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Ineffectiveness of Ethylene Biosynthetic and Action Inhibitors in Phenotypically Reverting the *Epinastic* Mutant of Tomato (*Lycopersicon esculentum* Mill.)

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Abstract. Five-day-old, dark-grown seedlings of the *Epinastic* (*Epi*) tomato mutant (*Lycopersicon esculentum* Mill.) and its parent, cultivar VFN8, were used as a system for assessing the role of ethylene in the *Epi* phenotype. The distinguishing features of *Epi* seedlings are an increase in hypocotyl diameter and reduced hypocotyl length. Treatment of VFN8 seedlings with 0.5 μl/liter ethylene closely mimicked the *Epi* phenotype. The rate of ethylene production by 5-day-old, dark-grown *Epi* seedlings was double that of VFN8 seedlings. Nevertheless, treatment of *Epi* seedlings with inhibitors of ethylene biosynthesis (aminoethoxyvinylglycine or Co^{2+}) or ethylene action (silver thiosulfate or norbornadiene) failed to normalize the *Epi* phenotype. *Epi* seedlings grown in sealed jars containing ethylene and CO_2 adsorbants also expressed the characteristic *Epi* phenotype. The results indicate that the physiological lesion resulting from the *Epi* gene mutation is not simply an overproduction of ethylene.

The *Epinastic* (*Epi*) tomato results from a single-gene, partially dominant mutation and is characterized by severe epinasty of the leaves, swelling of stem and petiolar cortex, and an abundance of lateral roots (Ursin 1987). During growth and development, the *Epi* phenotype becomes increasingly epinastic in conjunction with an elevation of tissue ethylene concentration (Fujino et al. 1988a). This increase in ethylene production results from elevated levels of the ethylene precursor, ACC, rather than greater EFE activity (Fujino et al. 1988a). Furthermore, the increase in ethylene levels in *Epi* does not appear to

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result from a difference in IAA content or sensitivity compared to its parent line, cultivar VFN8 (Fujino et al. 1988b). Therefore, 1 hypothesis would be that the *Epi* mutation has resulted in a constitutive overproduction of ethylene, which causes the characteristic phenotype.

We tested this hypothesis using dark-grown *Epi* and VFN8 seedlings. The *Epi* phenotype is expressed in seedlings as a thickened and shortened hypocotyl, compared to VFN8 seedlings. VFN8 seedlings treated with ethylene closely resemble *Epi* seedlings. We measured ethylene production by seedlings of the 2 genotypes and compared their morphologic responses to inhibitors of ethylene synthesis and action to determine the role of ethylene in the *Epi* phenotype.

Materials and Methods

Plant Material

Tomato (*Lycopersicon esculentum* Mill.) seeds of the *Epi* mutant and its parent (cultivar VFN8) were surface sterilized for 45 min in 2% NaOCl solution (40% bleach). Seeds were rinsed with 3 liters of distilled water, sown onto moistened blotter paper in 11 × 11 × 3 cm germination boxes, sealed and placed in the dark at 26°C. After 48 h, uniform seeds with 1 mm radicles were selected for the following experiments.

Ethylene Determination

Pregerminated seeds were sown onto moist blotter paper, placed in an 8 ml vial containing 1 ml of 2.5 mM KH_2PO_4 (pH 6.0), and kept in the dark at 26°C. On day 5, the vials were purged with ethylene-free air, sealed with rubber septa, and placed in the dark for 6 h. Ethylene accumulation within the vials was determined by gas chromatography as described by Bufler et al. (1980).

Ethylene Treatments

VFN8 and *Epi* seeds were sown on separate blotter papers, placed into 0.5 liter jars containing 10 ml of 2.5 mM KH_2PO_4 (pH 6.0), and sealed with lids containing rubber septa. Ethylene was injected into the jars to achieve final concentrations of 0.1, 0.2, and 0.5 μl/liter. The jars were placed in the dark at 26°C. On day 5, the seedlings were removed and photographed to compare the phenotypes of the 2 genotypes. Ethylene concentrations at the start and conclusion of the study were confirmed by gas chromatography.

