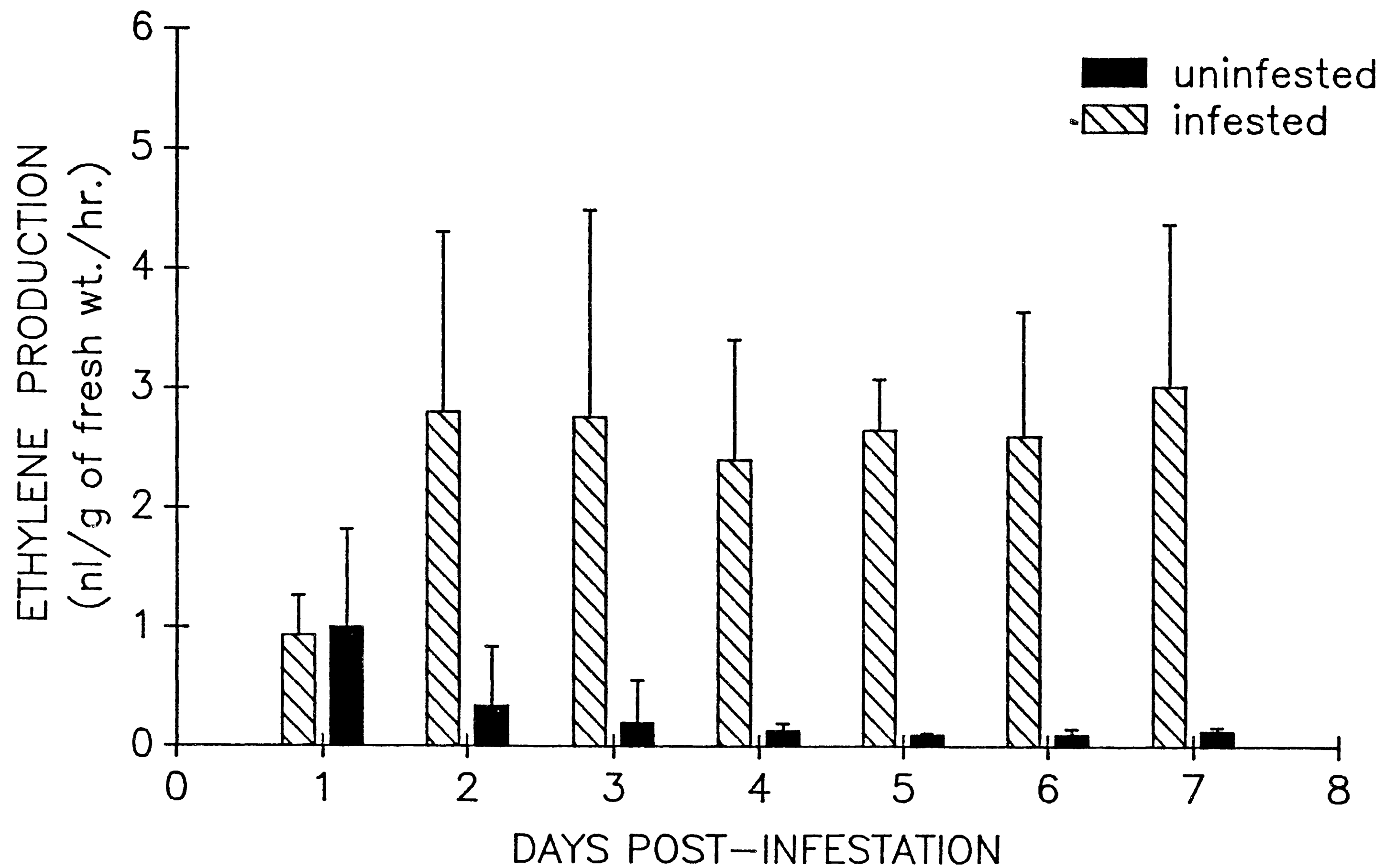


Figure 6. Ethylene production by SAA susceptible explants in response to SAA feeding

increased up to day two and a sustained level of production occurred up to seven days postinfestation. It should be noted that in this initial experiment no attempt was made to select leaves of a known age.

Two methods were used to verify that ethylene was being measured in these experiments. First the retention time of the unknown peak was compared to that of ethylene in an authentic standard gas mixture and was found to be identical (Figure 7). The second method was to treat the sample gas ith a 0.2 M solution of $Hg(ClO_4)_2$ which is known to quantitatively bind ethylene. This treatment completely removed the peak from a gas sample (Figure 8b). Further conformation was obtained by treating the $Hg(ClO_4)_2$ solution containing the trapped gas with chloride ion which is known to release bound ethylene (Sanders et al., 1989). This treatment resulted in the quantitative recovery (95%) of the ethylene in the original sample (Figure 8c). These results confirm that ethylene was the gas being produced by alfalfa in response to SAA feeding.

Ethylene Production and Age of Tissue

Aharoni et al. (1979) stated that ethylene was produced by mature green plant tissue, but whether ethylene could be produced by very old or very young plant tissue was not reported. Studies to determine if ethylene production by alfalfa infested with aphids is an age-dependent phenomenon were done and the results indicate in the alfalfa cultivar

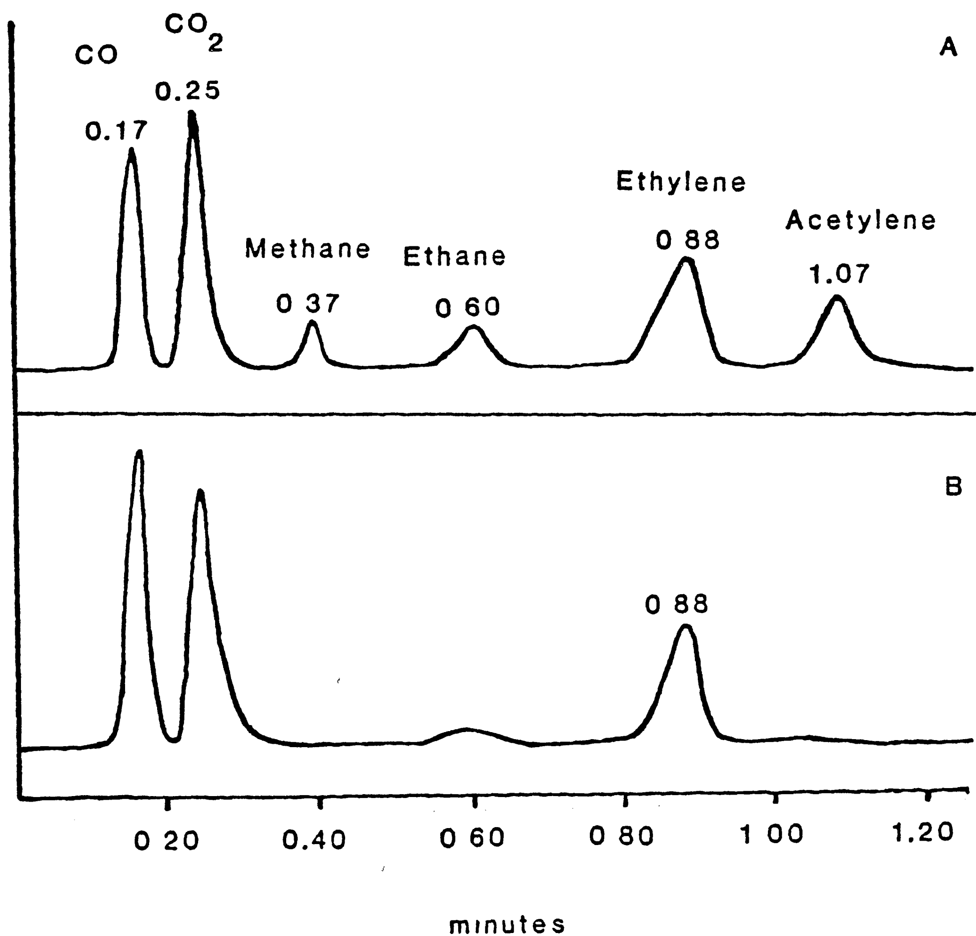


Figure 7. Comparison of retention times from a known gas standard (A) and sample gas (B)

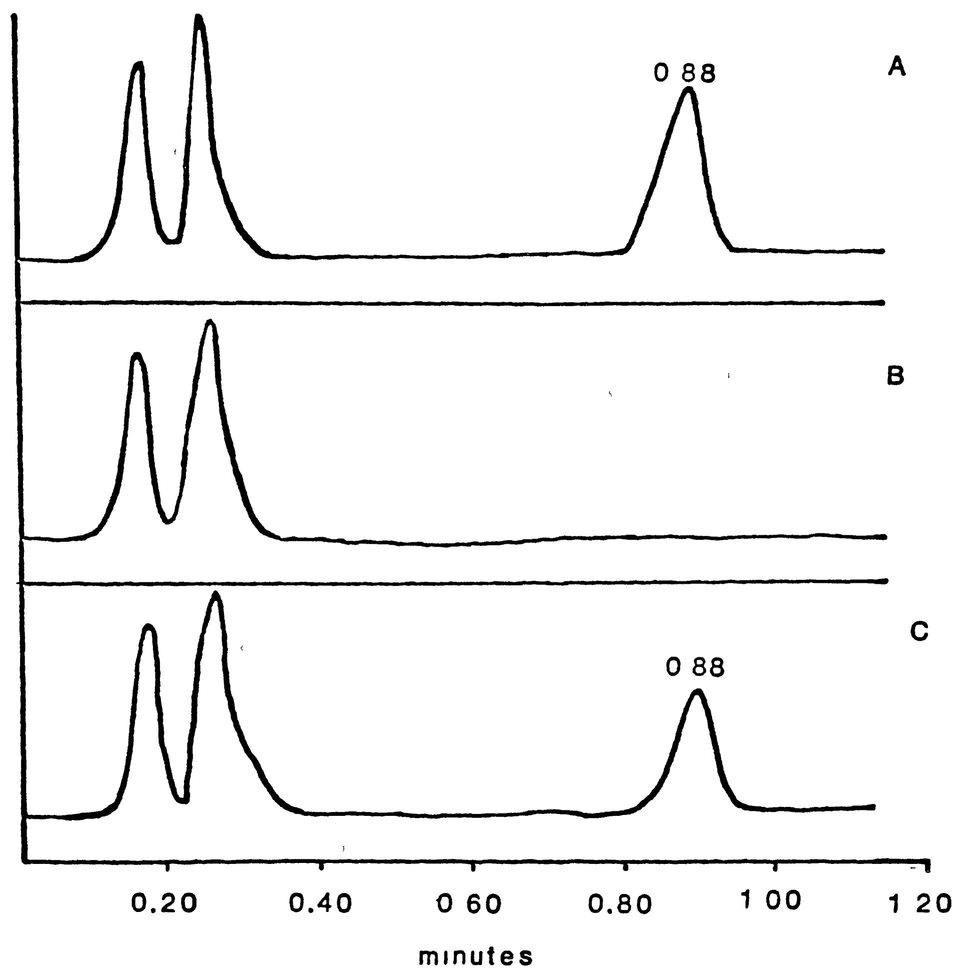


Figure 8. Trapping and release of ethylene: (A.) sample gas (B.) sample after treatment with 0.2M $Hg(ClO_4)_2$ (C.) release of ethylene from $Hg(ClO_4)_2$ solution by treatment chloride ion.

tested (OK-08), ethylene production differs according to the age of the leaves at infestation, with maximum production occurring in tissue eight days of age (Figure 9). In subsequent experiments eight day old trifoliolates were employed.

Ethylene Production and Age of Aphids

Earlier studies by Nickel and Sylvester (1959) indicated that the toxic symptoms produced by SAA feeding varied with the age of the aphid. Third and fourth instar, nymphs produced greater symptoms than younger, first and second instar nymphs. Spotted alfalfa aphids (SAA) three and seven days of age were placed on explants of susceptible alfalfa explants of eight days of age. Aphids three days of age (Figure 10) appeared to be unable to induce ethylene production at the levels observed for aphids seven days old (Figure 11). These studies seem to support the findings of Nickel and Sylvester (1959), however, due to large variation in the data the observed differences were not significant at the $P \leq 0.05$ level. More studies will be required to clarify this point. In order to eliminate possible complications of aphid age, only aphids of known age (7 days) were used in subsequent experiments.

Ethylene Production in Relation to Plant Genotype

Three alfalfa plant genotypes that were characterized earlier by Jimenez et al. (1988) were used in these studies.

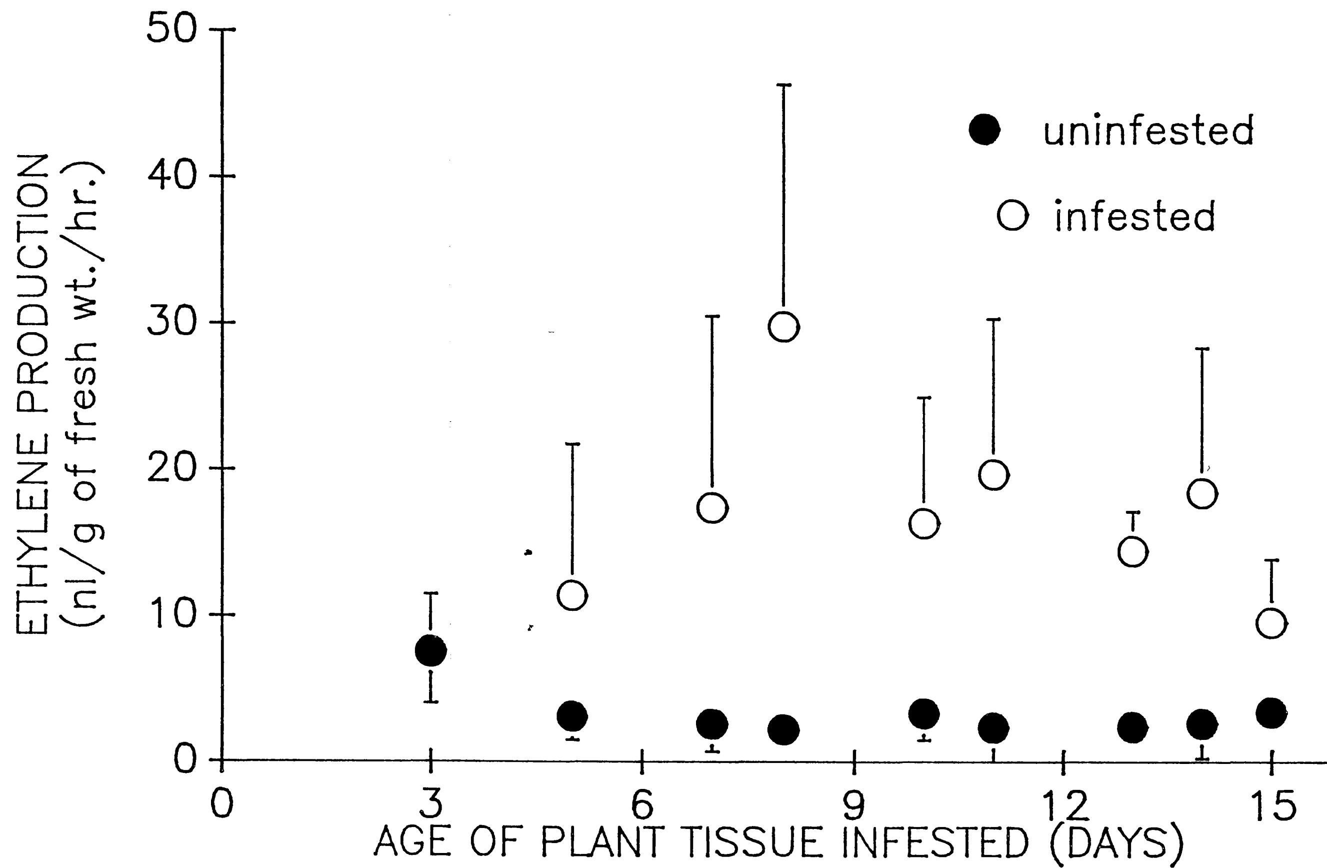


Figure 9 Ethylene production by susceptible alfalfa explants three days post-infestation

