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Ethylene Production during Development of Mustard (*Brassica juncea*) and Canola (*Brassica napus*) Seed[1]

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An open, continuous flow system was used to investigate ethylene production during degreening of maturing seed of mustard (*Brassica juncea* cv Cutlass and cv Lethbridge 22A) and canola (*Brassica napus* cv Westar and cv Alto). Isolated mustard seed evolved higher amounts of ethylene than those of canola, and this was particularly evident both early in embryogeny and later during the desiccation phase of seed maturation. The silique walls produced negligible amounts of ethylene in both species. The concentrations of ethylene surrounding seed as they matured within siliques were significantly higher in mustard than in canola, and this interspecies difference was greatest during the seed desiccation phase. In mustard, a 4-fold increase in silique internal ethylene levels was apparent during desiccation. In comparison, only a moderate increase in silique-derived ethylene occurred in canola.

The influence of ethylene on seed dormancy and germination has been extensively studied (e.g. Ross, 1984; Saini et al., 1989; Abeles et al., 1992). However, to our knowledge no one has investigated the possible roles of ethylene in seed maturation. This paper reports on the production of ethylene by seeds, by silique walls, and by intact siliques attached to the plant as they undergo maturation. Since CO_2 and ethylene have been shown to influence each others' metabolism and effects in other tissues (e.g. Dhawan et al., 1979; Dilley et al., 1993), CO_2 measurements were also made.

Brassica was chosen as the subject for investigation because of its agricultural importance and because of the relatively extensive amount of information available about its physiology and biochemistry. An added dimension to the investigation is that there is an economically significant "green seed problem." Chl content is an important factor influencing seed grading in many northern canola (*Brassica napus*) growing regions (Daun, 1985). The oil extracted from the seed contains Chl-type pigments that affect color, flavor, and oxidative stability (Levadoux et al., 1987). In many tissues ethylene affects the metabolism of Chl (Abeles et al., 1992), and it is possible that it may do so in seed as well.

Using an open, continuous flow system, determinations of ethylene evolution by isolated seed of mustard (*Brassica juncea*) and canola have been made at various stages of maturation. With this system (Bassi and Spencer, 1989) it is possible to prevent the accumulation of volatiles in the cuvette holding the seed. These include ethylene and CO_2, which may influence the rate of ethylene production. However, detachment per se may affect the rate of ethylene biosynthesis, as has been shown for yellowing leaves of tobacco (Alejar et al., 1988). Moreover, seed developing within a silique is, in effect, maturing within an environment that to some extent resembles a "closed" system. The silique wall may act to physically restrict the diffusion of gases, including those used and produced by the silique wall. Consequently, this study also reports on the in vivo concentrations of ethylene and CO_2 within the gas space of the siliques as they undergo maturation attached to the plant.

MATERIALS AND METHODS

Plant Growth Conditions

Certified seeds of *Brassica juncea* cv Cutlass and cv Lethbridge 22A, and *Brassica napus* cv Westar and cv Alto, were planted into 15-cm-diameter plastic pots containing a mixture of sand, perlite, and peat moss (1:1:1, v/v) enriched with a slow-release fertilizer. Pots were placed in a greenhouse under augmented lighting to provide a minimum irradiance (PAR) of 200 $\mu E\ m^{-2}\ s^{-1}$ (400-W high-pressure sodium vapor, Sylvania Lighting Producers Canada Ltd., Edmonton, Canada) or in a controlled-environment room (20°C days, 16°C nights) with a 16-h photoperiod. Illumination was provided by banks of 215-W Sylvania Gro-Lux lamps producing an irradiance (PAR) of 230 $\mu E\ m^{-2}\ s^{-1}$ at the top of the plant canopy. Seedlings were thinned to two per pot after 2 weeks.

Harvesting

Seed was harvested at regular intervals from the bottom third of the main raceme of plants that had been sown on the same date. Harvesting began when the average moisture content of the seed was around 80% and was continued throughout the period of seed maturation until moisture contents had dropped to approximately 45%. At each harvest around 8 g of seed was collected and subdivided to provide material for seed moisture and pigment content determinations and for the measurement of ethylene evolution. In addition, a chloroplast fraction was prepared and used to examine parallel changes in the in vitro Chlase and peroxidase activities; these results will be presented in a subsequent report. In separate experiments, flowers of *B. napus* and *B.*

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Abbreviation: DPA, days post-anthesis.

juncea were tagged for a developmental study. Individual siliques were collected over the seed maturation period and one representative seed was dissected. The remainder were used for fresh weight, dry weight, and moisture determinations.

