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The Role of Ethylene and Abscisic Acid in TMV-induced Symptoms in Tobacco

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Xanthi-*nc* tobacco leaves pre-treated with ABA or with Ethrel exhibited senescence and increased the number of TMV local lesions. Changes in local lesion numbers were not associated with similar changes in recoverable infective TMV. Endogenous ABA progressively increased in leaves as TMV lesions developed.

When BYMV-infected leaves of *Tetragonia expansa* were exposed to ethylene, necrotic spots developed in the normally chlorotic lesions. An inhibitor of ethylene action, namely CO_2, was able to inhibit the development of chlorotic lesions in infected *Tetragonia* leaves.

In a systemic host–pathogen combination (cultivar Samsun infected by TMV) both Ethrel and ABA caused dwarfing in healthy and in infected plants. Regarding the effect of ABA, it is proposed that an ABA-induced stimulation of ethylene release exists and this would be directly involved in the reduction of extension growth.

It is well known that abscisic acid (ABA) (e.g. BALÁZS *et al.*, 1973) and ethylene (e.g. BURG, 1962) can accelerate senescence when applied exogenously to plants and that enhanced senescence is often associated with the development of virus-induced local lesions and systemic symptoms. Virus-induced necrotic lesions may be a consequence of enhanced leaf senescence. This hypothesis is supported by data which show that local lesion formation is suppressed when juvenility is extended but increased when tissue senscence is promoted (e.g. KIRÁLY and SZIRMAI, 1964; KIRÁLY *et al.*, 1968; BALÁZS *et al.*, 1973). Increased ethylene production during local lesion development is well documented and there is evidence which points to the possible involvement of ethylene in virus localisation and/or concomitant host necrosis (BALÁZS *et al.*, 1969; NAKAGAKI *et al.*, 1970; GÁBORJÁNYI *et al.*, 1971; and PITCHARD and ROSS, 1975). In addition, BALÁZS *et al.* (1973) suggested a possible involvement of ABA by showing that the aging effect of ABA increased TMV lesion production and multiplication in tobacco.

Work with viruses which systemically invade host plants has shown that host ethylene production may be unaffected (BALÁZS *et al.*, 1969), or associated with virus-induced premature senescence (NAKAGAKI *et al.*, 1970). Increased ethylene production has also been correlated with cucumber mosaic virus (CMV)-induced stunting of cucumber hypocotyls and epinasty of cucumber cotyledons (MARCO *et al.*, 1976; LEVY and MARCO, 1976). Little attention has been paid

to the possible role of ABA in virus-induced stunting but BAILISS (1977) found no significant difference in the endogenous ABA content of healthy and CMV-infected cucumber cotyledons and leaves.

This paper reports further studies on the role of ethylene and ABA in virus-induced symptoms.

Materials and Methods

Viruses and hosts. Tobacco plants (*Nicotiana tabacum* cvs. Samsun, Xanthi and Xanthi-*nc*) were grown under glasshouse conditions and used at the 8–10 leaf stage. Experiments on chemically-induced stunting were made with two month old tobacco plants. The Ul strain of TMV was cultured in *N. tabacum* cv. Samsun and the virus purified using a modification of the methods described by FRAENKEL-CONRAT (1966) and GOODING and HEBERT (1967). Infectivity was determined by inoculating tobacco leaves (cv. Xanthi-*nc*) and counting the number of lesions produced. In some experiments chlorotic lesions were induced in leaves of *Tetragonia expansa* by infection with bean yellow mosaic virus (BYMV).

Chemical treatments. Aqueous solutions of ABA at concentrations of 1, 10 and 100 μg/ml were applied to tobacco leaves by either spraying half-leaves of attached leaves twice daily or by injection (KLEMENT, 1963). Ethrel (2-chloroethyl-phosphonic acid) (Amchem P. A., USA), dissolved in 0.06 *M* phosphate buffer (pH 6.5) to give a concentration of 200 μg/ml was sprayed daily onto half-leaves. Plants were exposed to CO_2 and ethylene by removing them from the soil and placing them into nutrient solution under three litre capacity bell jars. Ethylene gas or CO_2 was bubbled through water into the bell jar until a 1% concentration was reached.