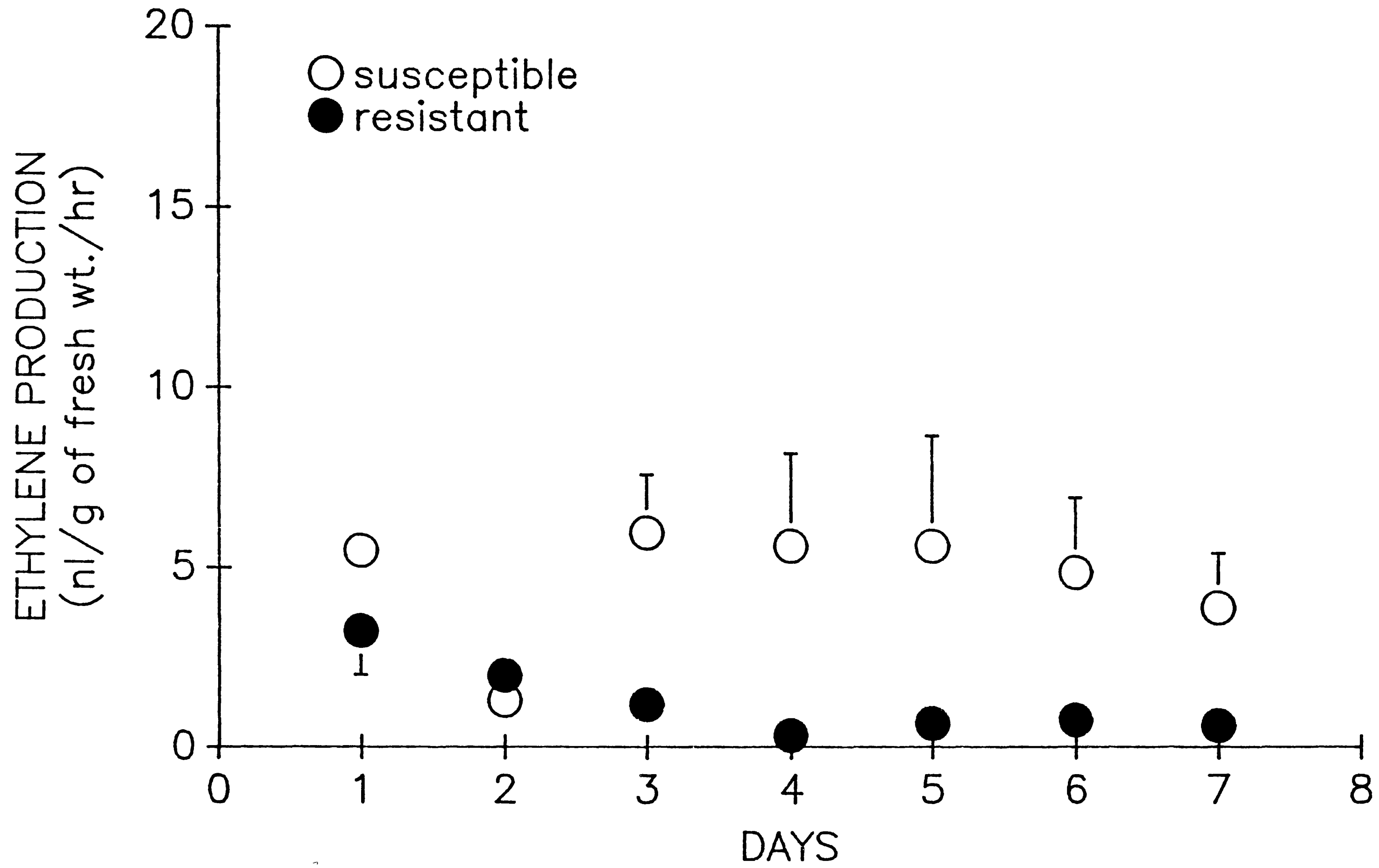


Figure 10. Ethylene production by SAA susceptible and resistant alfalfa explants in response to feeding by three day old SAA.

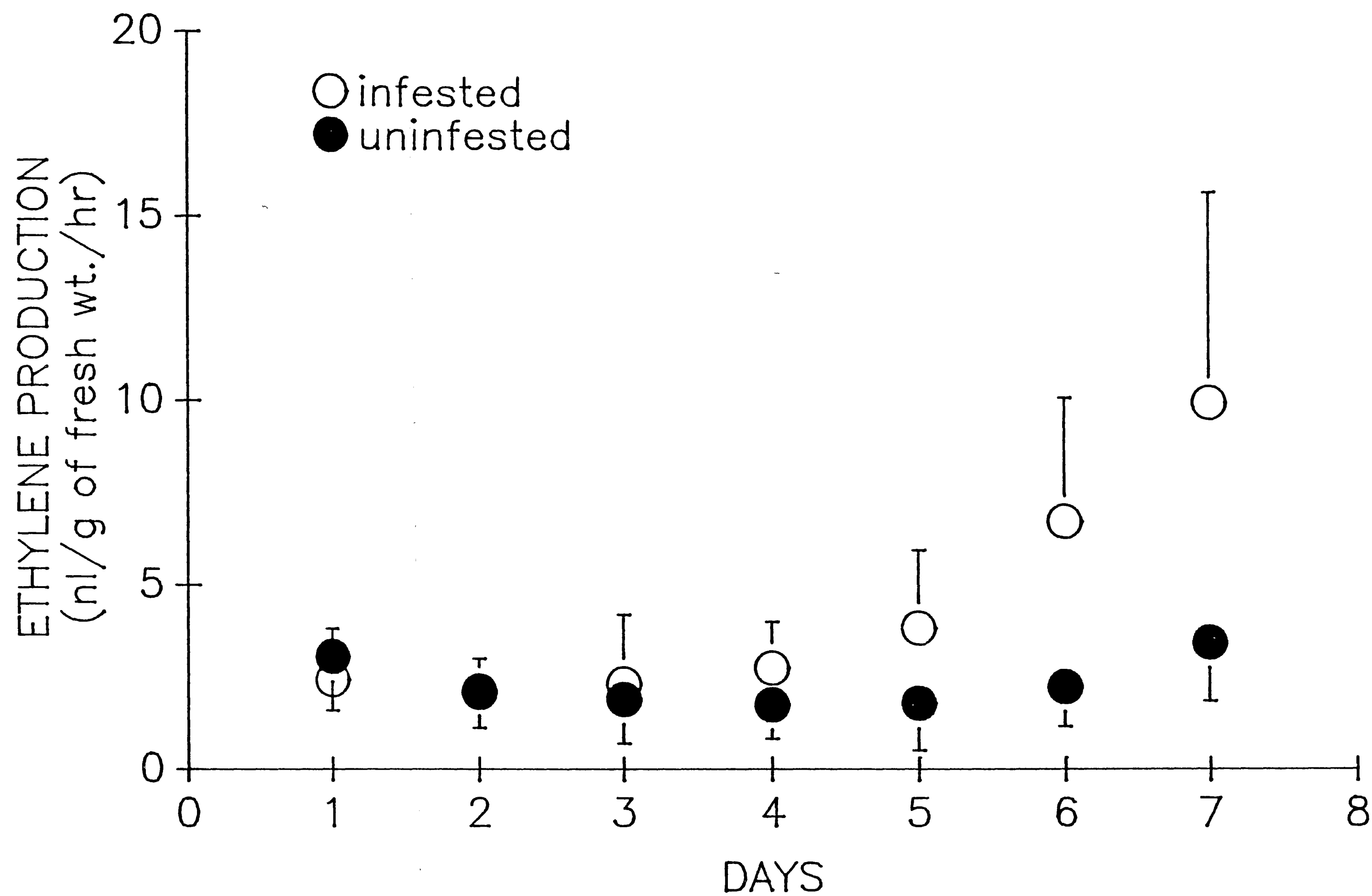


Figure 11 Ethylene production by SAA susceptible alfalfa explants, uninfested and infested with seven day old SAA, over a seven day time period

These plants which were selected from OK-08 were defined as SAA susceptible, tolerant, or resistant (antibiotic/anti-xenotic). Testing the effect of aphid feeding on ethylene production was based on findings in other systems using susceptible and resistant plants to pathogens (Gwinn et al. 1989) and insect pests (Shain & Hillis, 1972) where a high level of ethylene production was associated with an incompatible or resistant reaction to a particular parasite.

Ethylene production by SAA infested explants from the three plant genotypes was compared to uninfested explants from each of the respective plant genotypes. Results of these studies indicate that ethylene production by infested SAA susceptible explants is higher than in uninfested explants at three days postinfestation (Figure 11) and that the difference was significant to the $P \leq 0.05$ level six days postinfestation (Table III).

Production of ethylene by resistant explants was similar when infested or uninfested (Figure 12) and no differences to the $P \leq 0.05$ level were observed except for six days postinfestation (Table III).

Production of ethylene by tolerant explants with aphids was higher than uninfested explants (Figure 13). The differences in production were significant at the $P \leq 0.05$ except for one day postinfestation (Table III).

Ethylene evolution by infested susceptible and tolerant genotypes are approximately the same three days post-infestation, but the resistant gentotype was lower than the

TABLE III

ETHYLENE PRODUCTION[a] BY EXPLANTS FROM SAA SUSCEPTIBLE, TOLERANT, AND RESISTANT GENOTYPES WITH RESPECT TO APHID FEEDING OVER SEVEN DAYS

GENOTYPE	DAY	UNINFESTED	SAA INFESTED	PA INFESTED
Susceptible	1	3.06a	2.42a	2.10a
	2	2.12a	2.07a,b	1.19b
	3	1.90a	2.32a	2.11a
	4	1.75a	2.74a,b	6.16b
	5	1.79a	3.83a,b	5.13b
	6	2.24a	6.71b	7.31b
	7	3.44a	9.93b	9.42b
Resistant	1	1.88a	1.90a	1.31b
	2	1.54a	1.07a,b	0.87b
	3	1.20a	0.97a	1.81a
	4	0.84a	1.50a,b	1.98b
	5	0.77a	1.66a	3.09b
	6	0.89a	2.00b	2.92b
	7	1.96a	1.71a	3.10a
Tolerant	1	1.26a	1.79a	2.29a
	2	0.43a	1.27b	2.44c
	3	0.41a	2.23b	3.41b
	4	0.34a	1.92b	5.75c
	5	0.36a	2.02b	3.18b
	6	0.13a	1.60b	2.09b
	7	0.31a	1.45b	0.95a

[a]Expressed as nl/g of fresh wt./hr

Means in rows followed by the same letter are not significantly different (ANOVA,Scheffe's,$P \leq 0.05$)

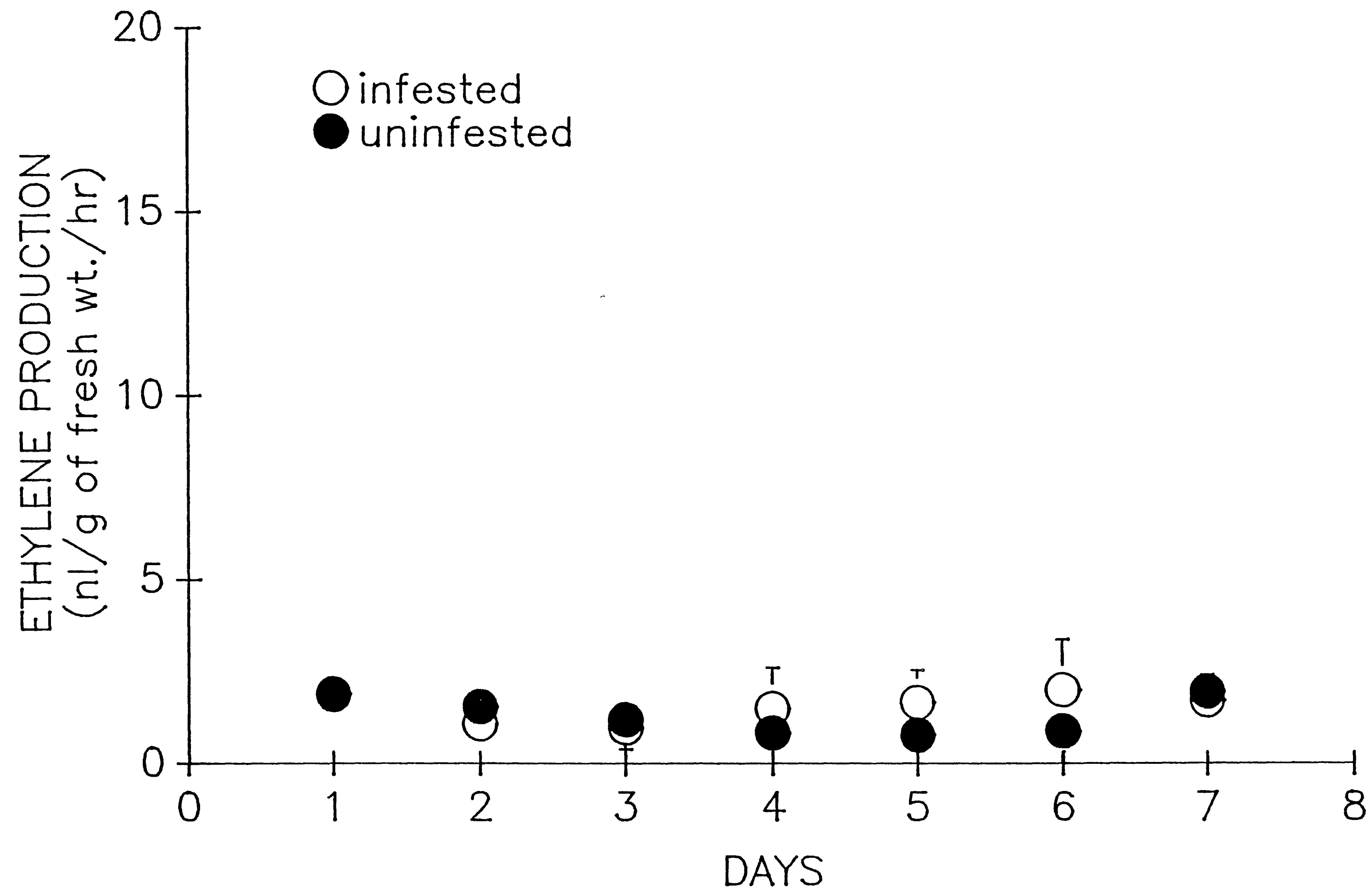


Figure 12   Ethylene production by SAA resistant alfalfa explants, uninfested and infested with seven day old SAA, over a seven day time period