Measurement of Ethylene Evolution

The evolution of ethylene from isolated seed, intact detached siliques, and the isolated silique walls was measured in an open, continuous flow system adapted from those used previously in this laboratory (Eastwell and Spencer, 1982; Bassi and Spencer, 1989; Saini et al., 1989). Compressed air containing 350 μL L^{-1} CO_2 (Linde Union Carbide, Edmonton, Alberta, Canada) was passed through a platinum-catalyzed thermal oxidizer to remove hydrocarbon contaminants and then through a humidifying bottle to prevent desiccation of the plant material. The gas flow was subsequently split to provide four independently regulated gas inlet lines that passed through the wall of a lightproof chamber to four lidded, airtight glass cuvettes (112 cm^3). Effluent gas from the cuvettes was diverted via exhaust channels through the side of the chamber to exterior sampling ports. Constant temperature conditions were maintained by partially submerging the cuvettes and the coiled copper inlet tubes in a water bath at 25°C. A bank of four 15-W cool-white fluorescent lamps attached to the ceiling provided a PAR of 38 μE m^{-2} s^{-1} at seed level, and air circulation within the chamber was maintained by an exhaust fan and vent.

In standard experiments, moist, hydrocarbon-free air flowing at 15 mL min^{-1} was passed through the system for 1 h prior to adding the plant material. A monolayer of freshly isolated seed (0.3 g), two intact siliques (0.6 g), or 0.3 g of isolated silique wall material was then placed at the bottom of each of the four cuvettes, the lids were securely fastened, and the first gas sample was taken after 30 min (95% turnover of the original gas in the cuvettes occurred in 24 min). Typically, 1-mL gas samples were taken with a syringe at 30-min intervals up to 5 h. Quantitative determinations of ethylene were performed on a photoionization detector (model 10A10, Photovac Inc., Thornhill, Ontario, Canada) using a Teflon column packed with Carbopak B (60–80 mesh, Supelco Canada Ltd.) or on a gas chromatograph (Hewlett-Packard 5880A) equipped with a Poropak Q column (80–100 mesh, Waters Associates, Inc., Milford, MA) and a flame-ionization detector. Limits of detection were around 0.5 and 50 nL L^{-1} for the photoionization and flame-ionization detectors, respectively (1-mL gas sample). The maximum rate of ethylene production in each cuvette was determined by averaging the three highest rates recorded during the peak period of ethylene evolution (see Tables I and II; Fig. 2).

Determination of the Ethylene Concentration within the Gas Space of Intact Attached Siliques

Measurements of the internal ethylene concentrations within attached siliques were made by inserting the tip of a 100-μL gas-tight syringe through the wall of the silique and into the space between the developing seed. Samples of gas (50 or 100 μL for mustard and canola, respectively) were withdrawn and analyzed on the photoionization detector. Possible dilution of the samples by external air entering the siliques was investigated by measuring internal ethylene levels in siliques held under water during gas collection and comparing them with those from siliques in air. With moist siliques, no dilution of samples with external air was detected, since results were very similar irrespective of whether the siliques were sampled in air or underwater. When the moisture content had decreased to 50%, it was technically difficult to obtain a gas sample from the siliques of either species without drawing water into the syringe. Therefore, internal gas concentrations, determined by sampling low-moisture-content siliques in air, are likely to be underestimated because of possible damage to the silique wall during sampling with a syringe.

CO_2 Measurements

CO_2 concentrations within attached siliques or within the cuvettes during ethylene evolution measurements were monitored by removing 50- to 100-μL (siliques) or 1-mL (cuvettes) gas samples as above. Analysis was performed on the gas chromatograph (Hewlett-Packard 5880A) equipped with a thermal conductivity detector.

