Planta (Berl.) 110, 227—235 (1973)

Role of Ethylene in Phytochrome-induced Anthocyanin Synthesis

Bin G. Kang and Stanley P. Burg
Fairchild Tropical Garden, and University of Miami, Miami, Florida 33156 USA*

Received October 16, 1972

Summary. Synthesis of anthocyanin pigments in etiolated cabbage seedlings is influenced by ethylene at concentrations higher than 10 ppb, and etiolated seedlings produce sufficient ethylene to influence their anthocyanin synthesis. When escape of endogenous ethylene from this tissue is enhanced by means of hypobaric treatment, anthocyanin synthesis is accelerated. Stimulation of anthocyanin synthesis by brief red illumination is completely prevented by applied ethylene and indoleacetic acid inhibits anthocyanin synthesis by stimulating ethylene production. Red light reduces endogenous as well as auxin-induced ethylene production and there is a close correlation between light-induced inhibition of ethylene synthesis and stimulation of anthocyanin formation. We suggest that in part photo-induced anthocyanin synthesis is due to a lowered ethylene content in light-treated tissue.

Introduction

Although more than one photosystem may be involved in light-induced formation of anthocyanin pigments (Grill and Vince, 1964; Downs *et al.*, 1965; Scherf and Zenk, 1967; Schneider and Stimson, 1971, 1972), a major portion of the photoresponse is mediated by phytochrome, at least in some cases (Lange *et al.*, 1971; Ku and Mancinelli, 1972). Among the various chemical factors that influence anthocyanin synthesis, auxins (Arnold and Albert, 1964; Stafford, 1968; Vince, 1968; Constabel *et al.*, 1971; Strickland and Sunderland, 1972) and ethylene (Morgan and Powell, 1970; Craker *et al.*, 1971; Craker and Wetherbee, 1972), under certain circumstances, appear to inhibit light-induced anthocyanin production. Numerous examples in which auxin action is mediated by auxin-induced ethylene production have been well established (Burg and Burg, 1966b, 1968a, b; Holm and Abeles, 1968; Kang and Ray, 1969; Apelbaum and Burg, 1972). The finding that anthocyanin synthesis may be stimulated by inhibitors of protein and RNA synthesis (Stafford, 1966) suggests the presence of a natural inhibitor, continuously synthesized in tissue by labile enzymes, such as those

* Mailing address: Fairchild Tropical Garden, Research Center, 11935 Old Cutler Road, Miami, Florida 33156, USA.

mediating ethylene biosynthesis in auxin-treated tissues (Kang *et al.* 1971).

Many etiolated seedlings normally produce ethylene at such a rate that the endogenous gas prevents, or inhibits to a certain extent, ethylene-sensitive physiological processes. Exposure of these seedlings to red light results in a reduction of ethylene production (Goeschl *et al.*, 1967; Kang *et al.*, 1967; Burg and Burg, 1968b; Kang and Ray, 1969; Imaseki *et al.*, 1971; Kang and Burg, 1972b, c), which in turn lessens the effect of endogenous ethylene. Such an interaction between red light and the ethylene-producing system in etiolated seedlings has been suggested to explain some phytochrome-mediated photomorphogenic responses (Goeschl *et al.*, 1967; Kang *et al.*, 1967; Burg and Burg, 1968b; Kang and Burg, 1972a, b, c). The present communication describes experiments demonstrating a causal relationship between ethylene, red light, auxin and anthocyanin synthesis in cabbage seedlings, in which ethylene mediates the action of red light and auxin.

Materials and Methods

Plant Material. Cabbage (*Brassica oleracea* L., cv. Red Acre) seeds were germinated and grown in wet vermiculite in complete darkness at 23° C for 5 days. Seedlings about 15–20 mm in height with well developed hypocotyl hooks were selected. Entire 1 cm apical segments including the cotyledons and upper part of the hypocotyl were excised and used for incubation or extraction of anthocyanin.