Epi Phenotype Reversion

Pregerminated seeds of VFN8 and *Epi* were sown onto blotter paper in 11 × 11 × 3 cm plastic boxes containing 13 ml of 2.5 mM KH_2PO_4 (pH 6.0), or

buffer containing either ethylene biosynthetic inhibitors (AVG or Co^{2+}), or the ethylene action inhibitor, 0.1 mM STS (0.1 mM $AgNO_3$ + 0.4 mM sodium thiosulfate). The boxes were covered and placed in the dark at 26°C. For norbornadiene treatment, seeds on moistened blotter paper were sealed in 0.5 liter jars. Norbornadiene was injected through a rubber septum to achieve a final concentration of 1000 μl/liter. Five days after seed germination, the seedlings were removed and photographed to compare the *Epi* phenotype to VFN8 controls.

Effect of CO_2- and Ethylene-Free Air

Pregerminated seeds of VFN8 and *Epi* were sown onto separate blotter papers and placed into 0.5 liter jars containing 10 ml of 2.5 mM KH_2PO_4 (pH 6.0). Soda lime, potassium permanganate (Purafil), or both soda lime and permanganate were added to the jars in separate vials. The jars were sealed and placed in the dark at 26°C. Levels of CO_2 and ethylene in the jars were monitored by gas chromatography. On day 5, the seedlings were removed and photographed to compare the phenotypes of the 2 genotypes.

Results

When 5-day-old dark-grown seedlings of VFN8 and *Epi* were compared, the *Epi* mutant was phenotypically different from its parent, VFN8 (Fig. 1).The hypocotyl was thicker and the total length of the hypocotyl was 62% less than that of VFN8 (Table 1). In addition, the ethylene production rate of *Epi* seedlings was twice that of VFN8 seedlings (Table 1). Treatment of VFN8 and *Epi* seedlings with various ethylene concentrations reduced hypocotyl elongation when compared with their untreated controls (Fig. 2). The percentage inhibition at each ethylene concentration did not differ significantly between *Epi* and VFN8 (Table 2), indicating that ethylene sensitivity of the 2 genotypes was essentially identical.

Treatment of VFN8 seedlings with 0.5 μl/liter ethylene phenotypically mimicked the *Epi* control seedlings (Fig. 2C). These results support the hypothesis that ethylene is responsible for the *Epi* phenotype, because the *Epi* mutant produces significantly more ethylene and treatment of VFN8 with exogenous ethylene mimicked the *Epi* phenotype.

Since the accelerated ethylene production rate appeared to be responsible for the *Epi* phenotype, a series of experiments were conducted using various inhibitors of ethylene biosynthesis or action in an attempt to revert the *Epi* phenotype. VFN8 and *Epi* seedlings were treated with the ethylene biosynthetic inhibitors AVG and Co^{2+} at various concentrations. Treatment with AVG at up to 2 μM was ineffective in normalizing the *Epi* phenotype (Fig. 3). Higher concentrations of AVG (10 and 50 μM) applied in sterile culture were also ineffective, although loss of normal root geotropism occurred in both ge-

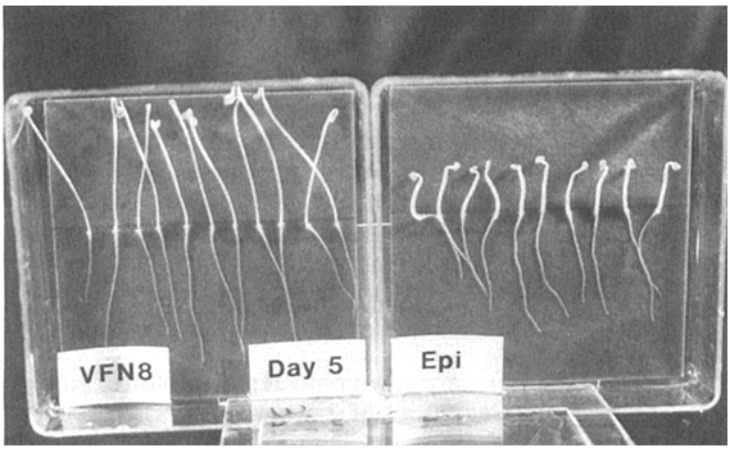

Fig. 1. Dark-grown seedlings of VFN8 and *Epi* 5 days after sowing.