RESULTS

In initial experiments, greenhouse-grown plants of *B. juncea* cv Lethbridge 22A and *B. napus* cv Westar were used to examine ethylene production by isolated seed, detached intact siliques, and isolated silique walls in a continuous flow system (Fig. 1). The time course of ethylene evolution was very similar in mustard and canola seed. Mustard seed,

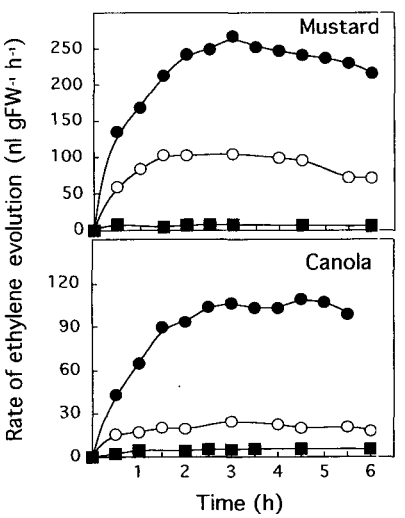

Figure 1. Ethylene evolution from isolated seed (●), intact detached siliques (○), and isolated silique walls (■) of mustard cv Lethbridge 22A and canola cv Westar as measured in a continuous flow system. Seed moisture contents were 67 and 76% for mustard and canola, respectively. Siliques contained seed at similar moisture contents. Each point is the mean value obtained from four (seed) or two (siliques and silique walls) cuvettes.

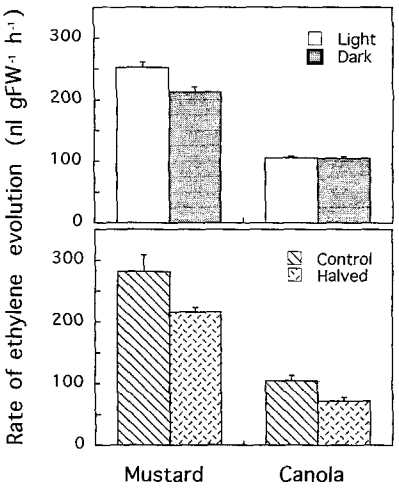

Figure 2. The effects of darkness and mechanical wounding on the maximum rates of ethylene evolution from isolated seeds of mustard cv Lethbridge 22A and canola cv Westar. Wounding was inflicted by cutting each seed in half with a scalpel. Seed moisture contents were 67 and 76% for mustard and canola, respectively. Duplicate cuvettes were monitored for each treatment and data presented are the means ± SE of the three highest rates in both cuvettes.

however, produced considerably more ethylene, with the maximum rate exceeding that in canola by more than 2-fold. The silique walls yielded negligible amounts of ethylene in both species (Fig. 1). Accordingly, ethylene evolution per gram fresh weight of the intact siliques was much lower than that of the isolated seed (Fig. 1). Mustard siliques, like their seed, exhibited higher rates of ethylene evolution than those of canola, and at the maximum rate, detached intact siliques of mustard yielded 5 times more ethylene per gram of silique fresh weight.

Darkness had no significant effect on the maximum rate of ethylene evolution from isolated canola seed and caused a small decrease in mustard (Fig. 2; *t* test, $P < 0.01$). Wounding, inflicted by cutting the seed in half with a sharp scalpel, resulted in slightly lower maximum rates of ethylene evolution in both species (Fig. 2; *t* test, $P < 0.02$). Seed subjected to a more severe crushing treatment produced maximum ethylene evolution rates that were approximately 20% that of the corresponding control (data not shown).

Batches of seed at the same moisture contents exhibited considerable variation in their actual rates of ethylene evolution when collected from plants grown in the greenhouse at different times of the year. Isolated seed of Lethbridge 22A, however, consistently produced several times more ethylene than those of Westar at the same moisture content. Nevertheless, to ensure reproducibility, all plants used for subsequent ethylene work were raised in an environmentally controlled growth room.

The different potentials for ethylene production in mustard and canola were further investigated by measuring the rates of ethylene evolution from batches of seed harvested at regular intervals throughout the period of seed maturation. The maximum rates of ethylene evolution from isolated seed of the mustard cultivars Lethbridge 22A and Cutlass were compared with those for the canola cultivars Westar and Alto in relation to seed moisture content (Fig. 3). In Westar, the maximum rate of ethylene evolution on a fresh weight basis showed a slow decline with seed moisture content. Similar results were found for Alto (not shown). In comparison, the two mustard cultivars exhibited a rapid decline in the maximum rates of ethylene evolution as seed moisture contents fell from 80 to 55%, followed by a second peak in ethylene production at around 47%, before declining to low levels in seed with less than 40% moisture content. Thus, isolated mustard seed evolved several times more ethylene than that of canola at an equivalent moisture content, particularly early and late in the maturation period examined. Similar trends were apparent when ethylene evolution was calculated on a dry weight basis.