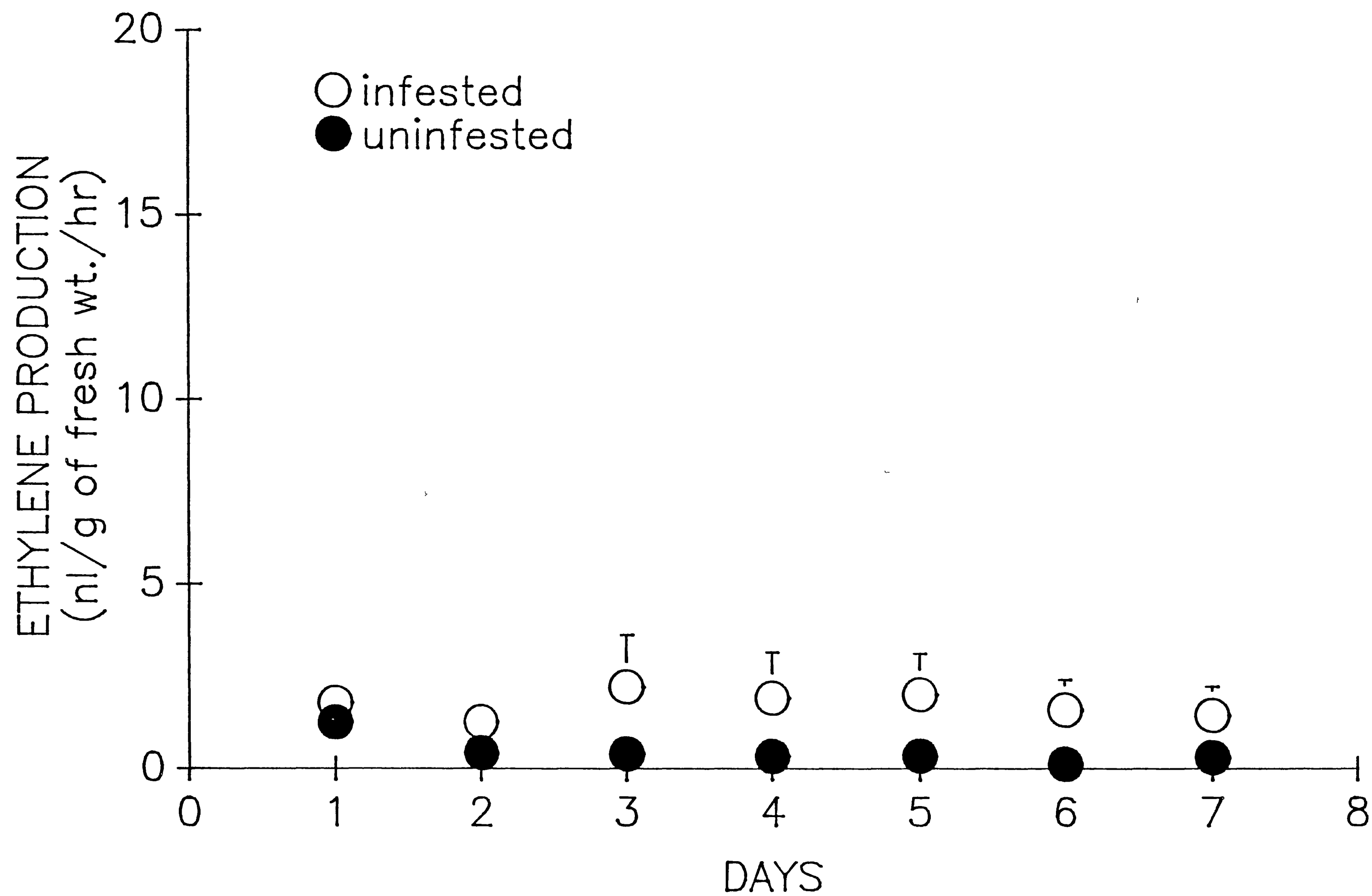


Figure 13 Ethylene production by SAA tolerant alfalfa explants, uninfested and infested with seven day old SAA, over a seven day time period

former (Figure 14). Seven days after infestation the tolerant and resistant plants produce similar amounts of ethylene, but the susceptible clone produces much more.

The mean production of ethylene for seven days post-infestation was significantly higher for the SAA infested explants than for the uninfested explants of the susceptible and tolerant lines (Table IV). Susceptible clones produced significantly greater amounts of ethylene than resistant plants infested for the same time period. For this time period, SAA infested tolerant and resistant plants produced about the same amount of ethylene. The means in this comparison are not significantly different. These results contradict the reports by Gwinn et al. (1989) and Shain & Hillis (1972) with respect to ethylene production by plants with resistance to various parasites. It is common for resistant plants to produce high amounts of ethylene in response to attack.

Results to this point indicated that the symptoms induced by SAA feeding might be senescence-like, in that ethylene production was greatly stimulated in susceptible plants where symptoms occur. In order to determine if this effect was specific to the SAA it was decided to examine the effects of another aphid on ethylene production. All of the genotypes used support PA. Unlike the SAA, the PA does not produce senescence-like symptoms in host plants. This aphid would allow us to compare feeding by a so-called toxic aphid (SAA) and with a non-toxic aphid (PA). In this study due to

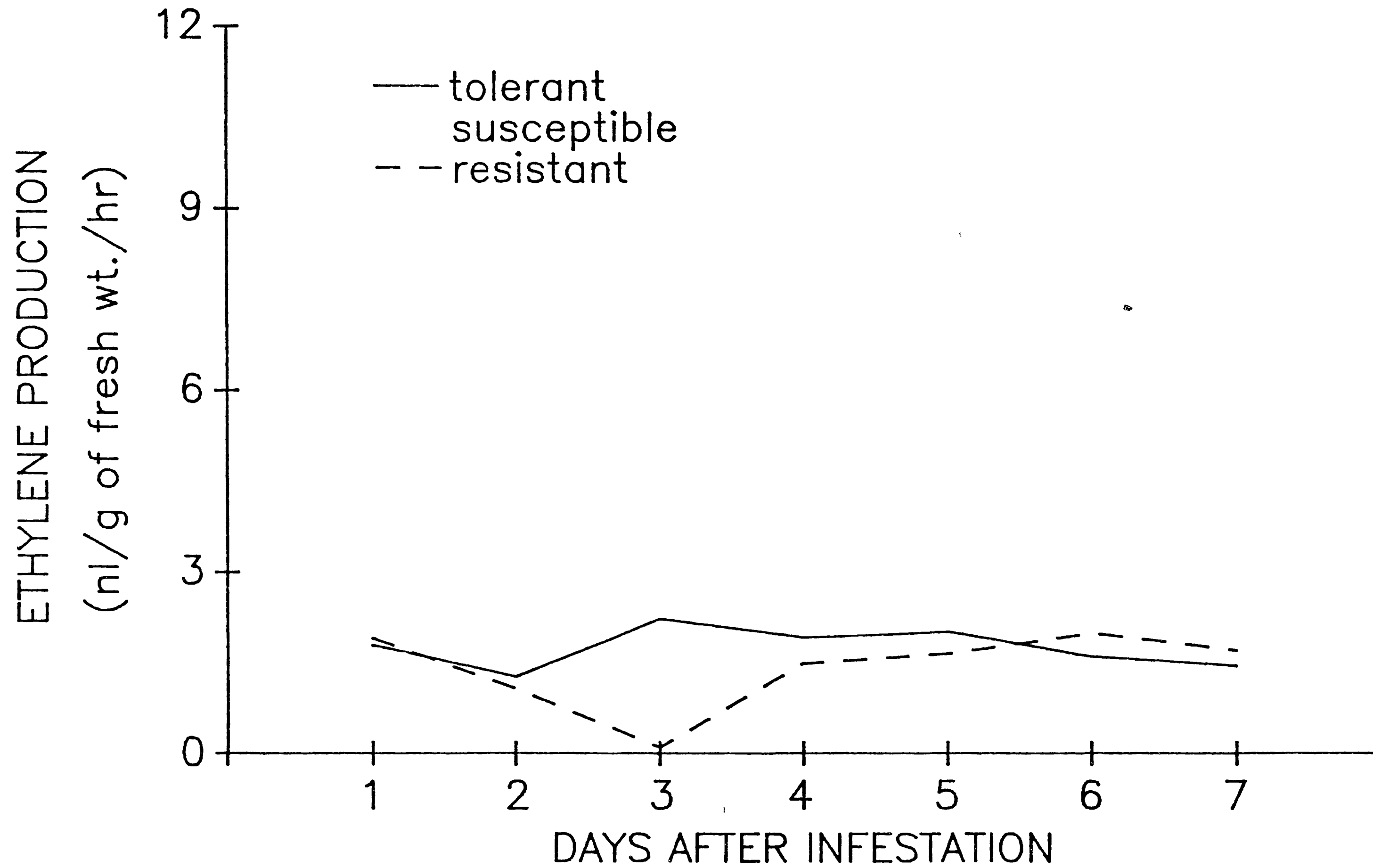


Figure 14 Kinetics of ethylene production by SAA susceptible, tolerant, and resistant alfalfa explants, infested with seven day old SAA, over a seven day time period.

TABLE IV

MEAN ETHYLENE PRODUCTION[a] BY EXPLANTS FROM THREE ALFALFA GENOTYPES FOR SEVEN DAYS POST-INFESTATION

GENOTYPE	UNINFESTED	SAA INFESTED	PA INFESTED
Susceptible	A 2.27a	A 4.36b	A 5.10b
Tolerant	B 0.46a	B 1.74b	B 2.81c
Resistant	C 1.30a	B 1.53a	B 2.18b

[a]Expressed as nl/g of fresh wt./hr

Means in rows followed by the same lower case letter are not significantly different. (ANOVA,Scheffe's,$P \leq 0.05$)

Means in columns preceded by the same upper case letter are not significantly different. (ANOVA,Scheffe's,$P \leq 0.05$)

time consideration, most of the studies considered only susceptible and resistant plant genotypes with respect to PA feeding.

Ethylene production by susceptible explants infested with PA exhibited a pattern similar to susceptible explants infested with SAA (Figure 15). The levels of ethylene increased over time in infested tissue, however, the differences between uninfested and infested became statistically significant earlier in the time course (Table III).

Again, ethylene production by resistant explants with PA showed the same type of kinetics as the SAA infested explants (Figure 16). The differences in means for each particular day were slight as compared to uninfested. However, there were only two days for which the differences were not significant (Table III).

Means for the seven day infestation period with PA indicated that the same pattern as when infested with SAA. There are significant differences in mean ethylene production by the susceptible, tolerant and resistant genotypes with the susceptible producing more than the tolerant and resistant (Table IV). According to the data, PA feeding induces the same response (ethylene production) in relation to plant genotype as the SAA; the aphid by which the genotypes were selected.

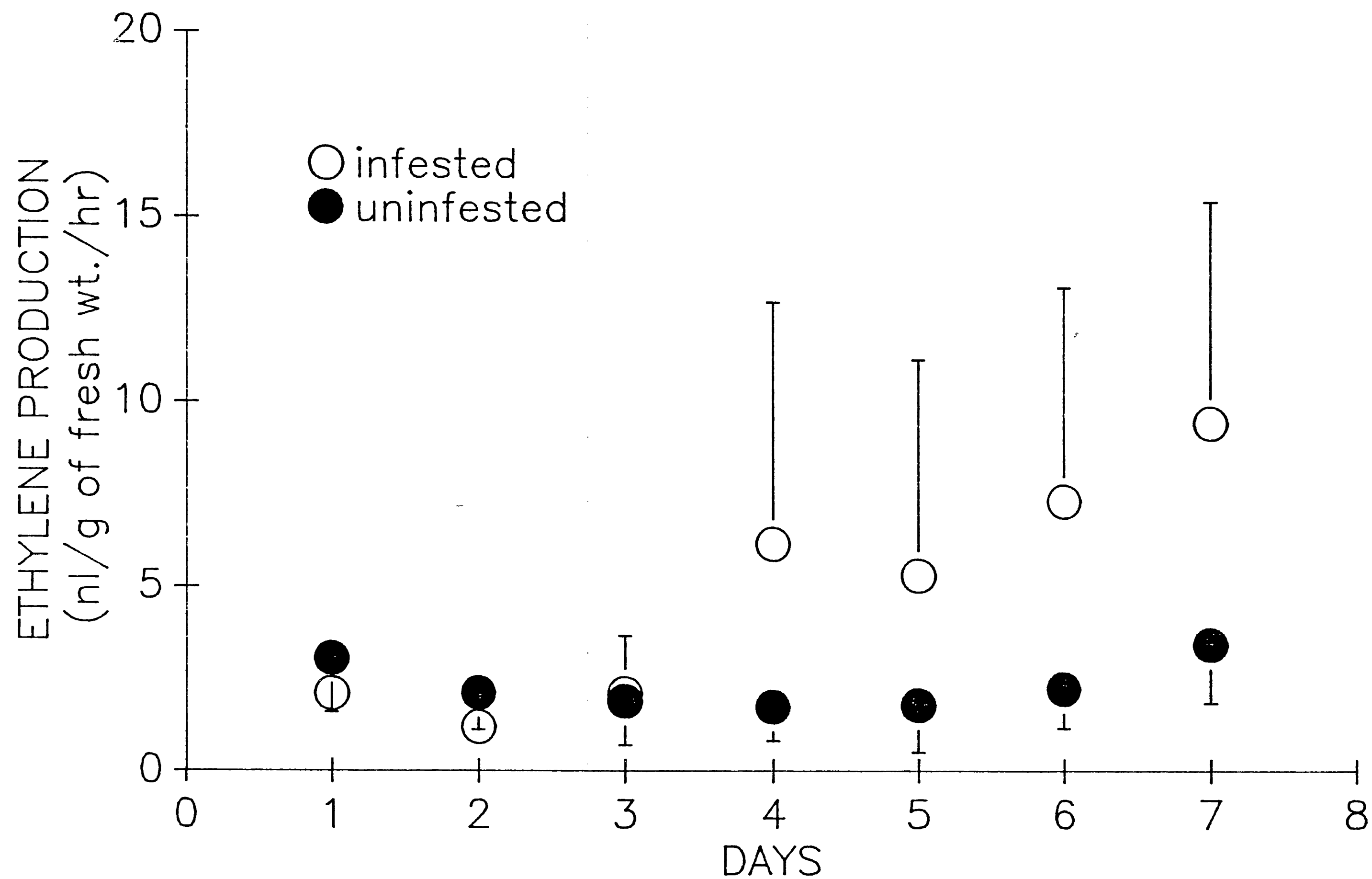


Figure 15. Ethylene production by SAA susceptible alfalfa explants, uninfested and infested with seven day old PA, over a seven day time period.