Ethylene determination. Plant ethylene production was estimated by collecting the gas from plants placed under bell jars with a continuous air stream in 0.25 *M* mercuric perchlorate in 2 *M* $HClO_4$ (YOUNG *et al.*, 1952) and the amount estimated by gas chromatography after releasing the ethylene by the addition of 2 *N* hydrochloric acid (BALÁZS *et al.*, 1969).

ABA determination. A modification of the methods of LENTON *et al.* (1971) and BAILISS (1977) was used to estimate the endogenous ABA content of plant tissue. Leaf tissue (*c.* 30 g fresh weight) was immersed immediately after sampling in 300 ml chilled 80% methanol and 1.5 g sodium bicarbonate added to the extract. The material was left at −15°C for five hours and then homogenized in a Waring blender at 4°C. The homogenate was left overnight at 4°C then centrifuged at 5000 *g* for 30 min at 4°C. The supernatant was reduced to the aqueous phase in a rotary evaporator at 40°C, adjusted pH 3.5 with 2 *N* HCl and extracted three times with equal volumes of diethyl ether. Bulked ether extracts were extracted three times with equal volumes of 5% aqueous sodium bicarbonate and, after adjusting the pH to 3.5, the combined aqueous extracts were extracted three times with equal volumes of diethyl ether. Combined ether extracts were evapo-

rated to dryness with a rotary evaporator, re-dissolved in a small volume of 96% ethanol and streaked onto Whatman No. 1 chromatography paper or Silufol 254 (Merck) thin-layer chromatography plates. Two solvent systems were used for further separation; a) *n*-butanol : ammonia : propanol : water, 2 : 1 : 6 : 2, and b) ethyl acetate : formic acid : benzene, 100 : 2 : 50. Authentic ABA markers were run on all chromatograms and their position located under UV light. Zones corresponding to the markers were eluted with ethanol and the ethanol evaporated off *in vacuo* in small vials. A 0.1 ml aliquot of Regisil reagent (Regis, Chicago, USA) (*bis*-trimethyl-silyl-trifluoroacetamide dissolved in 1% trimethyl-chlorosilane) was added to each vial. After sealing, the vials were placed in a water bath and exposed to 100°C for 10 min or longer. All determinations were made with a Packard Series 7400 chromatograph equipped with flame ionization detectors and with 60 cm × 4 mm glass columns containing Chromosorb G (60–80 mesh) coated with 1.5% SE 30. The column temperature was 200°C and the N_2 carrier gas flow rate was 48 ml/min.

Results

The effect of Ethrel and ABA-induced senescence on lesion numbers and virus infectivity. Half leaves of Xanthi-*nc* tobacco were sprayed with ABA and Ethrel (an ethylene producing agent) for varying periods before the entire leaves were

Table 1

The effect of Ethrel and ABA on local lesion production and TMV infectivity in Xanthi-*nc* tobacco

Experiment		Period of treatment (days)	Lesion number (per cent of untreated control leaves)*	Infectivity of ultracentrifuged extracts (per cent of untreated control leaves)*
ABA	I.	18–20	197.1**	120.9
	II.	14–17	153.8	113.1
	III.	10–13	126.6	103.9
Ethrel	I.	8–9	198.3	119.9
	II.	5–7	168.1	111.5
	III.	3–4	145.3	103.1

Each experiment was repeated 5 times. In each replicate 8 plants with 6–8 leaves were used. The ultracentrifuged extracts were assayed on 20 leaves of Xanthi-*nc* by half leaf comparisons.

* The correlation between lesion number and infectivity of the ultracentrifuged extract was significant ($P < 0.05$).

** Mean number of lesions per half leaves for the water-sprayed control was 138. ABA (100 μg/ml) or Ethrel (200 μg/ml in phosphate buffer pH 6.5) was sprayed onto half-leaves twice daily

inoculated with TMV. In a series of experiments such treatments increased the numbers of local lesions in the treated half-leaves compared with water-sprayed control half-leaves. The size of the increase in lesion numbers depended on the length of the treatment period. In every experiment the infectivity of ultracentrifuged leaf extracts was also determined (cf. BALÁZS *et al.*, 1976). Table 1 illustrates the results of typical experiments. Ethrel and ABA-induced increases in lesion numbers were pronounced after the longer treatment periods. However, the infectivity of ultracentrifuged extracts was always less than was expected on the basis of lesion production (Table 1). The data suggested that Ethrel and ABA treatment decreased the infectivity of the lesions which developed. This was further examined by determining the infectivity of virus recoverable from 100 lesions removed with a 2 mm diameter cork borer from the treated half-leaves and compared with 100 lesions similarly removed from the control half-leaves. The results (Table 2) confirmed that infectivity was reduced in lesions tested from treated leaves.