Light and Other Treatments. For studies with excised tissue sections, 12 apical segments were incubated in 1 ml of solution (5 mM potassium phosphate buffer at pH 6.8, 2% sucrose, and 5 μM cobalt chloride) in sealed 25-ml Micro-Fernbach flasks. IAA was added in the growth medium, and ethylene was applied by injecting the gas into flasks sealed with rubber septums. Red light (1250 erg cm^{-2} s^{-1}) treatments were given continuously to excised segments during the incubation period, or briefly to whole seedlings for 5 min. All experiments were carried in darkness, except for brief periods when a dim green safelight was used during handling or other test procedures. Hypobaric conditions were achieved by methods described previously (Apelbaum and Burg, 1972; Kang and Burg, 1972a, b, c).

Extraction and Measurements of Anthocyanin. 12 apical segments including the cotyledons and hypocotyl were placed in 5 ml of methanol containing 1% HCl, and kept in darkness for 24 h. At the end of this period, the methanol extract was filtered through glass wool, and the amount of anthocyanin determined by measuring absorbance of the extract at 525 nm with a Zeiss PMQ II spectrophotometer. Values are presented for each treatment comprising 12 segments in triplicate.

Measurements of Ethylene Production. Five ml air samples were withdrawn at appropriate times with a syringe from sealed 25-ml flasks containing 12 tissue segments, and the amount of ethylene determined by gas chromatography (Burg and Burg, 1968a).

Results

Dark-grown seedlings of Red Acre cabbage possess substantial amounts of anthocyanin, but exposure of these seedlings to red light

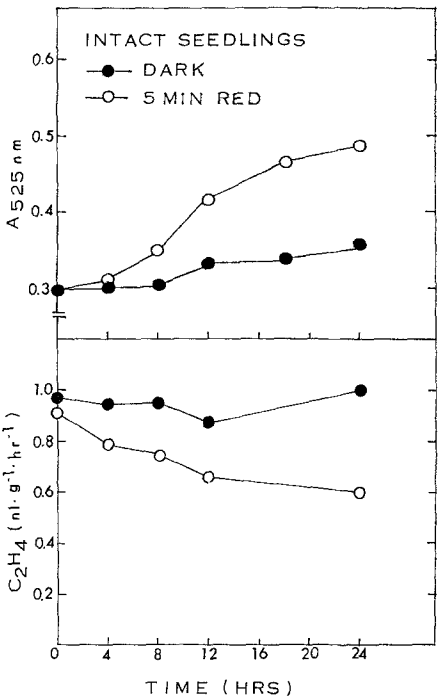

Fig. 1. Kinetics of anthocyanin synthesis and ethylene production following 5 min red illumination. Ethylene production was measured using excised 1-cm apical sections, cut at various times following illumination. Values are calculated from the total ethylene produced during a 12-h incubation period

for 5 min results in a subsequent stimulation of anthocyanin synthesis after about a 4-h lag period (Fig. 1). Production of ethylene from the upper portion of the seedlings, where most anthocyanin is accumulated, was measured by incubating tissue segments cut from the seedlings at various times following red illumination. Slight inhibition of ethylene production occurs during a 12-h dark incubation if segments are cut from seedlings immediately after illumination, but the inhibition intensifies progressively as the dark period following illumination and preceding excision of the sections is extended; by 24 h ethylene production is inhibited 40% as a result of previous illumination (Fig. 1).

Excised apical tissue segments respond to continuous red illumination during the incubation period. Anthocyanin synthesis is stimulated in excised control segments, compared to identical tissue in intact seedlings, but this may be due to exposure of the tissue to the green safelight during handling or to the sucrose in the growth medium.

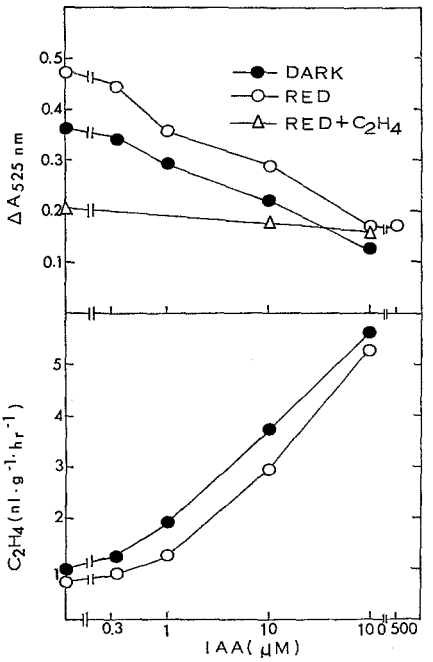

Fig. 2. Effects of various concentrations of IAA on anthocyanin synthesis and ethylene production by excised 1-cm apical sections in darkness and under continuous red illumination for a 24-h incubation period. The effect of IAA on red-light-induced anthocyanin production was also studied in the presence of 100 μl/l ethylene