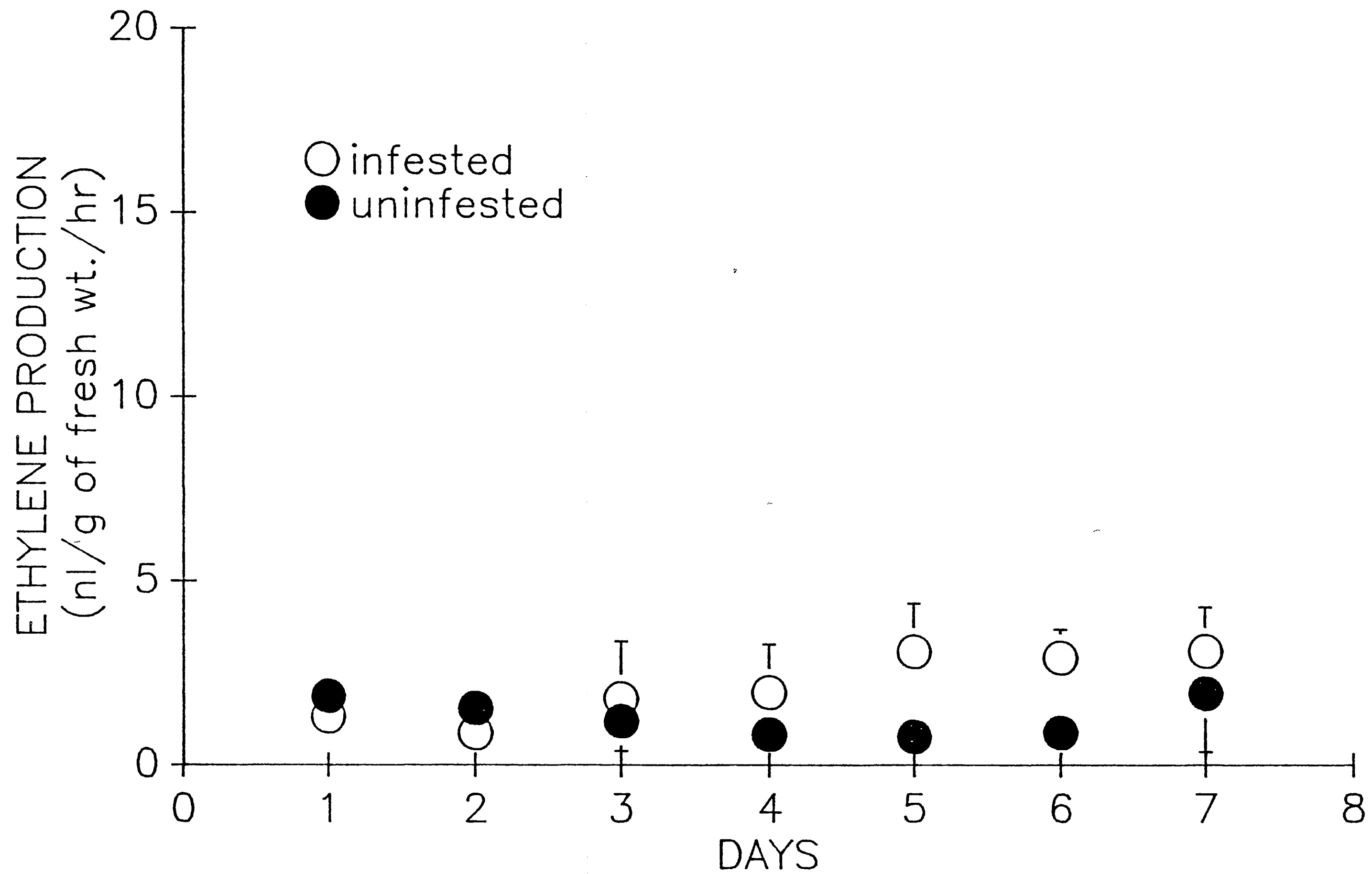


Figure 16 Ethylene production by SAA resistant alfalfa explants, uninfested and infested with seven day old PA, over a seven day time period

Ethylene Production in Relation to Whole Plant Infestation

Up to this point all controlled studies were conducted *in vitro* (except for preliminary studies) using explants. Another avenue to be explored is the effect of infesting whole plants and measuring ethylene production by these tissues. During of infestation, age of the plant tissue at infestation, position of the trifoliolate on the stem, and possible systemic effects were parameters investigated in this type of system.

The first study of the affects on whole plants involved infesting SAA susceptible, tolerant, and resistant plants, and comparing ethylene produciton to that in uninfested plants. The plants were heavily infested with 3000-4000 SAA per plant. All samples in this study were taken from the lowest node of stems having SAA and trifoliolates from uninfested plants were removed from the same relative positions. Ethylene production was measured immediately after removal of the leaves.

Figure 17 shows ethylene production by trifoliolates from SAA infested and uninfested plants of the susceptible type at three, seven, and ten days post infestation. Over time, the rate of ethylene production by these trifoliolates increased, while the rate for the uninfested tissue remained lower and relatively unchanged throughout the study. Ethylene production by infested plant material was significantly greater than uninfested for each of the sample

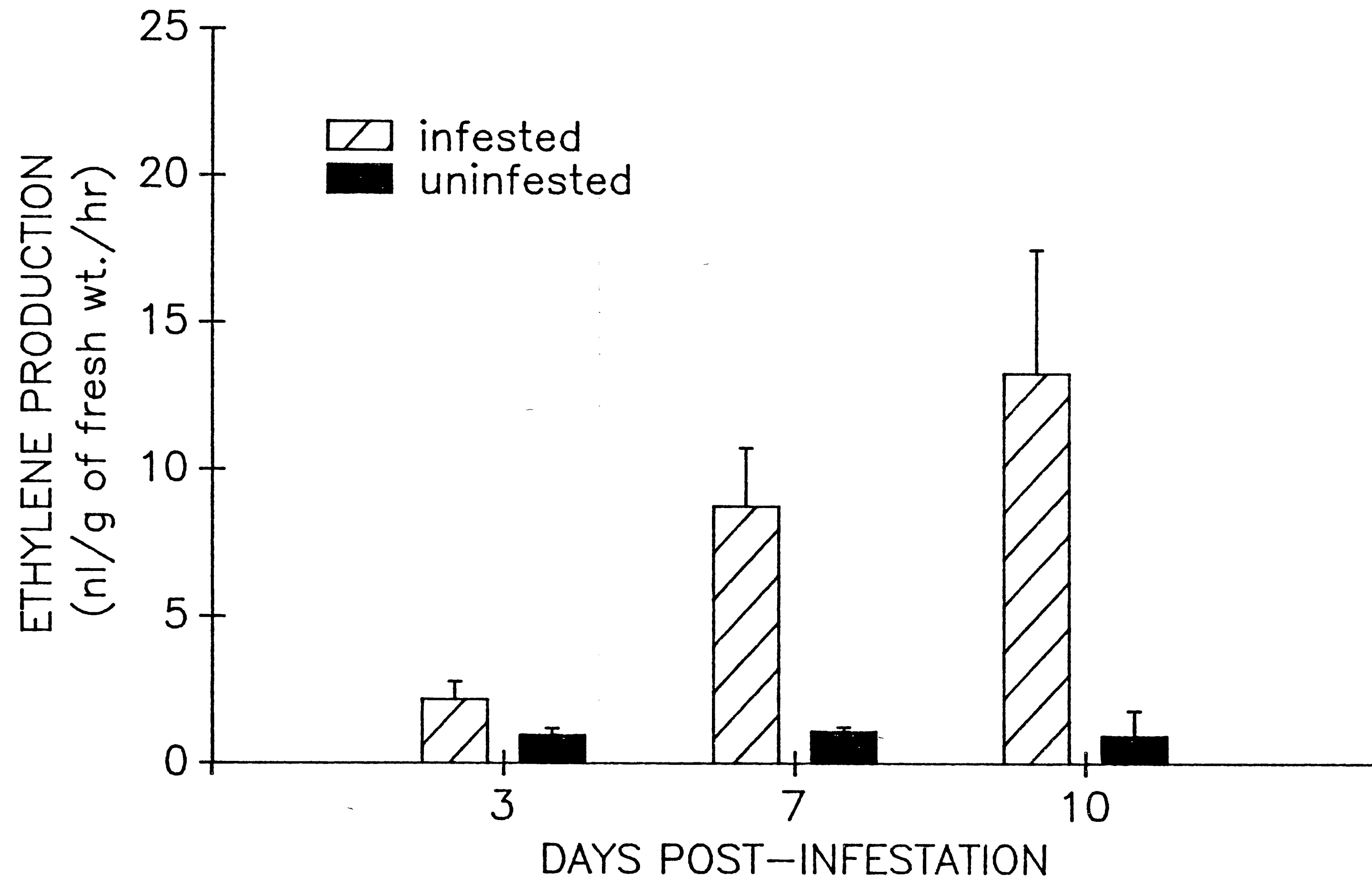


Figure 17. Ethylene production by SAA susceptible alfalfa. Trifoliolates were from uninfested and SAA infested plants. Trifoliolates were removed and ethylene measured four hours later

periods. Also, the amount of ethylene produced by tissue was significantly greater on ten days than seven days post-infestation, which in turn was significantly greater than the amount produced three days postinfestation (Table V).

Ethylene production for the resistant plant tissue increased significantly as the length of infestation increased (Figure 18). However, the amounts of ethylene produced by uninfested trifoliolates were not significantly different from the amounts produced by aphid infested material (Table V).

Contrary to observations for susceptible plants, in tolerant plants, the amount to ethylene produced by infested material decreased over time (Figure 19). But as in the susceptible and resistant clones, ethylene production by uninfested tolerant plants was lower than the infested and remained at a relatively constant rate. Ethylene production by infested material decreased significantly over time, while there was no significant change in the amount over time in the uninfested trifoliolates (Table V).

In a second experiment the effects of position on the stem, and the age of plant tissue were studied to determine if these parameters had any effect on ethylene production. Each trifoliolate in each position had a corresponding agethus allowing ethylene production to be evaluated in relation to age and position simultaneously. Single SAA adults were caged on trifoliolates of known age on susceptible, tolerant, and resistant plants. Aphids were

TABLE V

ETHYLENE PRODUCTION[a] BY TRIFOLIOLATES FROM SAA INFESTED PLANTS IN RELATION TO GENOTYPE AND LENGTH OF INFESTATION

Plant Status	Duration of Infestation Day 3	Day 7	Day 10
		Susceptible	
Infested	2.18a (0.61)	8.75b (1.98)	13.29c (4.21)
Uninfested	0.97d (0.23)	1.10d (0.15)	0.95d (0.86)
		Tolerant	
Infested	6.65h (2.65)	3.89i (1.66)	3.29j (1.54)
Uninfested	0.88d (0.33)	1.06d (0.36)	0.49d (0.85)
		Resistant	
Infested	0.86e (0.26)	1.41f (0.47)	1.37g (1.21)
Uninfested	0.88de (0.37)	1.16df (0.30)	1.05dg (0.52)

[a]Expressed as nl/g of fresh wt./hr, mean $\pm$ (s.d.)

Means followed by the same letter are not significanlty different. (ANOVA,Scheffe's,$P \leq 0.05$)

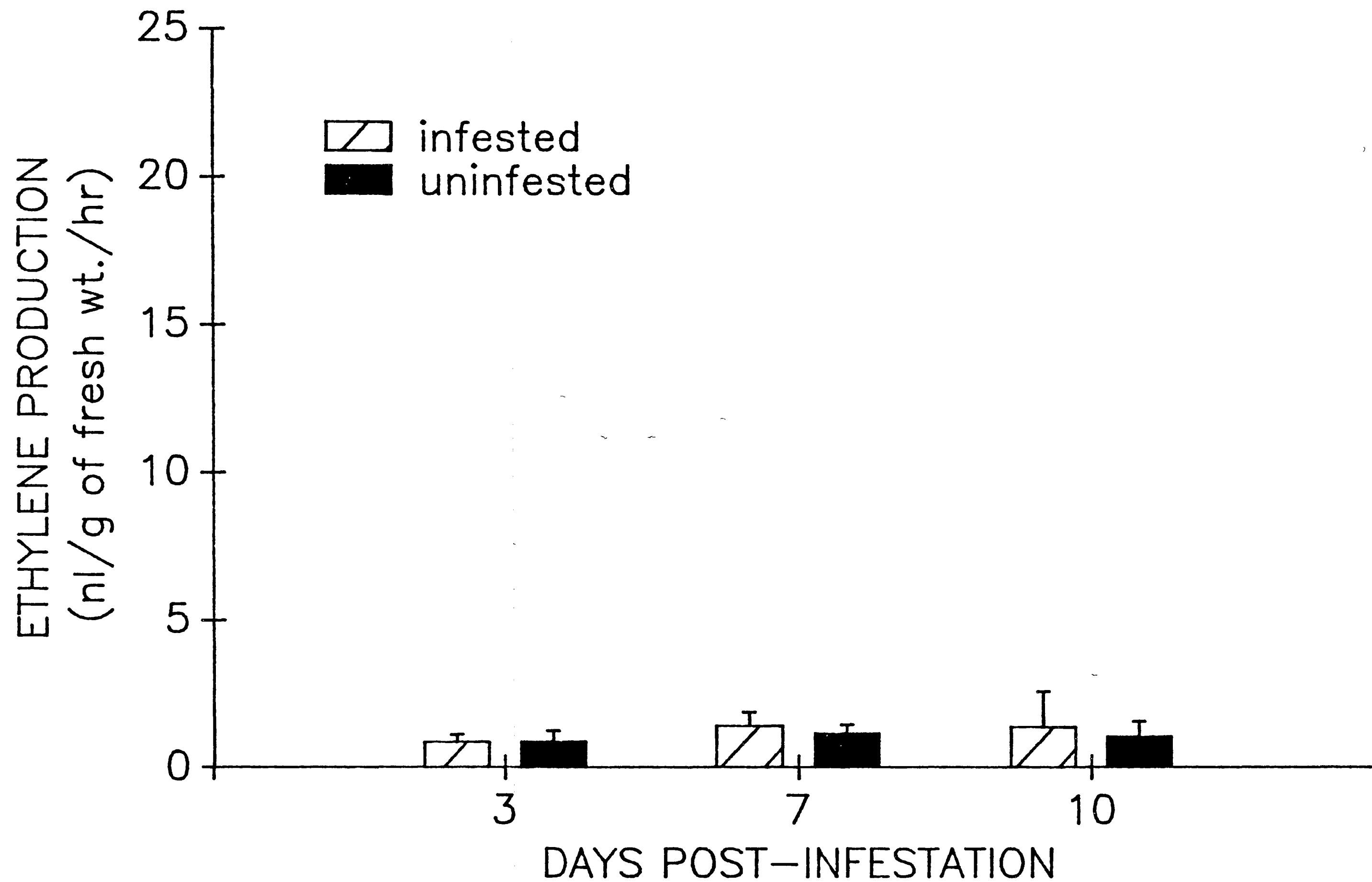


Figure 18. Ethylene production by SAA resistant alfalfa. Trifoliolates were from uninfested and SAA infested plants. Trifoliolates were removed and ethylene measured four hours later

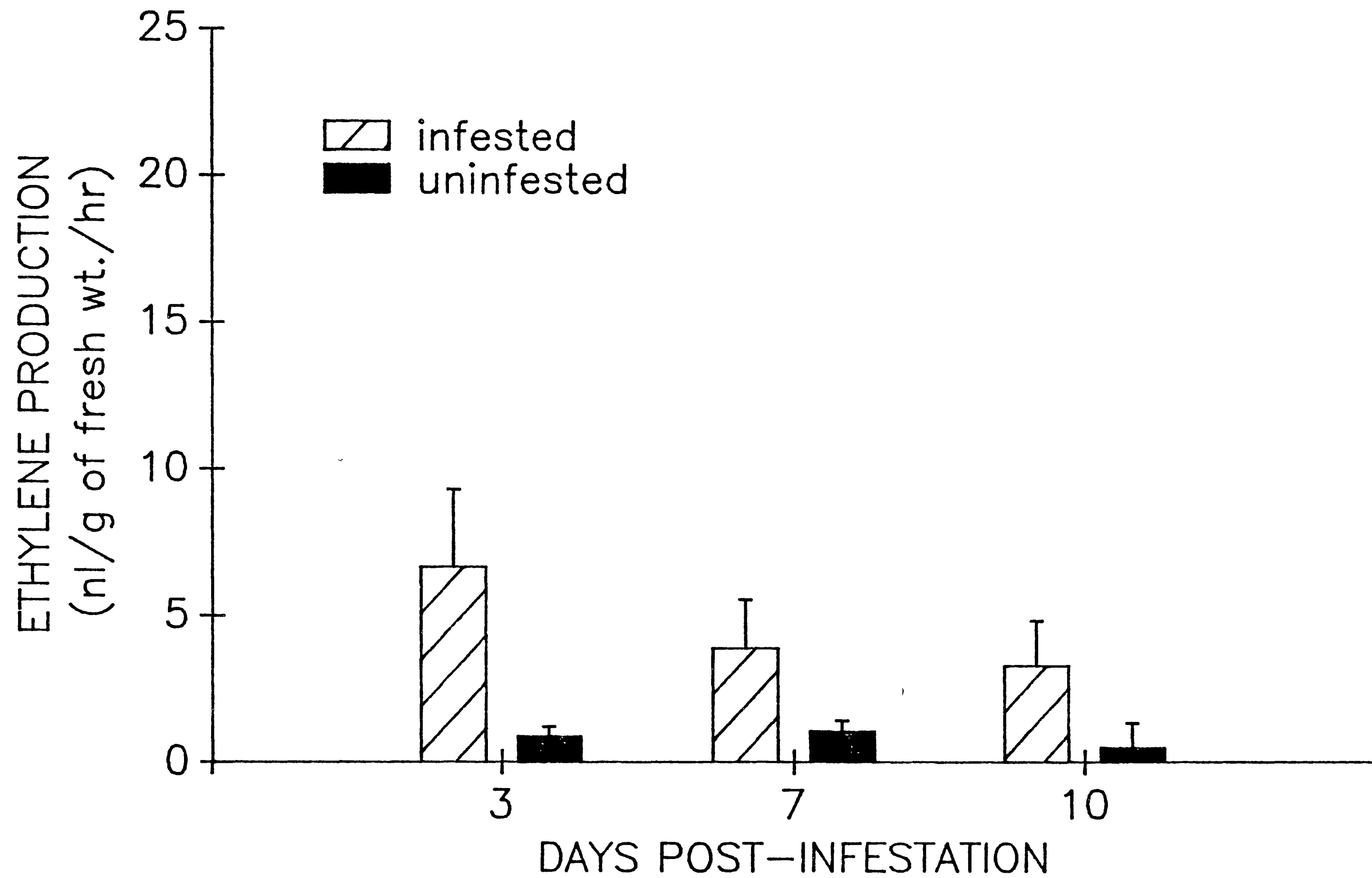


Figure 19. Ethylene production by SAA tolerant alfalfa. Trifoliolates were from uninfested and SAA infested plants Trifoliolates were removed and ethylene measured four hours later

allowed to feed and produce nymphs for one generation (130 cumulative degree days). Aphids were removed and trifoliolates were removed from plants along those adjacent, either one node above those infested. Corresponding trifoliolates from uninfested plants were harvested and the rate of ethylene production measured.