To ensure that moisture content was a valid measure of seed maturity, development in mustard and canola was compared (Fig. 4, top and bottom). Mustard seed was smaller and completed rapid dry matter accumulation 7 d earlier than did canola. Similarly, desiccation began at approximately 30 DPA in mustard versus 34 DPA in canola. This corresponded to approximately 58 and 60% seed moisture for mustard and canola, respectively. Seed color change began earlier in canola, whereas morphological development was slightly faster, relative to DPA, in mustard.

In addition to the studies using isolated seed of mustard and canola maintained in the continuous flow system, seed ethylene production in situ was also investigated. Direct sampling of the gas space within intact, attached siliques revealed that developing *Brassica* seed was surrounded by very high concentrations of both ethylene and CO_2 (Table I). Young, green mustard siliques (corresponding to approximately 70% seed moisture) were found to contain significantly higher concentrations of ethylene than those of canola. Moreover, as maturation progressed and the siliques began

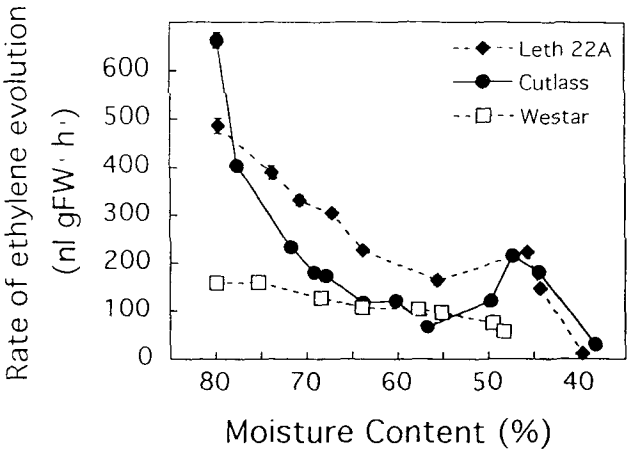

Figure 3. Maximum rates of ethylene evolution from isolated seeds of mustard (Lethbridge 22A and Cutlass) and canola (Westar) in relation to seed moisture content. Each point is the mean ± SE, based on four replicate cuvettes. Error bars are masked when they are smaller than the symbol size. Mean maximum rates were determined as outlined in "Materials and Methods."