Anthocyanin synthesis in both dark and illuminated segments is progressively inhibited as the concentration of IAA added to the medium is increased (Fig. 2). Such concentrations of IAA stimulate ethylene production, and red light reduces the amount of ethylene produced in response to the added IAA (Fig. 2). In the presence of a saturating concentration (100 μl/l) of added ethylene, anthocyanin synthesis is maximally inhibited with and without added IAA, and added IAA does not produce any further effect (Fig. 2). The data suggest that the effect of IAA on anthocyanin synthesis is due to ethylene production induced by the auxin, and indicate (Figs. 1, 2) a close correlation between synthesis of anthocyanin and production of ethylene.

The amount of anthocyanin synthesized by intact seedlings under continuous red illumination during a 24-h period is a function of the concentration of applied ethylene in the gas phase surrounding the tissue (Fig. 3). Very low concentrations of ethylene are effective, but even a saturating concentration does not completely abolish antho-

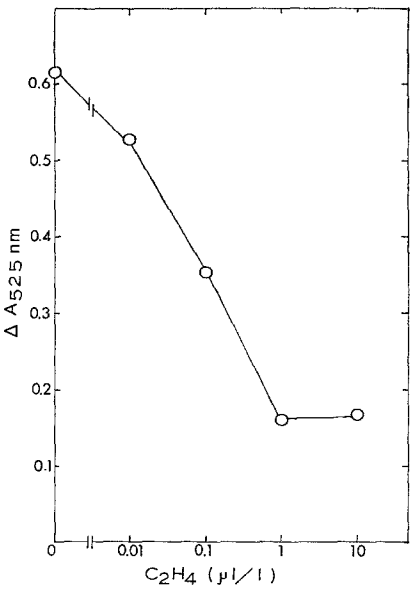

Fig. 3. Anthocyanin synthesis in intact seedlings continuously illuminated with red light in the presence of various concentrations of applied ethylene

cyanin synthesis induced by continuous red illumination. However, a saturating dose of ethylene almost completely suppresses anthocyanin synthesis induced by 5 min red illumination (Table 1).

Endogenous ethylene may be removed by enhancing outward diffusion at a subatmospheric pressure, supplementing the system with water-saturated oxygen to avoid anaerobiosis and desiccation. This method has been employed as a diagnostic test for identifying the action of endogenous ethylene produced by tissues (Burg and Burg, 1966a; Apelbaum and Burg, 1972; Byers *et al.*, 1972; Kang and Burg, 1972a, b, c).

Anthocyanin synthesis in intact seedlings is stimulated in darkness by a hypobaric treatment even when the partial pressure of oxygen is maintained about equal to that in the normal atmosphere (Table 1). This result suggests that accumulation of endogenous ethylene is inhibiting anthocyanin synthesis. Conversely, if seedlings are confined in a tightly closed container where escape of ethylene from the tissue is limited by the accumulation of the gas in the surrounding air, anthocyanin synthesis is severely suppressed. This result again suggests that seedlings produce sufficient ethylene to inhibit their anthocyanin synthesis.

Table 1. Formation of anthocyanin by intact seedlings under various conditions during a 24-h period

Treatment	$\Delta A_{525\ mm}$[a]
Dark	
Control	0.07
120 mm Hg O_2	0.17
Red light, 5 min	
Control	0.20
Closed air[b]	0.05
Ethylene, 100 µl/l	0.02
Red light, continuous	
Control	0.67
Ethylene, 100 µl/l	0.23

[a] Initial absorbance (seedlings at time zero) was 0.30.
[b] A pot having about 500 seedlings was confined in a sealed desiccator 2.5 l gas volume.