Table 1. Hypocotyl length and rate of ethylene evolution by 5-day-old, dark-grown seedlings of VFN8 and *Epi*

Genotype	Hypocotyl Length (mm)	Ethylene Production (nl/g/h)
VFN8	25.1 ± 1.6	0.31 ± 0.02
Epi	9.6 ± 0.4	0.64 ± 0.03

Hypocotyl lengths are the means of 10 seedlings ± 95% confidence interval. Ethylene evolution rates are means of 5 replicates of 6–8 seedlings per vial ± 95% confidence interval.

notypes (data not shown). Treatment of seedlings of both genotypes with Co^{2+} also had no effect on either *Epi* or VFN8 (data not shown).

A further attempt was made to normalize the *Epi* phenotype by treating seedlings with the ethylene action inhibitors, STS or norbornadiene. STS had no visible effect on the phenotypes of *Epi* or VFN8 seedlings (data not shown). In addition, when 19-day-old, light-grown *Epi* seedlings were treated continuously in solution culture with 100 μM STS for 14 days, only a partial reversion of the *Epi* phenotype was observed (Fig. 4). Similar results were obtained when light-grown seedlings (4-day-old) were treated with 1000 μl/liter norbornadiene (data not shown). Scrubbing the air surrounding the seedlings of CO_2 and ethylene also had no effect on the phenotype of either *Epi* or VFN8 seedlings (data not shown). Levels of CO_2 and ethylene were below the detectable range of the gas chromatograph (50 μl/liter and 5 nl/liter).

Discussion

Many features of the *Epi* phenotype suggest that the mutation has resulted in altered ethylene physiology. The rate of ethylene production is greater in both dark-grown (Table 1) and light-grown (Fujino et al. 1988a) seedlings of *Epi* than VFN8 seedlings. Increasing epinasty of the leaves is associated with elevated