Ethylene production decreased in susceptible trifoliolates from the upper to the lower positions of the stem. (Figure 20). Amounts produced by the trifoliolates were lower than the the amounts produced by caged trifoliolates (uninfested and infested). Ethylene production was lower by uninfested tissue. Differences inethylene production by infested and uninfested plants were significant, while differences in production by uncaged trifoliolates were not significant (Table VI). Table VI and Figures 21 and 22 show the same relationship between ethylene and position on the plant for tissues from tolerant and resistant plants respectively. Differences between each genotype were significant for each position including both infested and uninfested (Table VI).

Similar to what is seen with position, ethylene production in relation age of tissue decreased as the age of the tissue increased. As seen previously, the susceptible clones produce the most ethylene (Figure 23), followed by the tolerant (Figure 24), then lastly the resistant (Figure 25), all in relation to feeding by the SAA. The uninfested plants show the same relationship as the aphid infested

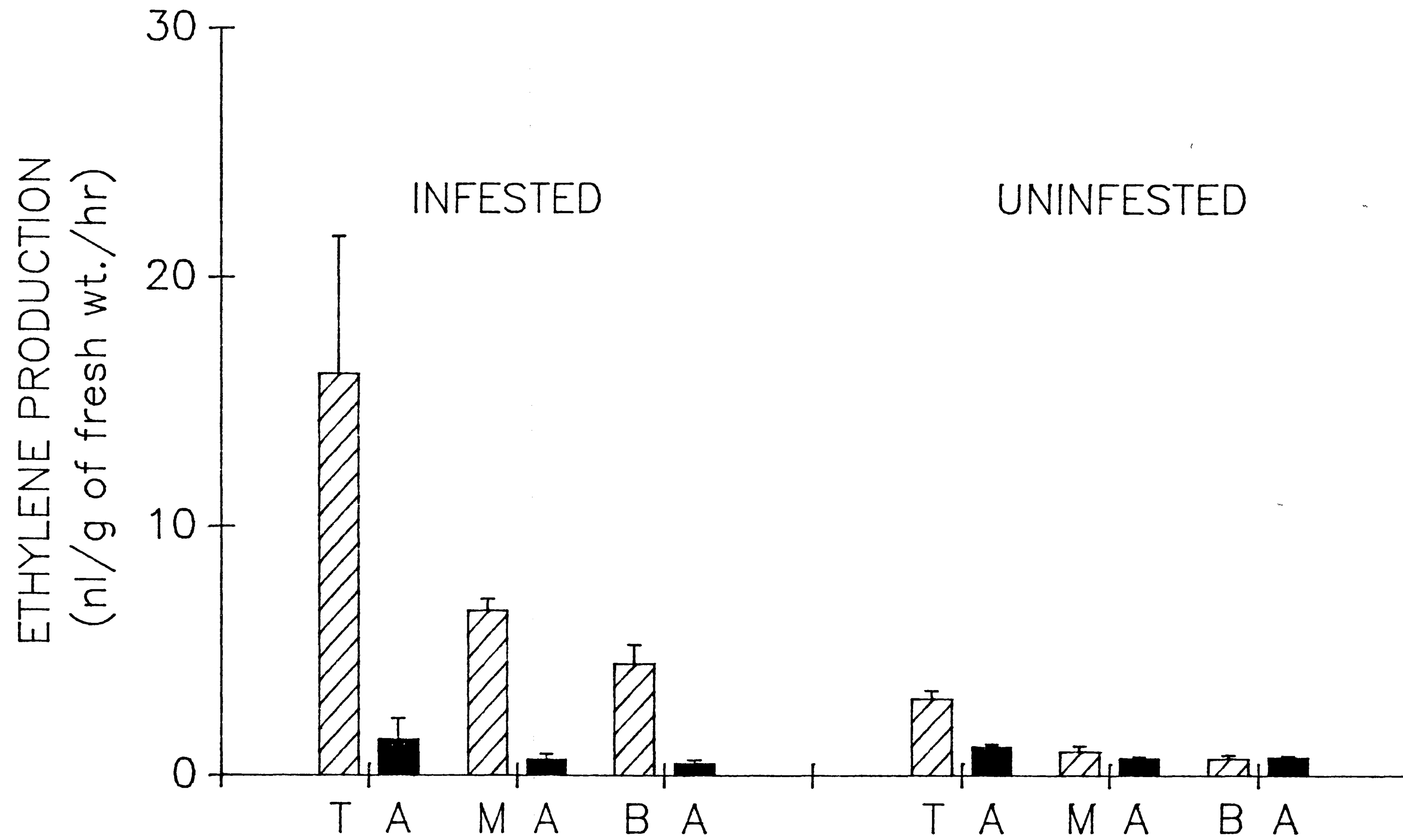


Figure 20. Ethylene production by SAA susceptible trifoliolates located at three positions on stems SAA infested and uninfested trifoliolates were caged. Adjacent trifoliolates were not caged Sampled seven days after infestation T=top, M=middle, B=bottom; A=adjacent

TABLE VI

ETHYLENE PRODUCTION [a] BY SAA SUSCEPTIBLE INFESTED AND UNINFESTED TRIFOLIOLATES IN RELATION TO POSITION AND GENOTYPE

Position of Cage on Stem	Genotype					
	Susceptible		Tolerant		Resistant	
	I[b]	U[c]	I	U	I	U
Top	16.12 (5.53)	3.09 (0.34)	4.60 (1.19)	1.76 (0.01)	1.78 (0.47)	1.23 (0.02)
Adj[d]	1.46a (0.84)	1.17a (0.12)	0.78a (0.09)	1.29a (0.86)	0.57a (0.10)	0.85a (0.35)
Mid	6.62 (0.48)	0.96 (0.24)	2.42 (0.77)	0.92 (0.01)	1.70 (0.96)	0.58 (0.01)
Adj	0.65b (0.22)	0.71b (0.07)	0.42b (0.02)	0.55b (0.04)	0.81b (0.16)	0.65b (0.35)
Bottom	4.49 (0.78)	0.67 (0.15)	1.45 (0.39)	0.45 (0.10)	0.54 (0.19)	0.35c (0.09)
Adj	0.49c (0.14)	0.75c (0.05)	0.44c (0.16)	0.57c (0.10)	0.18c (0.03)	0.35c (0.01)

[a]Expressed as nl/g of fresh wt./hr, mean ± (s.d.)
[b]Infested
[c]Uninfested
[d]Trifoliolates without cages; one node above or below caged trifoliolates at the Top, Middle, or Bottom

Means followed by the same letter are not significantly different. (ANOVA,Scheffe's,$P \leq 0.05$).

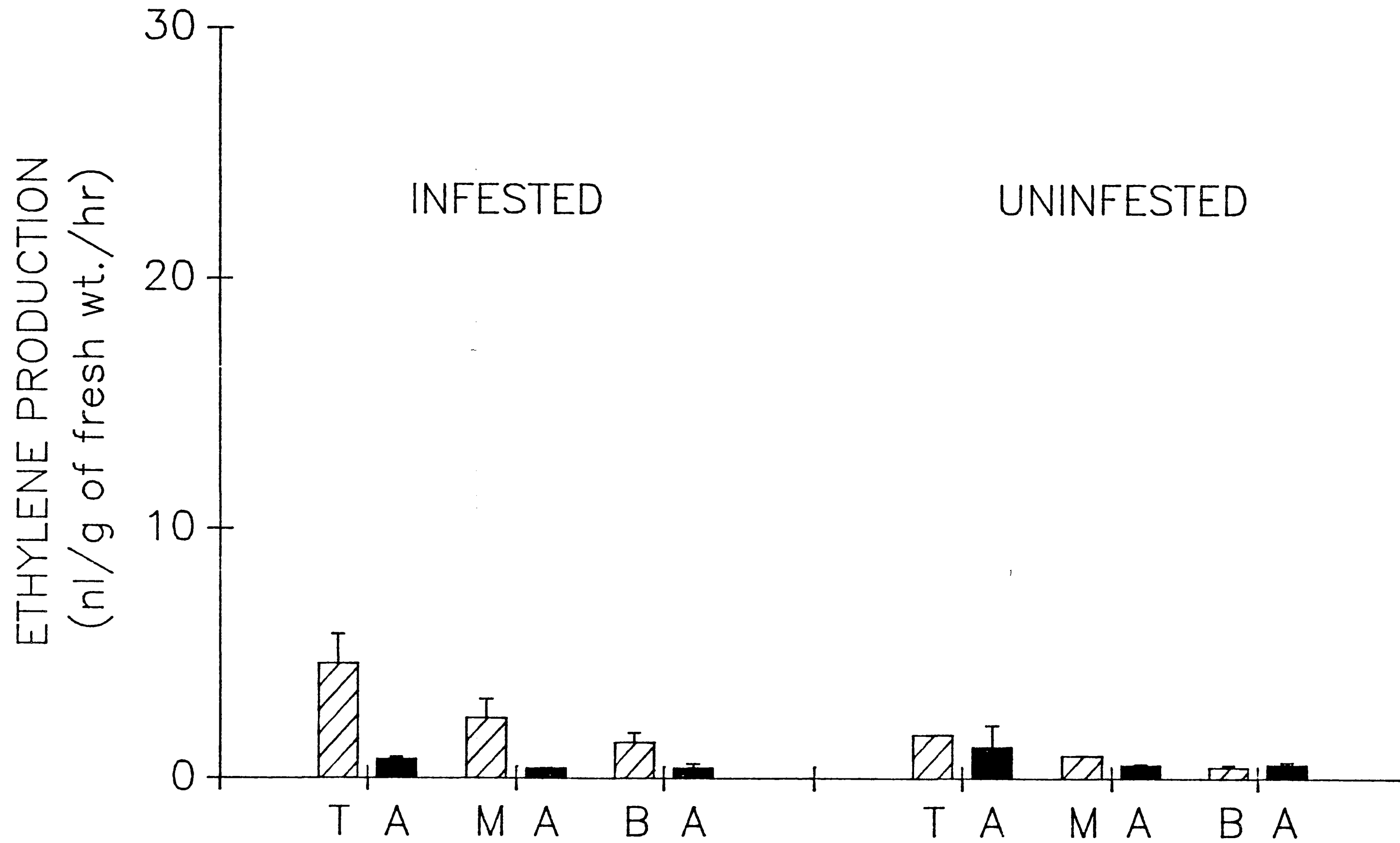


Figure 21. Ethylene production by SAA tolerant trifoliolates located at three positions on stems. SAA infested and uninfested trifoliolates were caged. Adjacent trifoliolates were not caged. Sampled seven days after infestation. T=top, M=middle, B=bottom; A=adjacent.

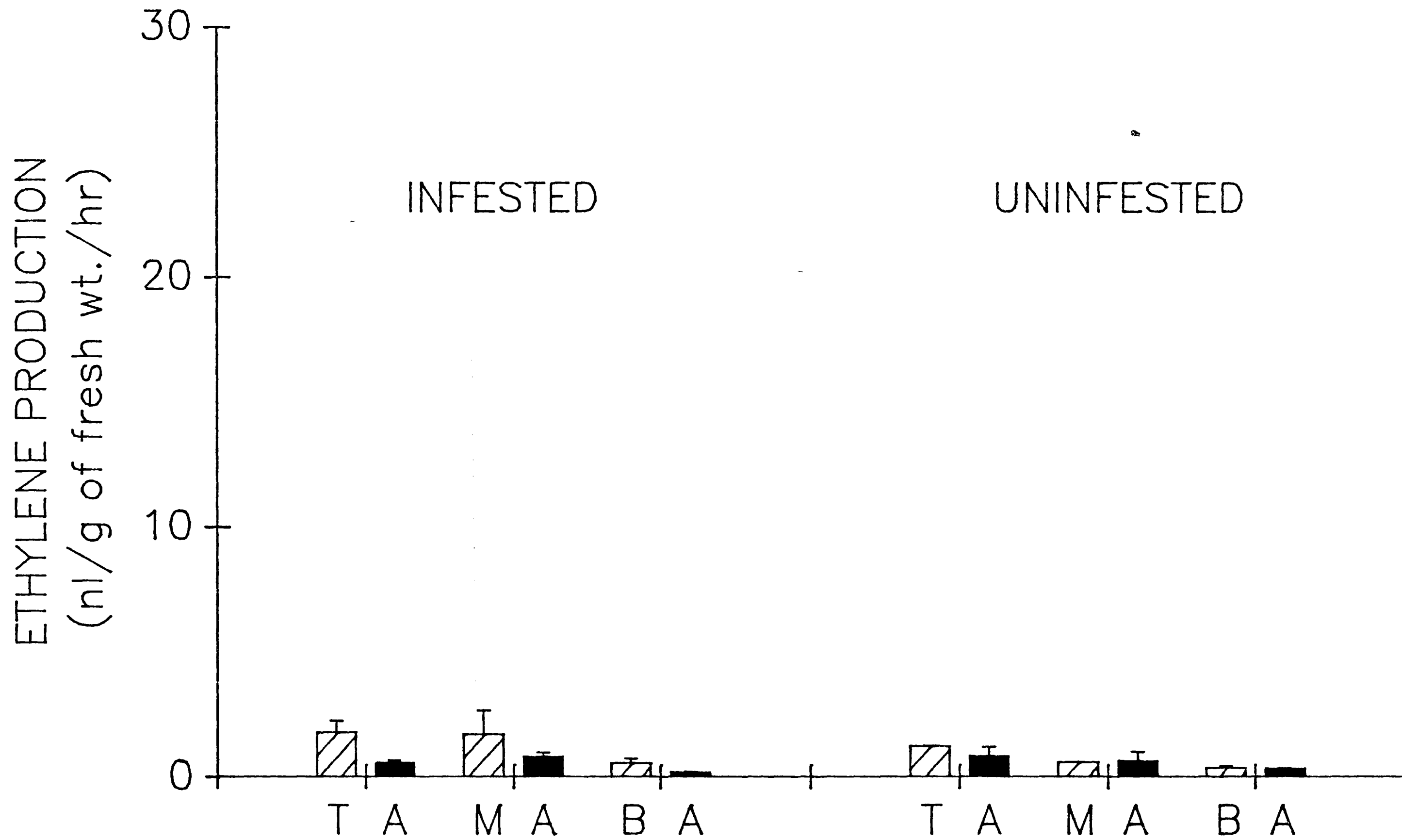


Figure 22 Ethylene production by SAA resistant trifoliolates located at three positions on stems SAA infested and uninfested trifoliolates were caged Adjacent trifoliolates were not caged Sampled seven days after infestation T=top, M=middle; B=bottom, A=adjacent