Discussion

Formation and accumulation of anthocyanin pigments is mediated by a complex system comprising several reaction sequences and enzymes producing different flavonoids. Numerous steps in the biosynthetic pathways could be affected by such factors as light, growth hormones, sugars, vitamins, or amino acids. Although phytochrome is the major regulatory photosystem in some etiolated seedlings, the "high-energy reaction" system also controls anthocyanin synthesis, and even photosynthesis may play a role in the photoresponse (Downs *et al.*, 1965; Scherf and Zenk, 1967; Schneider and Stimson, 1971, 1972). In sorghum seedlings, light-induced anthocyanin synthesis is suppressed by ethylene, but stimulated if seedlings are pre-exposed to ethylene and returned to an ethylene-free atmosphere (Craker *et al.*, 1971). Such stimulation of anthocyanin synthesis by ethylene might be related to ethylene-induced synthesis of phenylalanine ammonia-lyase, which is known to occur in response to applied ethylene in other plant systems (Imaseki *et al.*, 1968; Riov *et al.*, 1969; Hyodo and Yang, 1971). This enzyme, which catalyzes deamination of phenylalanine to form cinnamic acid, may function during the early phase of the synthetic sequence leading to anthocyanin formation. Stimulation of anthocyanin synthesis by ethylene also has been reported in *Cymbidium* orchids (Chadwick and Arditti, 1972). On the other hand, inhibition of anthocyanin accumulation by ethylene may result from either a direct inhibitory action of ethylene on one of the later reactions in the synthetic pathway of anthocyanin, or a stimulation of degradation of the pigments by the gas. The latter is the case in fading *Vanda* orchid blossoms, where loss of

anthocyanin is associated with induction of ethylene synthesis (Akamine, 1963; Burg and Dijkman, 1967). Fading of orchid blossoms involves peroxidation of anthocyanin pigments, which is catalyzed by a peroxidase whose activity is known to be enhanced by ethylene in several plant tissues (Stahmann *et al.*, 1966; Imaseki *et al.*, 1968).

Our data suggest that a reduced level of internal ethylene in tissue is associated with that portion of light-induced anthocyanin synthesis brought about by brief illumination with low intensity red light. A saturated inhibition of anthocyanin synthesis is attained with 100 μM IAA or 1 μl/l of ethylene (Figs. 2, 3), suggesting that the internal ethylene concentration in tissue treated with 100 μM of IAA is close to 1 μl/l. At this IAA concentration seedling sections produce ethylene at about 5 nl g^{-1} h^{-1}, giving a conversion factor of 0.2 μl/l per nl g^{-1} h^{-1}. This is in excellent agreement with previous calculations for epicotyl and root tissue of pea and hypocotyl hook tissue of bean (Burg and Burg, 1966b; Goeschl *et al.*, 1966; Chadwick and Burg, 1967; Kang and Ray, 1969). Accordingly, the concentration of endogenous ethylene in seedling tissue producing ethylene at 1 nl g^{-1} h^{-1} (Figs. 1, 2) is estimated to be about 0.2 μl/l. The concentration-dependence curve for inhibition of anthocyanin synthesis by applied ethylene in illuminated seedlings (Fig. 3) indicates half-maximal inhibition at around 0.1 μl/l of external ethylene. Illuminated seedlings also must contain approximately 0.1 μl/l of ethylene in the tissue air-phase (about half that in the dark) as a result of endogenous ethylene production, so the difference in anthocyanin synthesis between the control illuminated tissue and that exposed to 0.1 μl/l ethylene reflects in reality a response caused by increasing the internal ethylene level in the tissue air space from 0.1 to 0.2 μl/l. The difference in anthocyanin synthesis under these conditions should be, and in fact is close to the change caused by the red-light inhibition of ethylene synthesis. Therefore it is clearly demonstrated that the reduction in ethylene production caused by brief red illumination is sufficient in magnitude to induce the observed enhancement of anthocyanin synthesis. It is concluded that changes in the endogenous ethylene level of this tissue in part regulate the induction of anthocyanin synthesis caused by red light.