Table 2

Infectivity of TMV in lesions after Ethrel and ABA treatment

Experiment	Lesion number	
	control half-leaves	treated half-leaves
Ethrel	89	73
Abscisic acid	97	75

48 h after infection with TMV 100 lesions were cut from the leaves with a cork borer (2 mm in diameter), ground in 3 ml buffer pH 6.9 and assayed on Xanthi-*nc*.

Plants were sprayed twice daily with 200 ppm Ethrel and 100 ppm abscisic acid respectively, for 4 days. The ratio of lesion numbers in control and treated half-leaves was 131 : 100. The experiment was repeated ten times; the data are the means of 10 experiments

The possibility was considered that the chemicals used might have a direct effect on TMV infectivity. Therefore ethylene gas was bubbled through a purified preparation of TMV (100 μg/ml) for 1 h at 22°C. Air was bubbled through a second aliquot of the purified preparation to serve as a control. There was no difference in the infectivity of the two preparations as evidenced by lesion counts made on inoculated Xanthi-*nc* leaves. It was shown previously that ABA had no effect on the infectivity of TMV when mixed with the virus *in vitro* (BALÁZS *et al.*, 1973).

Endogenous ABA content of TMV-infected Xanthi-nc leaves. As ABA treatment affected lesion production it was decided to investigate the endogenous ABA content of healthy and infected half-leaves. Half-leaves of tobacco cv. Xanthi-*nc* were inoculated with TMV and the corresponding half-leaves with water. Samples were taken from inoculated and control half-leaves 24, 48 and 72 h after inocula-

tion and the ABA content estimated. The results (Table 3) showed a progressive increase in endogenous ABA in infected compared with control half-leaves. The rise in endogenous ABA was correlated with the appearance and expansion of local lesions.

Table 3

The endogenous ABA content of Xanthi-*nc* tobacco leaves infected or uninfected with TMV

Time between inoculation and sampling (h)	ABA content of half-leaves (μg/kg) fresh weight	
	control	infected
24	36	43
48	36	52
72	40	79

Half-leaves of Xanthi-*nc* plants (6—8 leaf stage) were inoculated with TMV. Twenty-four, 48 and 72 h after inoculation the infected and control half-leaves were collected separately and the ABA content estimated. Ten plants were used at each sampling time

The effect of ethylene and CO_2 on BYMV-induced symptoms in Tetragonia expansa. The effect of ethylene on symptom expression was further studied in a virus–host interaction resulting in the formation of chlorotic lesions. *T. expansa* plants were placed in bell jars containing 1 % CO_2 or 1 % ethylene two days after inoculation with BYMV for four days. Plants exposed to CO_2 failed to develop typical chlorotic lesions; the plants showed symptomless infection. BYMV-infected plants exposed to ethylene developed local necrotic spots within the chlorotic lesions. This effect was best shown in the older mature leaves. Necrotization could also be induced by spraying inoculated leaves with 1000 ppm Ethrel twice daily for four days after inoculation with BYMV.

The effect of ABA and Ethrel on the growth of healthy and TMV-infected Samsun tobacco. Further studies on the role of ABA and ethylene on virus symptom expression were made using systemically infected tobacco plants. Both ABA and Ethrel treatments caused further stunting in infected plants (Table 4). Untreated infected plants were stunted compared with control plants. The extension growth of plants sprayed once or twice daily virtually ceased. It was considered that the effects shown after ABA treatment could involve ABA-induced stimulation of ethylene release rather than a simple direct effect of ABA. Therefore, healthy Samsun leaves were injected with a range of ABA concentrations and control plants injected with water. As shown in Table 5, ABA treatment enhanced ethylene production and the magnitude of ethylene release depended on the concentration of ABA supplied.