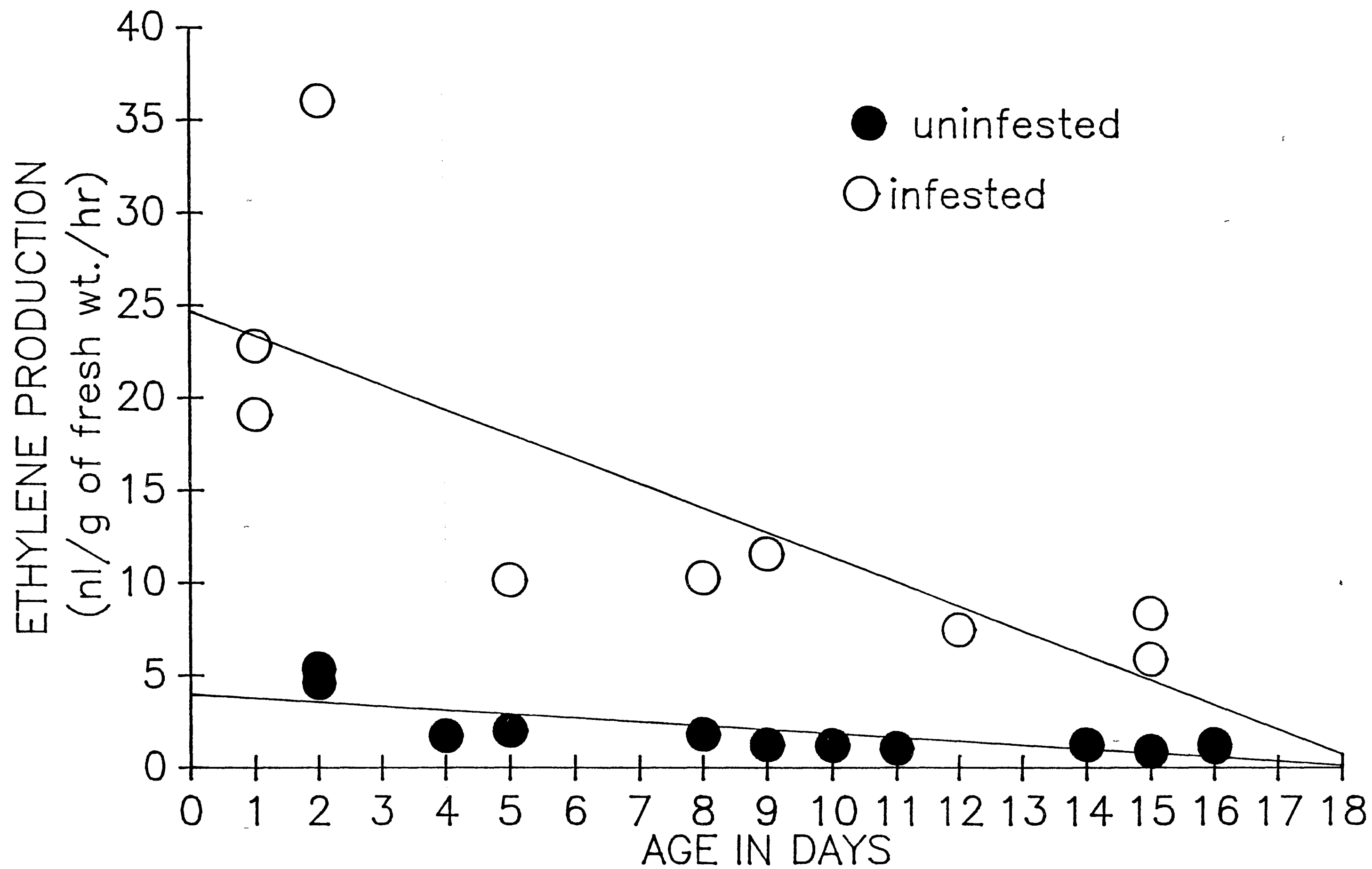


Figure 23 Ethylene production by SAA susceptible alfalfa trifoliolates of various ages from uninfested and SAA infested plants

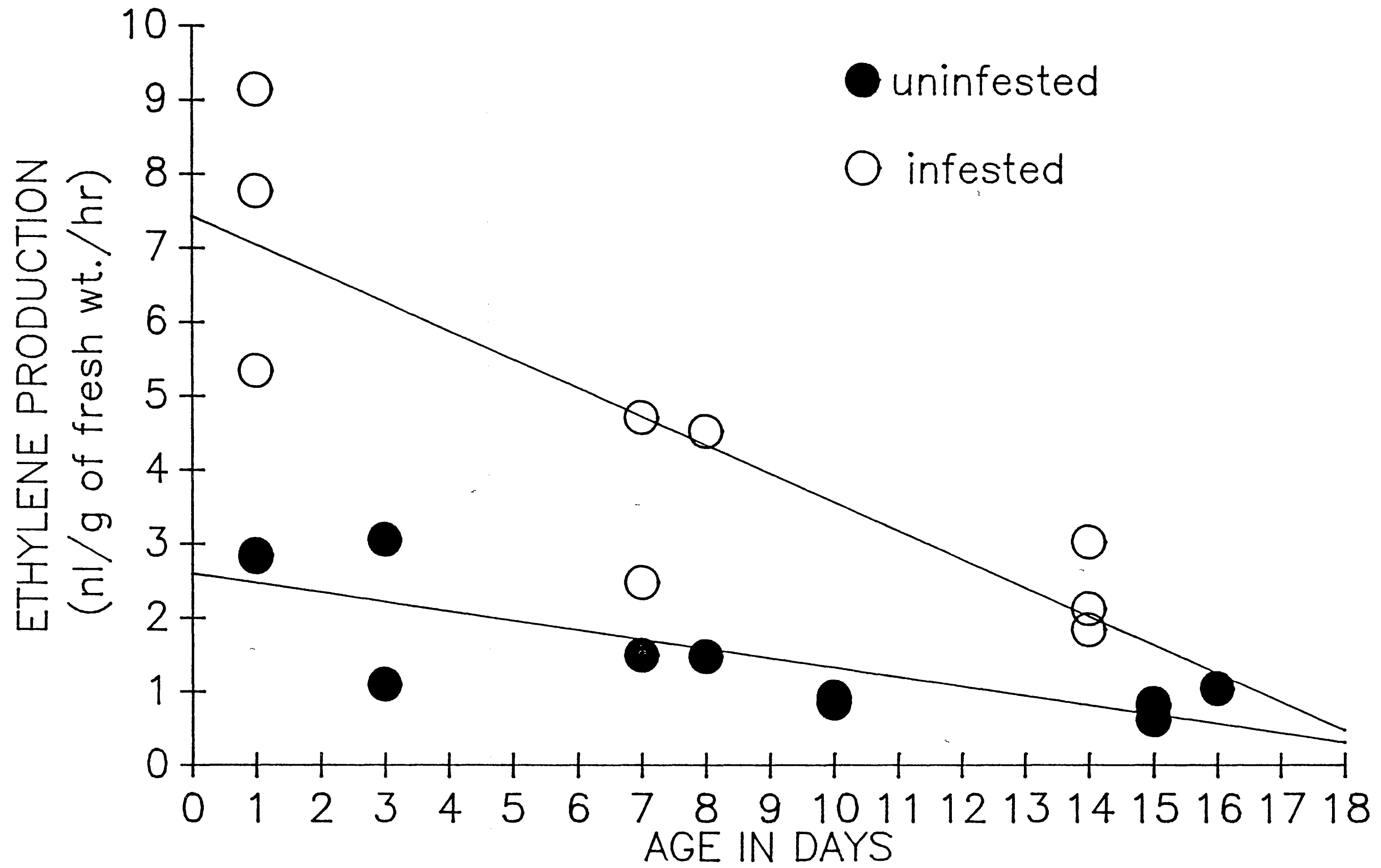


Figure 24 Ethylene production by SAA tolerant alfalfa trifoliolates of various ages from uninfested and SAA infested plants

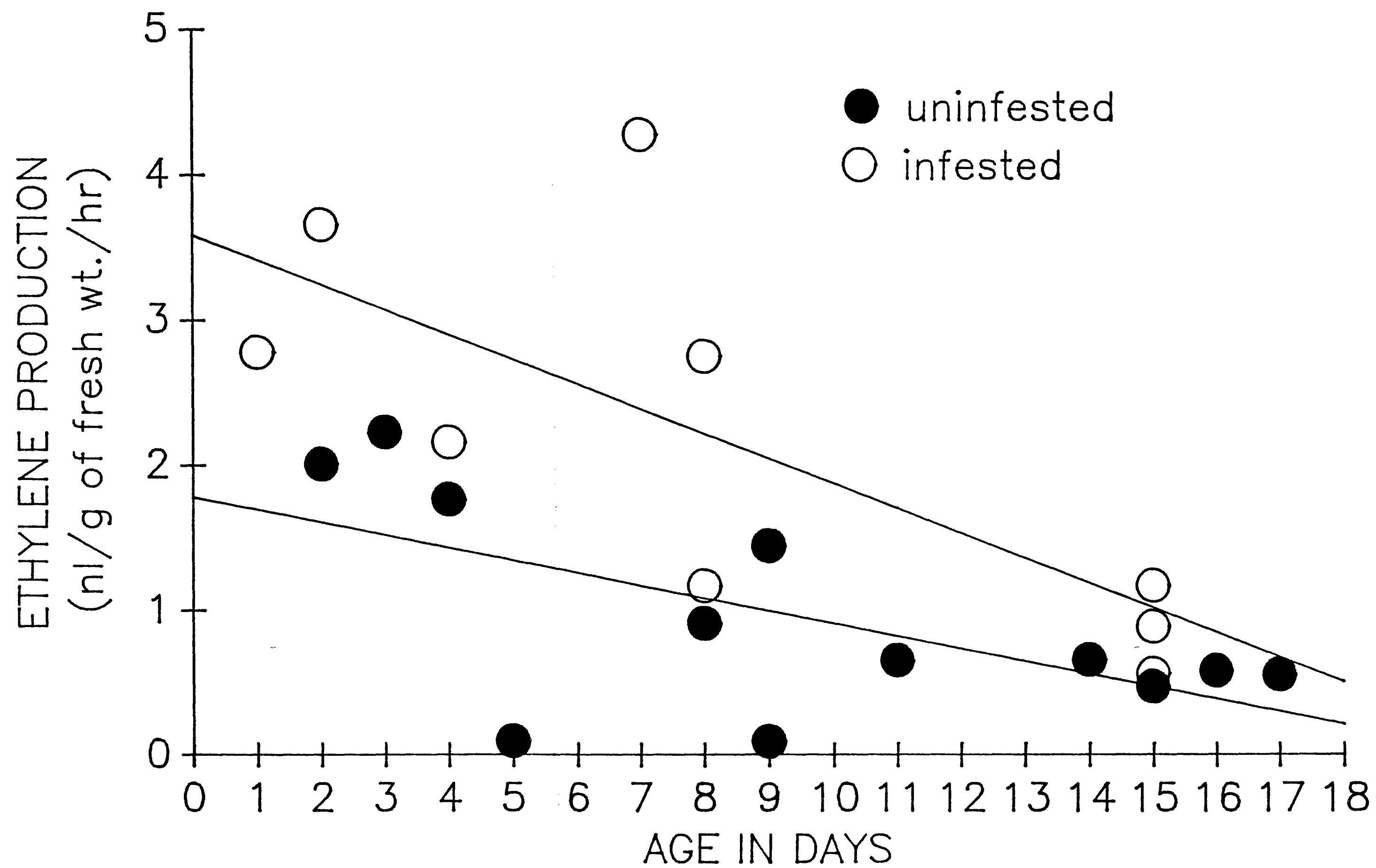


Figure 25. Ethylene production by SAA resistant alfalfa trifoliolates of various ages from uninfested and SAA infested plants.

tissue, but produce ethylene at much lower levels. This indıcates that ethylene production by alfalfa is dependent upon the age of the tissue; younger tissue producing more ethylene than older. These results differ from that seen in explants, in which trifoliolates of intermediate age produce the most ethylene.

Aphid Feeding and Lipid Peroxidation

Hilderbrand et al. (1986) observed that in soybeans lipid peroxides increase in response to feeding by the twospotted spider mite *Tetranychus urticae* Koch. Spotted alfalfa aphid colony food plants infested with SAA were samples by removing at random trifoliolates from stems. Using a modification of the assay by Dhindsa et al., (1980), malondialdehyde, a product of lipid peroxidation, was measured in the plant samples. In susceptible alfalfa, lipid peroxides, measured as malondialdehyde, increased in response to aphid feeing. The amount measured in aphid infested foliage was significantly greater than the amount measured in uninfested controls (Figure 26). The presence of high levels of lipid peroxides may indicate that SAA feeding may induce a senecence-like process in susceptible alfalfa.

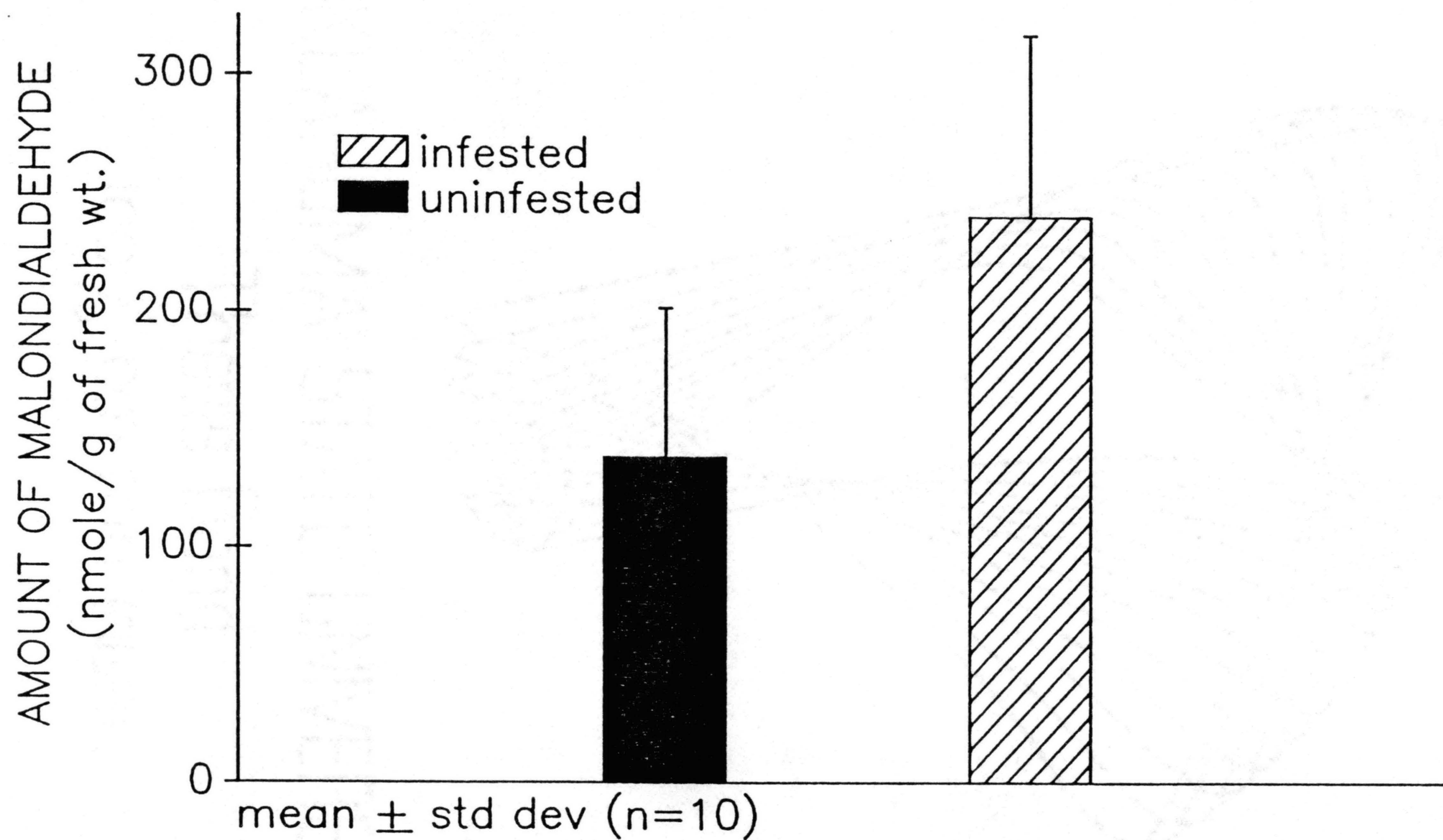


Figure 26. Mean amount of malondialdehyde (MDA) as a measure of lipid peroxidation in uninfested and SAA infested susceptible alfalfa.