This work was supported by National Science Foundation Grant GB 27424 to S. P. Burg.

References

Akamine, E. K.: Ethylene production in fading of *Vanda* orchid blossoms. Science **140**, 1217–1218 (1963).

Apelbaum, A., Burg, S. P.: Effects of ethylene and 2,4-dichlorophenoxyacetic acid on cellular expansion in *Pisum sativum*. Plant Physiol. **50**, 125–131 (1972).

Arnold, A. W., Albert, L. S.: Chemical factors affecting anthocyanin formation and morphogenesis in cultured hypocotyl segments of *Impatiens balsamina*. Plant Physiol. **39**, 307–312 (1964).

Burg, S. P., Burg, E. A.: Fruit storage at subatmospheric pressures. Science **153**, 314–315 (1966a).

Burg, S. P., Burg, E. A.: The intsraction between auxin and ethylene and its role in plant growth. Proc. nat. Acad. Sci. (Wash.) **55**, 262–269 (1966b).

Burg, S. P., Burg, E. A.: Ethylene formation in pea seedlings: Its relation to the inhibition of bud growth caused by indole-3-acetic acid. Plant Physiol. **43**, 1069–1074 (1968a).

Burg, S. P., Burg, E. A.: Auxin stimulated ethylene formation: Its relationship to auxin inhibited growth, root geotropism and other plant processes. In: Biochemistry and physiology of plant growth substances, F. Wightman and G. Setterfield, eds., p. 1275–1294. Ottawa: Runge Press 1968(b).

Burg, S. P., Dijkman, M. J.: Ethylene and auxin participation in pollen induced fading of *Vanda* blossoms. Plant Physiol. **42**, 1648–1650 (1967).

Byers, R. E., Baker, L. R., Sell, H. M., Herner, R. C., Dilley, D. R.: Ethylene: A natural regulator of sex expression of *Cucumis melo* L. Proc. nat. Acad. Sci. (Wash.) **69**, 717–720 (1972).

Chadwick, A. V., Arditti, J.: Ethylene evolution and anthocyanin synthesis by *Cymbidium* (Orchidaceae) flowers. (Abstr.) Plant Physiol. **48**, S-111 (1972).

Chadwick, A. V., Burg, S. P.: An explanation of the inhibition of root growth caused by indole-3-acetic acid. Plant Physiol. **42**, 415–420 (1967).

Constabel, F., Shyluk, J. P., Gamborg, O L : The effect of hormones on anthocyanin accumulation in cell cultures of *Haplopappus gracilis* Planta (Berl.) **96**, 306–316 (1971).

Craker, L. E., Standley, L. A., Starbuck, M. J.: Ethylene control of anthocyanin synthesis in *Sorghum*. Plant Physiol. **48**, 349–352 (1971).

Craker, L. E., Wetherbee, P. J.: Ethylene, light, and anthocyanin synthesis. (Abstr.) Plant Physiol. **49**, S-112 (1972).

Downs, R. J., Siegelman, H. W., Butler, W. L., Hendricks, S. B.: Photoreceptive pigments for anthocyanin synthesis in apple skin. Nature (Lond.) **205**, 909–910 (1965).

Goeschl, J. D., Pratt, H. K., Bonner, A. B.: An effect of light on the production of ethylene and the growth of the plumular portion of etiolated pea seedlings. Plant Physiol. **42**, 1077–1080 (1967).

Goeschl, J. D., Rappaport, L., Pratt, H. K.: Ethylene as a factor regulating the growth of pea epicotyl subjected to physical stress. Plant Physiol. **41**, 877–884 (1966).

Grill, R., Vince, D.: Anthocyanin formation in turnip seedlings (*Brassica rapa* L.): Evidence for two light steps in the biosynthetic pathway. Planta (Berl.) **63**, 1012 (1964).

Holm, R. E., Abeles, F. B.: The role of ethylene in 2,4-D-induced growth inhibition. Planta (Berl.) **78**, 293–304 (1968).

Hyodo, H., Yang, S. F.: Ethylene-enhanced synthesis of phenylalanine ammonia-lyase in pea seedlings. Plant Physiol. **47**, 765–770 (1971).