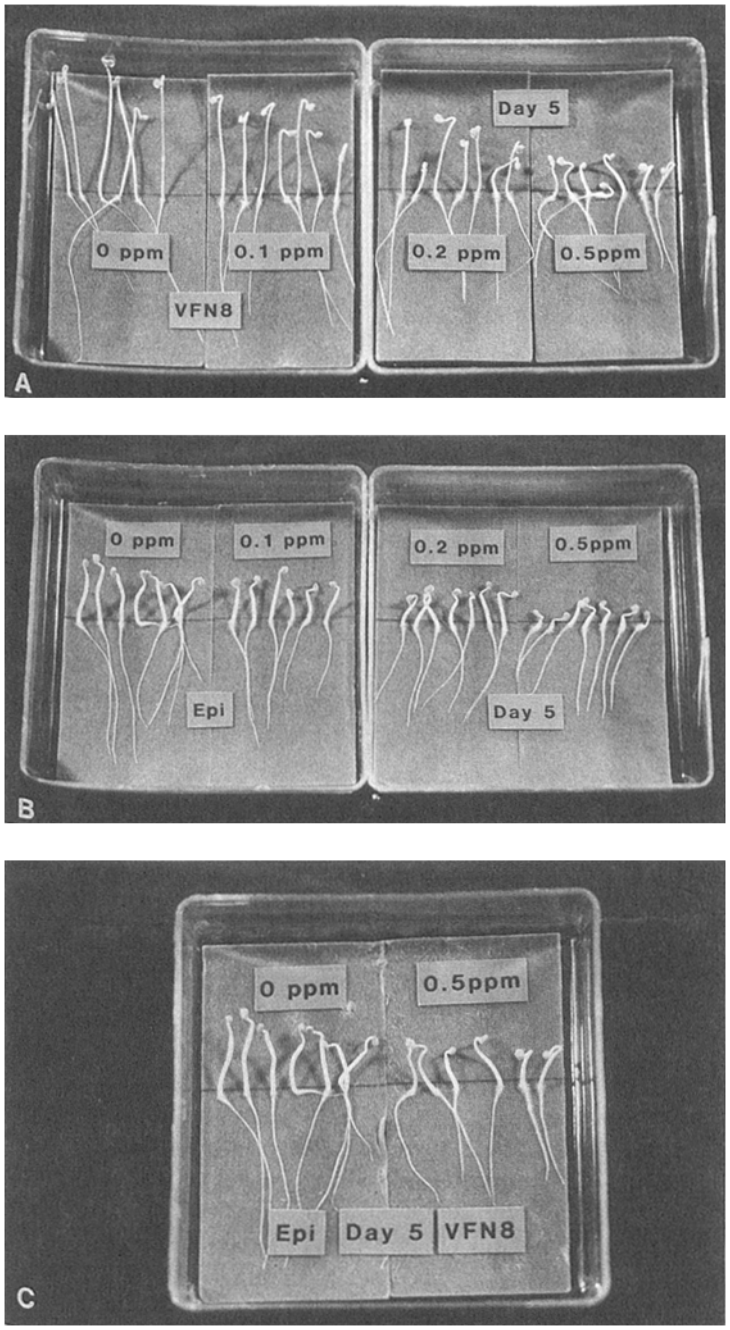

Fig. 2. Dark-grown seedlings of VFN8 and *Epi* germinated for 2 days, then treated for 3 days with the indicated ethylene concentrations. **(A)** VFN8; **(B)** *Epi*; **(C)** comparison of *Epi* untreated control to VFN8 treated with 0.5 μl/liter ethylene.

Table 2. Effect of exogenous ethylene on hypocotyl elongation of 5-day-old, dark-grown seedlings of VFN8 and *Epi*

Genotype	Ethylene Concentration (μl/liter)	Hypocotyl Length (mm)
VFN8	0.0	20.3 ± 2.5 (100)
	0.1	16.3 ± 1.9 (80)
	0.2	11.0 ± 1.4 (54)
	0.5	6.7 ± 0.7 (33)
Epi	0.0	9.7 ± 1.2 (100)
	0.1	7.5 ± 1.4 (77)
	0.2	5.7 ± 0.4 (59)
	0.5	2.8 ± 0.8 (29)

Data are the means of 6 seedlings ± 95% confidence interval. The percentages of the control elongation for each genotype are shown in parentheses.

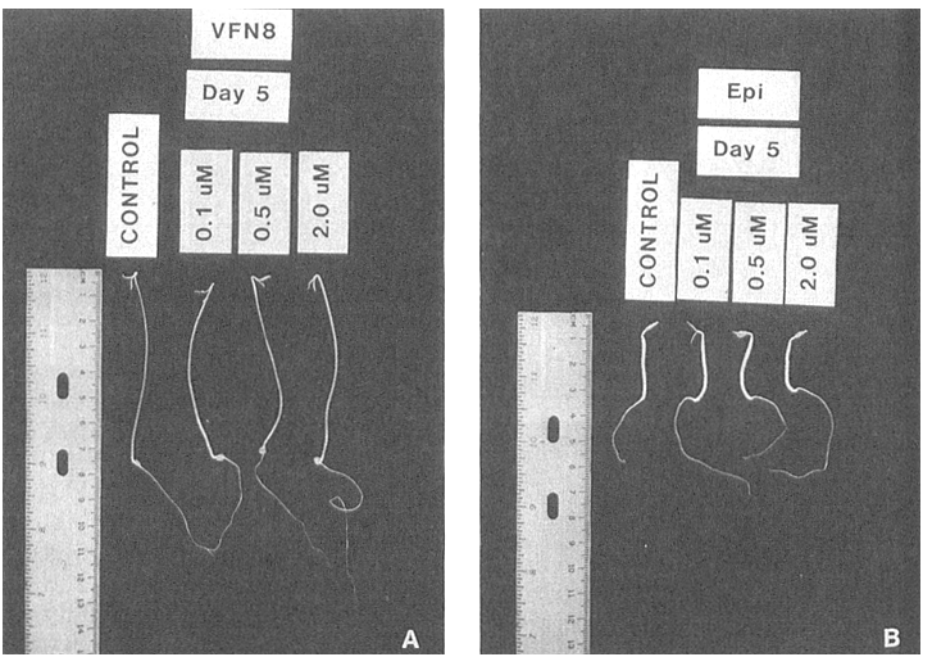

Fig. 3. Dark-grown seedlings of VFN8 and *Epi* germinated for 2 days, then treated for 3 days with AVG at the indicated concentrations. A representative seedling is shown for each concentration. **(A)** VFN8; **(B)** *Epi*.

ethylene levels in *Epi* plants (Fujino et al. 1988a). Petiole epinasty, cortical stem swelling, inhibition of elongation, and root branching are well-known responses to ethylene (Abeles 1973). Inhibition of hypocotyl (Table 2) and root growth (Fujino et al. 1988a) by ethylene is similar in VFN8 and *Epi* seedlings, indicating that the response to ethylene has not been altered by the mutation. *Epi* tissues produced ethylene at a greater rate than those of VFN8 (Table 1), perhaps accounting for the reduction in hypocotyl growth in *Epi* seedlings (Fig.