Table 4

The effect of ABA and Ethrel on stem length and leaf fresh weight of healthy and TMV-infected Samsun tobacco

Plant material	Stem length (cm)	Leaf weight (g)
Healthy Samsun	12.8 ± 1.0	5.7 ± 0.5
Healthy Samsun plus ABA (100 μg/ml)	6.0 ± 1.0	3.7 ± 0.5
TMV-infected Samsun	5.6 ± 0.9	2.5 ± 0.6
TMV-infected Samsun plus ABA (100 μg/ml)	3.3 ± 0.5	2.0 ± 0.4
Healthy Samsun	14.3 ± 0.8	—
Healthy Samsun plus Ethrel (200 μg/ml)	6.4 ± 0.7	—
TMV-infected Samsun	6.0 ± 0.9	—
TMV-infected Samsun plus Ethrel (200 μg/ml)	3.1 ± 0.5	—

Plants were measured one month after inoculation with TMV and the data are the mean of 20 measurements. Plants were inoculated when two months old with a purified preparation of TMV (120 μg/ml) by using a glass rod. No abrasive was added to the inoculum. The ABA treatment was applied by spraying twice daily for one month after inoculation. The Ethrel treatment was applied similarly but once daily

Table 5

The effect of ABA on ethylene production in healthy Samsun tobacco plants

ABA treatment (μg/ml)	Ethylene production (μl/24 h/g tissue) time after treatment	
	24—48 h 2 days	48—72 h 3 days
0	1.5	1.5
1	1.7	1.9
10	1.6	2.9
100	4.2	5.8

ABA was injected with a hypodermic syringe into the tobacco leaves. Control plants were injected with water

Discussion

Pre-treatment of tobacco (cv. Xanthi-*nc*) leaves with ABA and Ethrel induced senescence and increased the number of TMV local lesions visible to the naked eye. These results contrasted markedly with those obtained by BALÁZS *et al.* (1976) who pre-treated tobacco leaves with kinetin and found senescence was delayed and TMV lesion numbers decreased. In both experiments, however, changes in lesion numbers were not associated with similar changes in recoverable

infective TMV. Results reported here for Ethrel and ABA treatment support the possibility that virus replication is reduced in lesions which are formed. In experiments in which kinetin caused a reduction in lesion numbers there was a concomitant increase in single necrotic cells which, in terms of virus infectivity, probably compensated for the decrease in macro lesions (BALÁZS *et al.*, 1976). The demonstration that kinetin, ABA and ethylene treatments affect lesion numbers strengthens earlier observations (GÁBORJÁNYI *et al.*, 1971) that plant growth regulators are probably involved in local lesion development.

The role of ethylene in lesion development has been considered to be a consequence of enhanced host tissue senescence caused or followed by ethylene production (BALÁZS *et al.*, 1969; NAKAGAKI *et al.*, 1970; GÁBORJÁNYI *et al.*, 1971; PITCHARD and ROSS, 1975). ABA may also be involved for it accelerates leaf senescence in Xanthi-*nc* tobacco (BALÁZS *et al.*, 1973) and evidence is presented here that endogenous ABA progressively increases in tobacco leaves as TMV lesions develop, ABA treatment increases ethylene production in Samsun tobacco and, as discussed above, exogenous ABA treatment increases lesion production.

The precise roles of ABA and ethylene in virus-induced necrosis remain unclear. However, the experiments reported with BYMV infection of *Tetragonia expansa* provide further information regarding the role of ethylene. BYMV-induced chlorotic lesions represent localized areas of intense tissue senescence without necrosis. BYMV infection stimulates ethylene production (GÁBORJÁNYI *et al.*, 1971) and indicates tissue necrosis may not be a prerequisite for enhanced ethylene production. However, when infected plants were exposed to ethylene, necrotic spots developed in the chlorotic lesions whereas exposure to CO_2 (an inhibitor of ethylene action) inhibited chlorotic lesion development. Clearly ethylene is involved in chlorotic lesion formation and perhaps in the necrotization process, although the latter may be very much dependent on the ethylene concentration supplied and the time when the plant is exposed to the gas relative to inoculation.

In the systemic TMV/tobacco cv. Samsun system both Ethrel and ABA treatments caused dwarfing in healthy and infected plants. As ABA treatment increased ethylene production, it is possible that ethylene may have the more direct effect on extension growth. However, further work is required to clarify the roles of ethylene and ABA in virus-induced effects on extension growth, particularly in view of, for example, the absence of endogenous ABA imbalance in CMV-stunted cucumber (BAILISS, 1977) and the variable effects of systemic infections on host ethylene production (e.g. BALÁZS *et al.*, 1969; MARCO *et al.*, 1976).

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