Imaseki, H., Pjon, C. J., Furuya, M.: Phytochrome action in *Oryza sativa* L. IV. Red and far red reversible effect on the production of ethylene in excised coleoptiles. Plant Physiol. **48**, 241–244 (1971).

Imaseki, H., Uchiyama, M., Uritani, I.: Effect of ethylene on the inductive increase in metabolic activities in sliced sweet potato roots. Agr. Biol. Chem. **32**, 387–389 (1968).

Kang, B. G., Burg, S. P.: Involvement of ethylene in phytochrome-mediated carotenoid synthesis. Plant Physiol. **49**, 631–633 (1972a).

Kang, B. G., Burg, S. P.: Relation of phytochrome-enhanced geotropic sensitivity to ethylene production. Plant Physiol. **50**, 132–135 (1972b).

Kang, B. G., Burg, S. P.: Ethylene as a natural agent inducing plumular hook formation in pea seedlings. Planta (Berl.) **104**, 275–281 (1972c).

Kang, B. G., Newcomb, W., Burg, S. P.: Mechanism of auxin-induced ethylene production. Plant Physiol. **47**, 504–509 (1971).

Kang, B. G., Ray, P. M.: Ethylene and carbon dioxide as mediators in the response of the bean hypocotyl hook to light and auxins. Planta (Berl.) **87**, 206–216 (1969).

Kang, B. G., Yocum, C. S., Burg, S. P., Ray, P. M.: Ethylene and carbon dioxide; mediation of hypocotyl hook opening response. Science **156**, 958–959 (1967).

Ku, P., Mancinelli, A. L.: Photocontrol of anthocyanin synthesis. I. Action of short, prolonged, and intermittent irradiation on the formation of anthocyanins in cabbage, mustard, and turnip seedlings. Plant Physiol. **49**, 212–217 (1972).

Lange, H., Shropshire, W., Jr., Mohr, H.: An analysis of phytochrome-mediated anthocyanin synthesis. Plant Physiol. **47**, 649–655 (1971).

Morgan, P. W., Powell, R. D.: Involvement of ethylene in responses of etiolated bean hypocotyl hook to coumarin. Plant Physiol. **45**, 553–557 (1970).

Riov, J., Monselise, S. P., Kahn, R. S.: Ethylene-controlled induction of phenylalanine ammonia-lyase in citrus fruit peel. Plant Physiol. **44**, 631–635 (1969).

Scherf, H., Zenk, M. H.: Induction of anthocyanin and phenylalanine ammonia-lyase formation by a high energy light reaction and its control through the phytochrome system. Z. Pflanzenphysiol. **56**, 203–206 (1967).

Schneider, M. J., Stimson, W. R.: Contribution of photosynthesis and phytochrome to the formation of anthocyanin in turnip seedlings. Plant Physiol. **48**, 312–315 (1971)

Schneider, M. J., Stimson, W. R.: Phytochrome and photosystem. I. Interaction in a high energy photoresponse. Proc. nat. Acad. Sci. (Wash.) **69**, 2150–2154 (1972).

Stafford, H. A.: Regulatory mechanisms in anthocyanin biosynthesis in first internodes of *Sorghum vulgare:* Effect of presumed inhibitors of protein synthesis. Plant Physiol. **41**, 953–961 (1966).

Stafford, H. A.: Relationship between the development of adventitious roots and the biosynthesis on anthocyanins in first internodes of *Sorghum*. Plant Physiol. **43**, 318–326 (1968).

Stahmann, M. A., Clare, B. G., Woodbury, W.: Increased disease resistance and enzyme activity induced by ethylene and ethylene production by black rot infected sweet potato tissue. Plant Physiol. **41**, 1505–1512 (1966).

Strickland, R. G., Sunderland, N.: Production of anthocyanins, flavonols, and chlorogenic acids by cultured callus tissues of *Haplopappus gracilis*. Ann. Bot. **36**, 443–457 (1972).

Vince, D.: Growth and anthocyanin synthesis in excised *Sorghum* internodes. I. Effects of growth regulating substances. Planta (Berl.) **82**, 261–279 (1968).