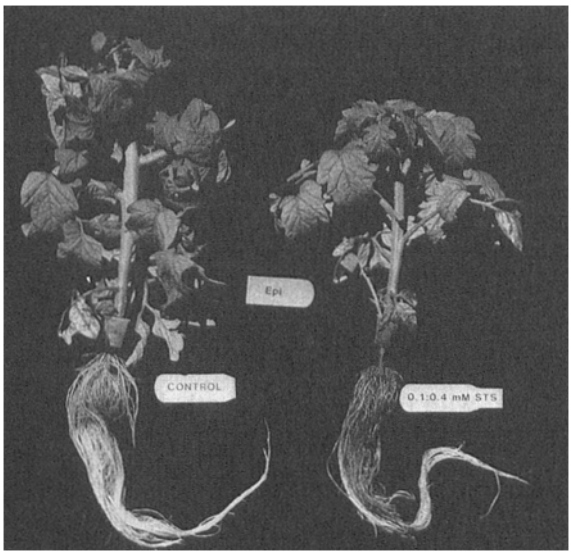

Fig. 4. *Epi* plant grown in the greenhouse for 19 days in half-strength Hoagland's solution #2, then grown for an additional 14 days in the same solution (*left*) or solution supplemented with 0.1 mM STS (*right*).

l; Table 2). If so, inhibition of ethylene production or action should phenotypically revert *Epi* seedlings to the parent morphology.

However, AVG, Co^{2+}, STS, and norbornadiene had no effect on the *Epi* phenotype. The inhibitors used have been shown to be potent inhibitors of ethylene biosynthesis or action (Beyer 1976; Sisler and Yang 1984; Yu and Yang 1979). In those experiments using ethylene synthesis inhibitors, the concentration of AVG was 10 times higher than the reported K_i for tomato fruit (Boller et al. 1979), while the Co^{2+} concentration was 5 times higher than the concentration used for inhibiting IAA-induced ethylene by mung bean hypocotyls (Yu and Yang 1979). Higher concentrations of AVG (5, 10, and 50 μM) severely affected root geotropism without affecting phenotype in 4-day-old seedlings of VFN8 and *Epi* (data not presented), indicating that the inhibitor was absorbed and was active. Ursin (1987) showed that AVG was equally effective in inhibiting ethylene synthesis by VFN8 or *Epi*. Phytotoxicity symptoms were observed at 5 mM Co^{2+} for both genotypes. For the ethylene action inhibitors, the norbornadiene concentration used was almost 10 times greater than the reported K_i (Sisler and Yang 1984). The STS concentration was within the range of concentrations used for overcoming ethylene effects in plant tissue (Veen 1983). Partial reversion of the *Epi* phenotype was observed following continuous STS treatment (Fig. 4; Fujino 1987) and STS blocked the ethylene component of petiole epinasty induced by auxin in *Epi* (Ursin 1987), indicating that STS is active in *Epi* tissue. Failure of inhibitors of ethylene biosynthesis and of ethylene action to revert the *Epi* phenotype strongly suggests that ethylene is not directly responsible for the differences in phenotype between VFN8 and *Epi*.

Supraoptimal auxin levels can inhibit bud and stem growth (Michener 1942), although it has been suggested that the response is mediated via stimulation of

ethylene production (Burg and Burg 1968). It is possible that the reduced hypocotyl growth of *Epi* seedlings is due to elevated levels of endogenous IAA, which would be consistent with its higher rates of ethylene synthesis (Table 1). However, no difference was found in IAA content of dark-grown apices of VFN8 and *Epi* (Fujino et al. 1988b).