Development of an Assay for ACC in Alfalfa

Quantifying the amount of ACC in alfalfa tissue was done based on a method by Concepcion et al. (1979). This method converts ACC to ethylene, which can be measured by gas chromatography. Figure 27 shows a standard curve indicating the amount of ethylene produced from a known concentration of an ACC standard. In a preliminary study, it was shown that foliage from plants with heavy SAA infestations, contain up to 2 nmoles/gm of fresh wt. of ACC, whereas uninfested plant material contained less than 1 nmole/gm of fresh wt. These results are preliminary, however, they are consistant with the higher levels of ethylene production in aphid infested tissues. Further investigation of ACC levels in relation to aphid feeding and plant genotype will need to be done.

Inhibition of Ethylene Production

Meijer and Brown (1988) used inhibitors of ethylene synthesis (AVG and AOA) in alfalfa cell culture to inhibit somatic embryogenesis. However, the concentrations used did not inhibit ethylene biosynthesis in cell culture. Ethylene production by susceptible alfalfa explants when infested with SAA was shown to be inhibited by the use of AVG (aminoethoxyvinylglycine) and AOA (aminooxyacetic acid) (Figure 28) at concentrations listed in Tables VII and VIII respectively. Dose response curves for AVG (Figure 29) and

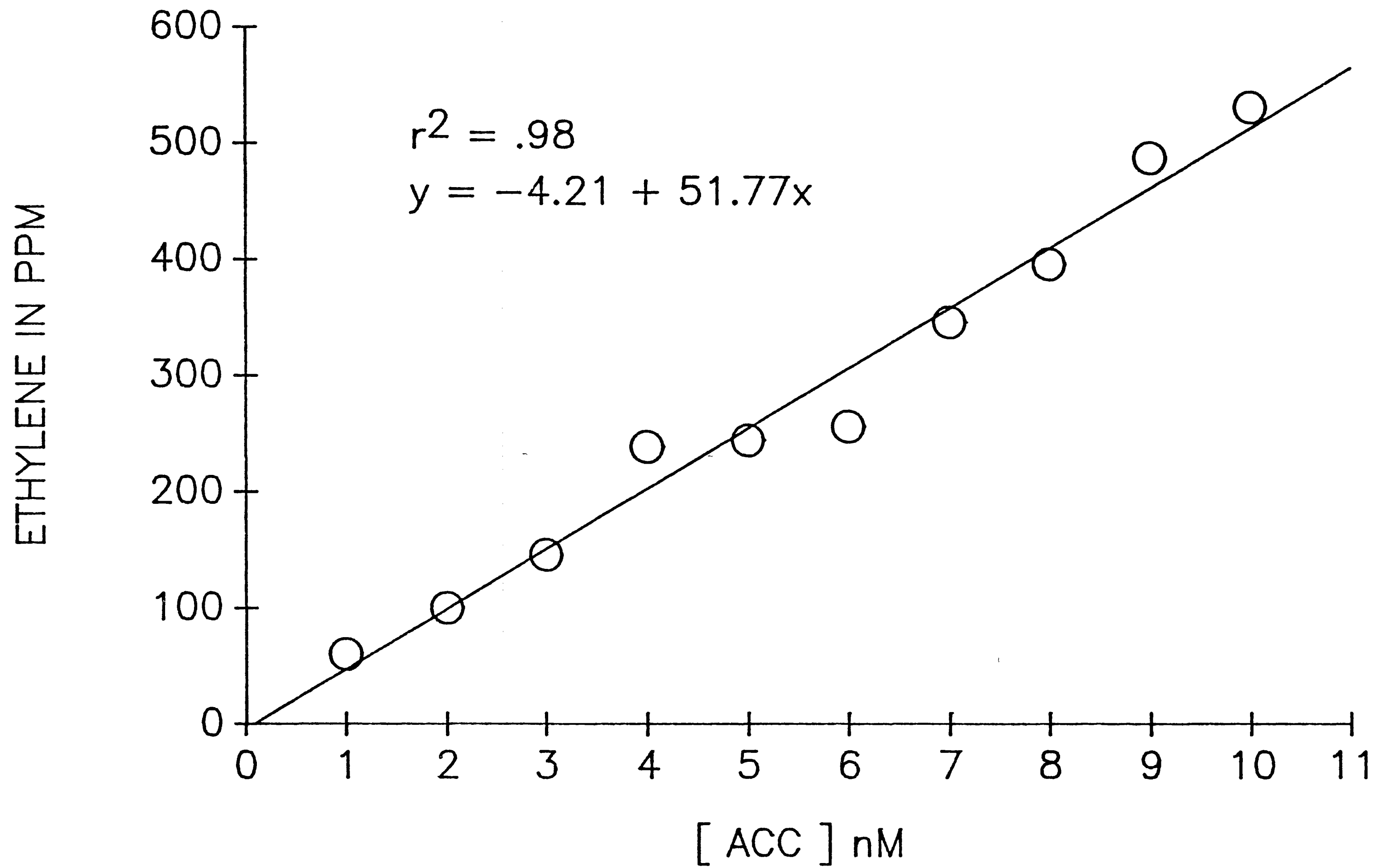
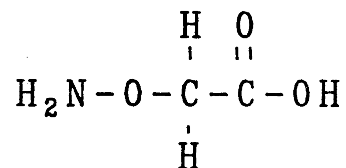
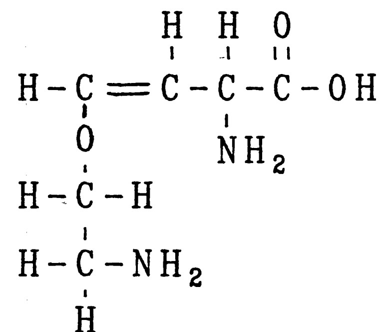


Figure 27 Curve showing the amount of ethylene produced from an ACC standard at concentrations ranging from 1 to 10 nM.



aminooxyacetic acid

(carboxymethoxylamine)



aminoethoxyvinylglycine

Figure 28. Chemical structure of AOA (aminooxyacetic acid) and AVG (aminoethoxyvinylglycine), inhibitors of biosynthesis.

TABLE VII

EFFECT OF AMINOETHOXYVINYLGLYCINE (AVG) ON ETHYLENE PRODUCTION IN EXCISED TRIFOLIOLATES

AVG Concentration (micromolar)	% of Control ± std. dev.	% Inhibition
670	18.15 ± 4.04	81.15
500	32.74 ± 11.21	67.26
330	44.78 ± 22.40	55.22
170	53.73 ± 11.32	46.27
100	78.49 ± 20.22	21.51
80	80.39 ± 25.89	19.61
40	96.27 ± 36.56	3.73
20	97.61 ± 39.93	2.39

TABLE VIII

EFFECT OF AMINOOXYACETIC ACID (AOA) ON ETHYLENE PRODUCTION IN EXCISED TRIFOLIOLATES

AOA Concentration (millimolar)	% of Control ± std. dev.	% Inhibition
33.3	23.40 ± 5.85	76.60
16.7	29.26 ± 7.23	70.74
6.7	42.53 ± 19.72	57.47
3.3	98.97 ± 32.72	1.03
1.0	126.82 ± 25.37	-26.28
0.8	165.28 ± 46.63	-65.28
0.6	176.04 ± 77.74	-76.04
0.4	111.56 ± 51.36	-11.56
0.2	119.94 ± 32.02	-19.94
0.1	92.68 ± 45.24	7.32

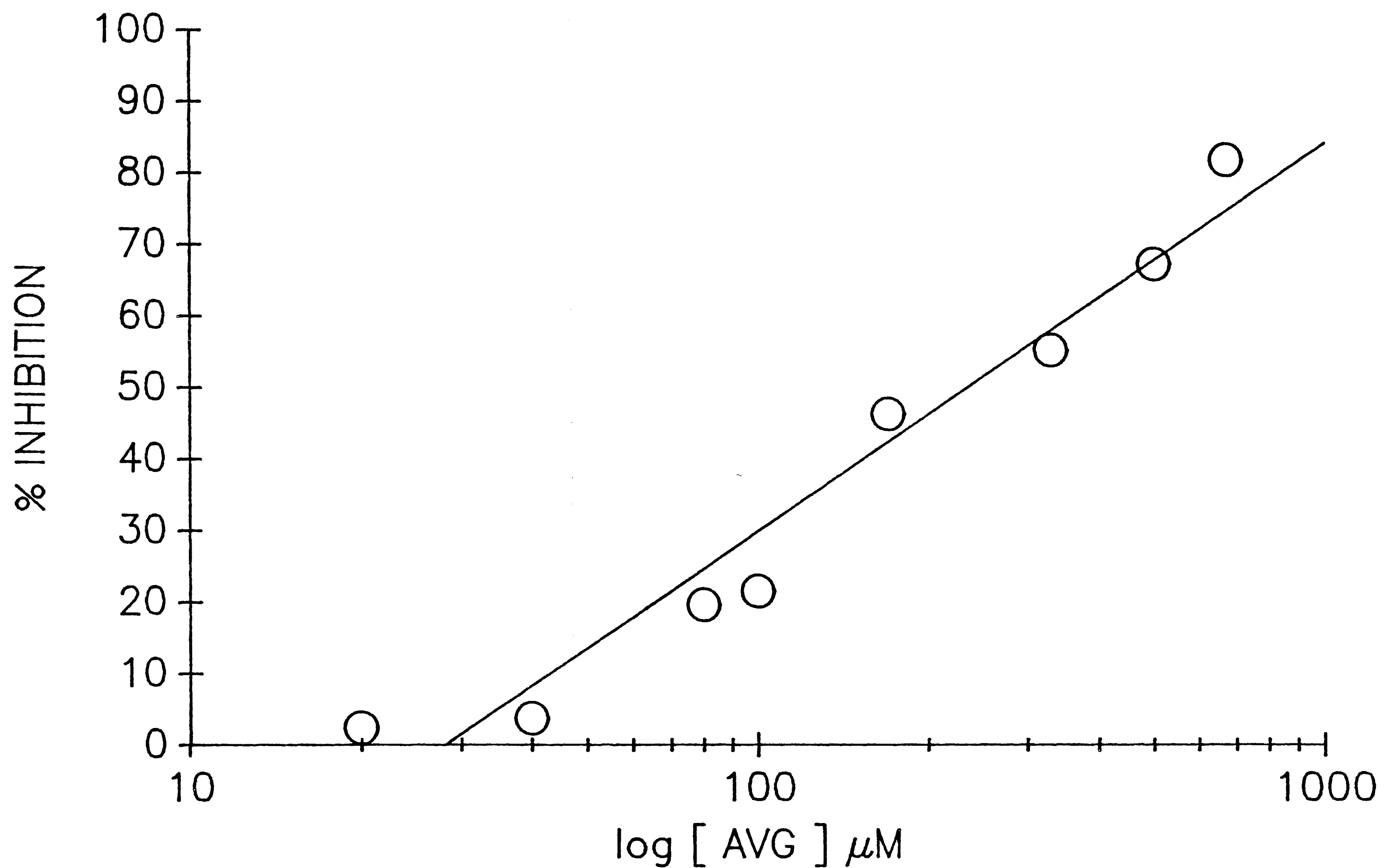


Figure 29 Dose response curve indicating inhibition of ethylene production at μM concentrations of AVG. Percent inhibition calculated as the amount of ethylene produced as a percent of uninhibited control subtracted from ethylene produced by uninhibited control (100%).

AOA (Figure 30) indicate an ID_{50} (dosage at which there is a 50% inhibition of ethylene production) for each inhibitor. The ID_{50} for AVG is approximately 240 μM and for AOA it is approximately 10 mM. An interesting note is that at concentrations lower than 3.3mM, AOA actually stimulates ethylene production, indicating that these concentrations are exhibiting some stress on the plant tissue, and not inhibiting ethylene biosynthesis. Inhibitor concentrations used had no appearent effect on aphid longevity and fecundity.

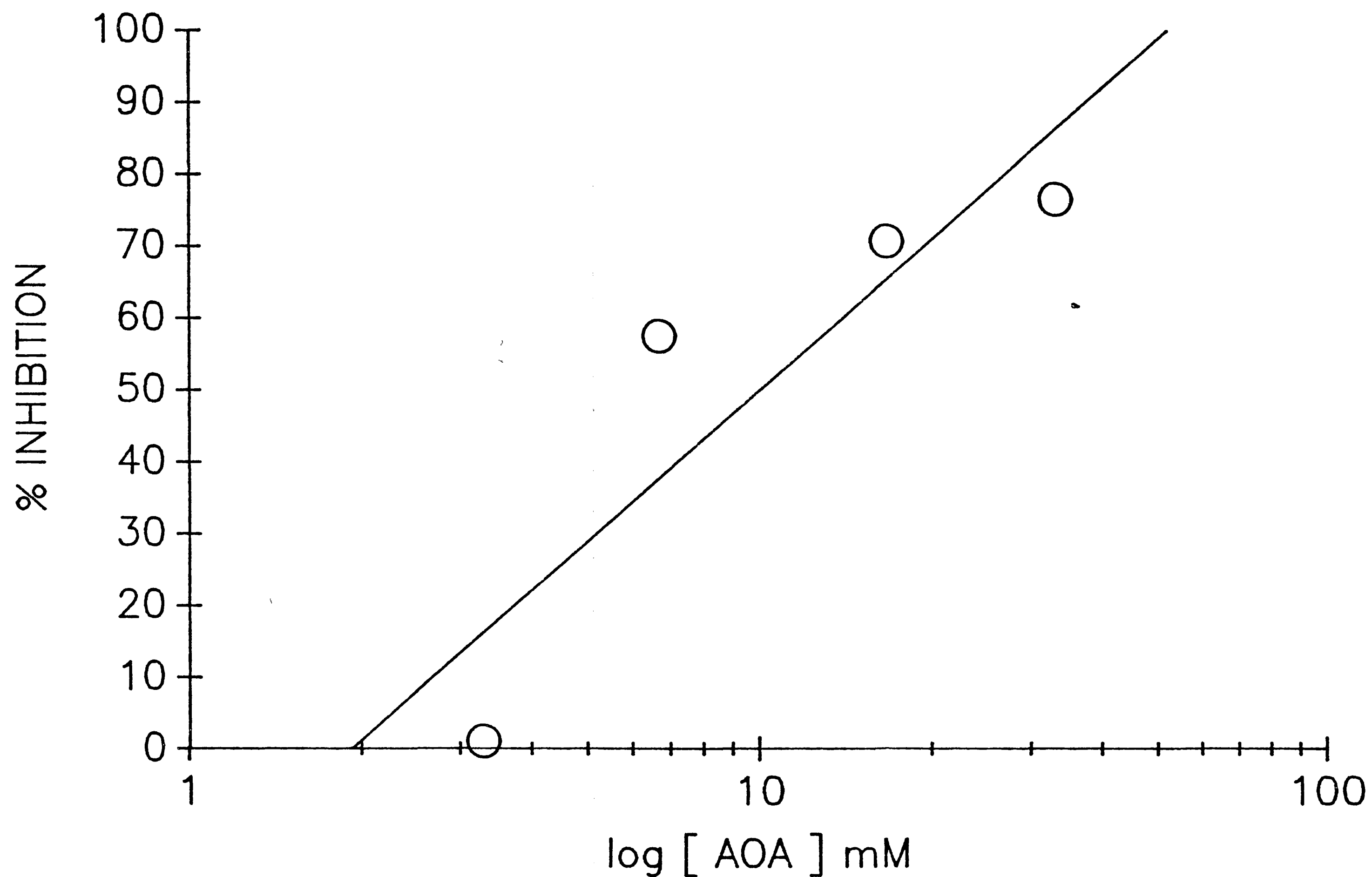


Figure 30. Dose response curve indicating inhibition of ethylene production at mM concentrations of AOA. Percent inhibition calculated as the amount of ethylene produced as a percent of uninhibited control subtracted from ethylene produced by uninhibited control (100%).