Since ethylene production and ethylene perception, per se, seem not to be responsible for the *Epi* phenotype, an alternative hypothesis is that the cellular responses to ethylene and/or auxin may be perturbed. Osborne (1982) proposed that the orientation of cellular expansion in auxin- and ethylene-responsive tissues is based on target cells with differential sensitivity to the 2 growth regulators. Tomatoes possess at least 3 distinct target cell types that differ in their growth responses to auxin and ethylene. The type III cell, as defined by Osborne (1982), is located in the petiole region of tomato and elongates in response to both auxin and ethylene, with the response to ethylene requiring the presence of auxin. Using an excised petiole system involving the *dgt* and *Epi* tomato mutants, Ursin (1987) reexamined this definition of the type III cell. She showed that petiole target cells apparently do not require the presence of auxin to respond to ethylene. In addition, she advanced the hypothesis that the *Epi* mutant has an altered target cell specificity. Unlike target cells in VFN8 petioles, where a growth response to auxin is completely dependent upon the stimulation of ethylene synthesis, *Epi* petioles possess a unique target cell type that responds to auxin independently of the presence of ethylene.

The hypocotyl cells responsible for the *Epi* phenotype in this paper have been classified as type I cells (Osborne 1982), which expand in length in response to auxin, but swell laterally in response to ethylene (Eisinger 1983). Our results are consistent with the model proposed by Ursin (1987), since *Epi* hypocotyls still exhibit the swollen phenotype characteristically induced by ethylene even when ethylene synthesis and action are blocked. In this sense, *Epi* appears to have a constitutive ethylene-like response regardless of whether the hormone is actually present. The reason for the higher ethylene production rates in *Epi* tissues remains unexplained, but they would tend to exacerbate this constitutive tendency, as indicated in Figure 2.

In conclusion, the primary physiological lesion resulting from the *Epi* gene mutation apparently does not result from ethylene overproduction or increased ethylene sensitivity, but from altered cell types that exhibit ethylene responses even when ethylene is not present. The *Epi* mutation represents a unique class of hormonal mutant in which the cellular response to a hormone, rather than synthesis or perception of the hormone, has been altered. Identification of the function of the *Epi* gene should provide insight into the mechanisms by which hormonal signals orient the direction of cell expansion.

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References

Abeles FB (1973) Ethylene in plant biology. Academic Press, New York

Beyer EM (1976) A potent inhibitor of ethylene action in plants. Plant Physiol 58:268–271

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

Bufler G, Y Mor, MS Reid, SF Yang (1980) Changes in 1-aminocyclopropane-1-carboxylic acid content of cut carnation flowers in relation to their senescence. J Plant Growth Regul 1:1–8

Burg SP, EA Burg (1968) Ethylene formation in pea seedlings: its relation to the inhibition of bud growth caused by indole-3-acetic acid. Plant Physiol 43:1069–1074

Eisinger W (1983) Regulation of pea internode expansion by ethylene. Annu Rev Plant Physiol 34:225–240

Fujino DW (1987) Hormonal characterization of a single-gene mutant of tomato (*Lycopersicon esculentum* Mill.). Ph.D. Dissertation, University of California, Davis

Fujino DW, DW Burger, SF Yang, KJ Bradford (1988a) Charcterization of an ethylene overproducing mutant of tomato (*Lycopersicon esculentum* Mill. cultivar VFN8). Plant Physiol (In press)

Fujino DW, SJ Nissen, AD Jones, DW Burger, KJ Bradford (1988b) Quantification of indole-3-acetic acid in dark-grown seedlings of the *diageotropica* and *Epinastic* mutants of tomato (*Lycopersicon esculentum* Mill.). Plant Physiol (In press)

Michener HD (1942) Dormancy and apical dominance in potato tubers. Am J Botany 29:558–568

Osborne DJ (1982) The ethylene regulation of cell growth in specific target tissues of plants. In: P. F. Wareing (ed) Plant growth substances 1982. Academic Press, New York, pp 279–290

Sisler EC, SF Yang (1984) Ethylene, the gaseous plant hormone. BioSci 34:234–238

Ursin VM (1987) Morphogenetic and physiological analyses of two developmental mutants of tomato (*Lycopersicon esculentum* Mill.), *Epinastic* and *diageotropica*. Ph.D. Dissertation, University of California, Davis

Veen H (1983) Silver thiosulfate: an experimental tool in plant science. Sci Hort 20:211–224

Yu YB, SF Yang (1979) Auxin-induced ethylene production and its inhibition by aminoethyoxyvinylglycine and cobalt ion. Plant Physiol 64:1074–1077