CHAPTER V

SUMMARY AND CONCLUSIONS

Spotted alfalfa aphid susceptible, tolerant, and resistant explants produced ethylene in response to SAA and PA infestation. Newly excised trifoliolates and explants from susceptible alfalfa produce ethylene in response to SAA feeding. Ethylene production in explants peaked at two days postinfestation, and maintained a relatively constant level for the remaining five days. Explants eight days of age when removed from the plant displayed the highest rate of ethylene production in response to SAA infestation. Susceptible explants produced more ethylene than tolerant, resistant and uninfested explants in response to feeding by both aphid species. Tolerant explants were found to produce more ethylene than resistant explants.

Samples removed from plants infested for three days produced less ethylene than from plants infested for seven days; these in turn produced less ethylene than samples from plants infested for ten days. Trifoliolates from resistant plants infested with SAA produced ethylene at a constant low rate over the three sample dates. Tissue from tolerant plants infested with SAA produced ethylene at significantly different rates on each of the sample dates with samples

three days postinfestation producing the most ethylene, seven days postinfestation producing at the intermediate rate, and ten days postinfestation producing the least amount of ethylene. Trifoliolates from all the uninfested plants produced the same low level of ethylene over all of the sample dates.

Ethylene production by SAA susceptible, tolerant, and resistant trifoliolates infested with SAA decreased going from top to bottom of the stem. Ethylene production was greatest by susceptible clones, followed by the tolerant clones, then resistant producing the least amount of ethylene. The position on the stem (top to bottom) corresponds to the age of the tissue (young to old) which indicated that ethylene production decreased in relation to an increase in age of tissue. Tissue which was uninfested showed the same relationship between ethylene production and position as the infested trifoliolates, except the level of ethylene production was much lower. The trifoliolates in adjacent positions (without cages) produced ethylene at lower levels than the uninfested tissue, however the relationship between position and ethylene production, was not as clear.

Lipid peroxidation in susceptible plant material was shown to be greater in SAA infested material than in material that did not have aphids feeding upon it. Lipid peroxidation was measured as the amount of malondialdehyde present, which is a product of lipid peroxidation.

Inhibition of ethylene biosynthesis was shown in SAA susceptible tissue by the use of AVG (aminoethoxyvinylglycine), an inhibitor of ACC synthase and AOA (aminooxyacetic acid), a potent transaminase inhibitor. Ethylene production by trifoliolates treated with inhibitors produced less ethylene than controls, but was not completely blocked.

Several conclusions can be made about the affects of SAA feeding on alfalfa from the findings of these studies. It can be concluded that SAA feeding on alfalfa induce ethylene production in alfalfa at a higher rate than seen in uninfested alfalfa. Ethylene production and the generation of lipid peroxides are evidence supporting the the theory that SAA feeding induces a senescence-like process. Lipid peroxides are formed at greater levels in susceptible alfalfa fed upon by SAA than in alfalfa not fed upon.

Ethylene production by alfalfa is an age related phenomenon. Older plant tissues produce less ethylene than younger. This corresponds to ethylene production by trifoliolates in relation to position on the stem; lower foliage producing less ethylene than foliage on the upper part of the stem.

Length of infestation affects the level of ethylene production. In susceptible alfalfa, as the length of infestation increases, ethylene production increases up to ten days. The opposite effect is seen in tolerant alfalfa. In the resistant clones, ethylene production does not change within the ten days.

These studies indicate that the production of ethylene by alfalfa is via methionine metabolism. The inhibitor AVG specifically inhibits ACC synthase. Conversion of S-adenosylmethionine to ACC blocked, thus inhibiting ethylene biosynthesis. AOA is a potent inhibitor of transamination. The inhibition prevents the resynthesis of methionine and thus the synthesis of S-adenosylmethionineis blocked. Ethylene production in not completely inhibited, but is much lower than aphid infested controls. This indicates that ethylene production via the breakdown of lipid peroxides may be present.

LITERATURE CITED

Abeles,F.B. (1985). Ethylene and plant development: An introduction. In: Ethylene and Plant Developement. J.A. Roberts and G.A. Tucker (eds). Butterworths, London. pp 1-8.

Adam,Z.A. and Mayak,S. (1984). Solubilization and partial purification of an enzyme converting 1-aminocyclopropane-1-carboxylic acid to ethylene in plants. FEBS Lett. 172:47-50.

Adams,D.O. and Yang,S.F. (1979). Ethylene biosynthesis: Identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc. Nat. Acad. Sci. USA. 76:170-174.

Aharoni,N.M., Lieberman,M. and Sisler,H.D. (1979). Patterns of Ethylene Production in Senescing Leaves. Plant Physiol. 64:796-800.

Amrhein,N., Brueing,F., Eberle,J., Skorupka,H., and Tophof,S. (1982). In: Plant Growth Substances 1982. P.F. Wareing (ed). Academic Press, London. pp. 249-258.

Amrhein,N., Dorzok,U., Kionka,C., Kondiziolka,U., Skorupka,H. and Tophof,S. (1984). In: Biochemical, physiological and applied aspects of ethylene. Y.Fuchs and E.Chaultz (eds). Martinus Nijhoff Publ. The Hauge. pp. 11-20.

Apelbaum,A. and Yang,S.F. (1981). Biosynthesis of stress ethylene induced by water deficit. Plant Physiol. 68:594-596.

Bleeker,A.B. (1987). The regulation of ethylene biosynthesis in plants; properties of ACC synthase. Curr. Topics Plant Bioch. Physiol. 6:15-24.

Boller,T., Herner,R.C., and Kende,H. (1979). Assay for and enzymatic formation of an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. Planta. 145:293-303.

Bousquet,J.F. and Thimann, K.V. (1984). Lipid peroxidation forms ethylene from 1-aminocyclopropane-1-carboxylic acid and may operate in leaf senescence. Proc. Nat. Acad. Sci. USA. 81:1724-1727.

Brady,C.J., Scott,N.S., and Munns,R. (1974). Mechanisms of regulation of plant growth. In: Bulletin 12 of the Royal Society of New Zealand. R.L. Bieleska, A.R. Ferguson and M.M. Cresswell (eds). pp. 403-409.

Burden,B.J., Morgan,P.W., and Sterling,W.L. (1989). Indoleacetic acid and the ethylene precursor, ACC, in the cotton fleahopper (Hemiptera:Miridae) and their role in cotton square abscission. Ann. Entomol. Soc. Am. 82:476-480.

Burroughs,L.F. (1957). 1-aminocyclopropane-1-carboxylic acid: A new amino-acid in perry pears and cider apples. Nature. 179:360-361.

Burroughs,L.F. (1960). The free amino acids of certain british fruits. J. Sci. Food Agric. 11:14-18.

Cameron,A.C., Fenton,C.A.L., Yu,Y.B., Adams,D.O. and Yang,S.F. (1979). Increased production of ethylene by plant tissues treated with 1-aminocyclopropane-1-carboxylic acid. HortSci. 14:178-180.

Concepcion,M., Lizada,C., and Yang,S.F. (1979). A simple assay for 1-aminocyclopropane-1-carboxylic acid. Anal. Biochem. 100:140-145.

Crocker,W. and Knight,L.I. (1908). Effect of illuminating gas and ethylene upon flowering carnations. Bot. Gaz. 46:259-276.

Dhindsa,R.S., Plumb-Dhindsa,P., and Thorpe,T.A. (1980). Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. 126:93-101.

Duffey,J.E. and Powell,R.D. (1979). Microbial induced ethylene synthesis as a possible factor of square abscission and stunting in cotton infested by cotton fleahopper. Ann. Entomol. Soc. Am. 72:599-601.

Ecker,J.R. and Davis,W.D. (1987). Plant defense genes are regulated by ethylene. Proc. Nat. Acad. Sci. USA. 84:5202-5206.

Even-Chen,Z.C., Atsmon,D. and Itai,C. (1978). Hormonal aspects of senescence in detached tobacco leaves. Physiol. Plantarum. 44:377-382.

Ferguson,I.B., Watkins,C.B. and Harman,J.R. (1983). Inhibition by calcium of senescence of detached cucumber cotyledons. Plant Physiol. 71:182-186.

Galliard,T. (1979). The enzymatic degredation of membrane lipids in higher plants. In: Advances in Biochemistry and Physiology of Plant Lipids. L.A. Appelqvist and C. Liljenberg (eds). Elsevier North Holland, Amsterdam. pp 121-132.

Gane,R. (1934). Production of ethylene by some ripening fruits. Nature. (London). 134:1008

Gepstein,S. and Thimann,K.V. (1981). Role of ethylene in the senescence of oat leaves. Plant Physiol. 68:349-354.

Gwinn,K.D., Stelsig,D.A., and Bhatia,S.K. (1989). Differential ethylene production by potato tuber tissue with a compatible or an incopmatible race of Phytophthora infestans. Amer. Potato J. 66:417-423.

Hilderbrand,D.F., Rodriguez,J.G., Brown,G.C., Lu,K.T. and Volden,C.S. (1986). Peroxidative responses of leaves in two soybean genotypes injured by twospotted spider mites. J. Econ. Entomol. 79:1459-1465.

Hoagland,D.R. and Arnon,D.I. (1938). The water culture method for growing plants without soil. Bull. Cir. #347. Univ. Calif. Exp. Sta.

Jimenez,H.O., Caddel,J.L., and Berberet,R.C. (1988). Selection and characterization of tolerance to the spotted alfalfa aphid in alfalfa. J. of Econ. Entomol. 81:1768-1774.

Kao,C.H. and Yang,S.F. (1983). Role of ethylene in the senescence if detached rice leaves. Plant Physiol. 73:881-885.

Kappel,F., Proctor,J.T.A., and Murr,D.P. (1987). Effect of spotted tentiform leafminer injury on ethylene production and ACC content in apple leaves. Hort Sci. 22:469-471.

Kar,M. (1986). The effect of photoperod on chlorophyll loss and lipid peroxidation in excised senescing rice leaves. J. Plant Physiol. 123:389-393.

Legge,R.L. and Thompson,J.E. (1983). Involvement of hydroperoxides and an ACC-derived free radical in the formation of ethylene. Phytochem. 22:2161-2166.

Leopold,A.C. (1961). Senescence in plant development. Science. 134:1727-1732.

Leshem,Y.Y., Halevy,A.H., and Frenkel,C. (eds.) (1986). Membranes and senescence. In: processes and control of plant senescence. Elsiver Publishing, New York. pp.53-84.

Mack,W.B. (1927). Action of ethylene in accelerating the blanching of celery. Plant Physiol. 2:103.

Martin,W.R., Morgan,P.W., Sterling,W.L., and Meola,R.W. (1988). Stimulation of ethylene production in cotton by salivary enzymes of the cotton fleahopper (Heteroptera:Miridae). Environ. Ent. 17:930-935.

McGlasson,W.B., Poovaiah,B.W., and Dostal,H.C. (1975). Ethylene Production and Respiration in Aging Leaf Segments and in Disks of Fruit Tissue of Normal and Mutant Tomatoes. Plant Physiol. 56:547-549.

Meijer,E.G.M. and Brown,D.C.W. (1988). Inhibition of somatic embryogenesis in tissue cultures of Medicago sativa by aminoethoxyvinylglycine, aminooxyacetic acid, 2,4-dinitrophenol, and salicyclic Acid at concentrations which do not inhibit ethylene biosynthesis and growth. J. Exp. Bot. 39:263-270.

Nickel,J.L. and Sylvester,E.S. (1959). Influence of feeding time, stylet penetration, and developmental instar on the toxic effect of the spotted alfalfa aphid. J. Econ. Entomol. 52:249-254.

Nilsen,K.W. and Hodges,C.F. (1983). Hypobaric control of ethylene-induced leaf senescence in intact plants of Phaseolus vulgaris L.. Plant Physiol. 71:96-101.

Pauls,K.P. and Thompson,J.E. (1980). In vitro simulation of senescence-related membrane damage by ozone-induced lipid peroxidation. Nature. 283:504-506.

Pitkanen,O.M., Hallman,M., and Andersson,S.M. (1989). Determination of ethane and pentane in free oxygen radical-induced lipid peroxidation. Lipids. 24:157-159.

Roberts,J.A. and Osborne,D.J. (1981). Auxin and the control of ethylene production during the development and senescence of leaves and fruit. J. of Exp. Bot. 32:875-887.

Sanders,I.O., Smith,A.R., and Hall,M.A. (1989). The measurement of ethylene binding and metabolism in plant tissue. Planta. 179:97-103.

Shain,L. and Hillis,W.E. (1972). Ethylene production in *Pinus radiata* in response to *Sirex*-*Amylostereum* attack. Phytopath. 62:1407-1409.

Smith,A.R. and Hall,M.A. (1984). Biosynthesis and metabolism of ethylene. In: The biosynthesis and metabolism of plant hormones. A.Crozier and J.R. Hoffman (eds.). Cambridge Univ. Press, Cambridge. pp. 201-209.

Sorensen,E.L., Byers,R.A., and Horber,E.K. (1988). Breeding for insect resistance. In: Alfalfa and alfalfa improvement. A.A. Hanson, D.K. Barnes and R.R. Hill (eds.). American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wisconsin. pp. 859-902.

Verhagen,J., Veldink,G.A., Egmond,M.R., Vliegenhart,J.F.C., Boldingh,J. and Van der star,J. (1978). Steady state kinetics of the anaerobic reaction of soybean lipoxygenase-1 with linoleic acid. Biochem. Biophys. Acta. 529:369-379.

Vliegenthart,J.F.G. and Veldink,G.A. (1982). Lipoxygenases In: Free radicals in biology. Academic Press, New York. pp. 29-64.

Yang,S.F. (1981). Biosynthesis of ethylene and its regulation. In: Recent Advances in the Biochemistry of Fruits and Vegetables. J. Friend and M.J.C. Rhodes (eds.) Academic Press, London. pp. 89-106.

Zimmerman,P.W. and Wilcoxon,F. (1935). Several chemical growth subtances which cause initiation of roots and other responses in plants. Contributions of the Boyce Thompson Institute. 7:209-229.

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