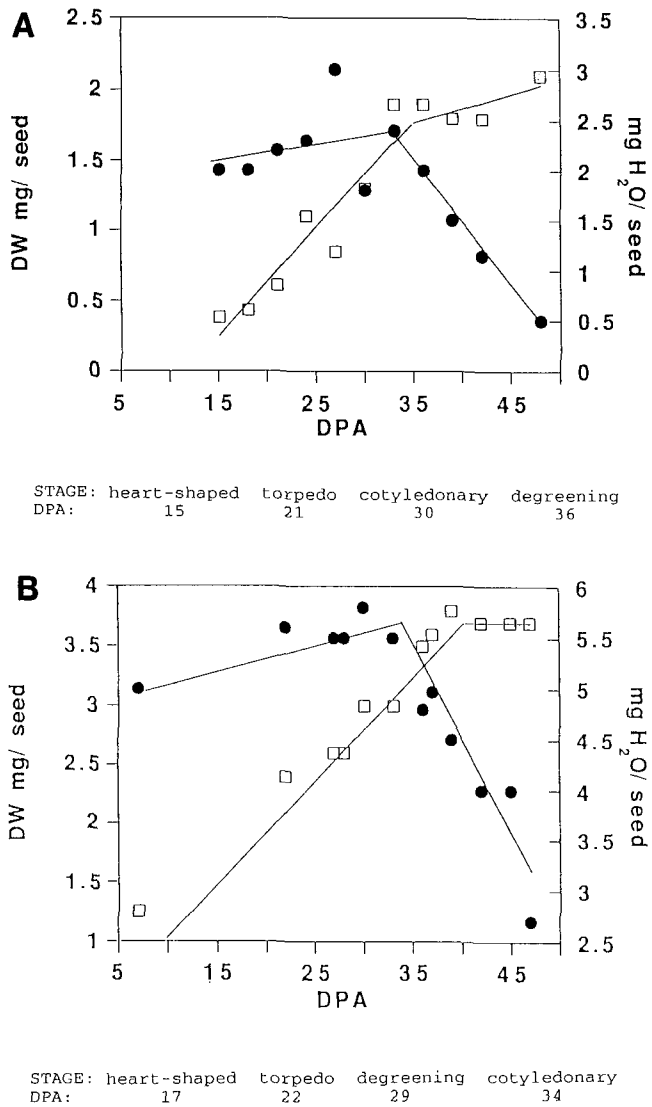

Figure 4. Development of mustard and canola seed. Values are the average of 12 to 23 mustard seeds and over 80 canola seeds (from four separate experiments). A, Mustard (●) dry weight (mg/seed), r = 0.97 for 15 to 33 DPA and r = 0.69 for 36 to 48 DPA. Water (□) content (mg/seed), r = 0.18 for 15 to 33 DPA and r = 0.99 for 36 to 48 DPA. Seed morphology is indicated below the graph. B, Canola (●) dry weight (mg/seed), r = 0.99 for 7 to 33 DPA and r = 0.52 for 36 to 47 DPA. Water (□) content (mg/seed), r = 0.83 for 7 to 33 DPA and r = 0.90 for 36 to 47 DPA. Seed morphology is indicated below the graph.

to yellow (approximately 55% seed moisture), the internal ethylene concentration more than doubled in mustard.

A more detailed examination of internal ethylene concentrations in relation to seed moisture content was performed by sampling the gas space of siliques on the same plants at intervals several days apart (Table II). Although both species exhibited a general rise in internal ethylene at moisture contents below 50%, a pronounced increase in ethylene accumulation was observed in mustard siliques containing seed at 46% moisture content, which was consistent with the results obtained with isolated seed (Fig. 3).

Table I. *Ethylene and CO_2 concentrations within siliques of mustard and canola*

Gas samples were removed from 15 intact, attached siliques of mustard (cv Lethbridge 22A) or canola (cv Westar) at a similar stage of development as determined by visual inspection. Five siliques were analyzed on each of three plants. Values are the means ± SE, n = 15.

Silique Color	Species	Internal Silique Concentration	
		Ethylene	CO_2
		$\mu L/L^{-1}$	mL/L^{-1}
Green	Mustard	3.42 ± 0.42	6.56 ± 0.42
	Canola	2.42 ± 0.35	10.21 ± 0.84
Yellow/green	Mustard	7.11 ± 0.80	4.58 ± 0.44
	Canola	1.11 ± 0.21	4.54 ± 0.75

DISCUSSION

Developing seed of mustard and canola were found to produce significant amounts of ethylene during embryogenesis; indeed, ethylene production levels at all stages of maturation exceeded those commonly accepted as "threshold levels" for activity (Abeles et al., 1992). Early in the maturation phase (around 20 DPA or 75% moisture content), isolated seed of the two canola cultivars yielded as much as 100 to 150 nL ethylene g^{-1} fresh weight h^{-1} (Fig. 3). In mustard seed at an equivalent stage of development (approximately 22 DPA or 75% moisture), ethylene evolution rates were 3 to 4 times higher than in canola. Such ethylene production, comparable to that exhibited during the climacteric in fruits such as pear (Brennan and Frenkel, 1977) and tomato (Saniewski et al., 1987), specifically characterized the early stages of the predesiccation phase of *Brassica* seed development. Thereafter, ethylene evolution declined in mustard as moisture contents fell toward 55%. The transition from predesic-

Table II. *Internal ethylene concentrations within siliques of mustard and canola in relation to seed moisture content*

Sampling was performed on the same plants at intervals several days apart. Gas samples were removed from intact, attached siliques of mustard (cv Lethbridge 22A) or canola (cv Westar), the siliques were then detached, and the moisture content of the enclosed seed was determined for each silique. Results from siliques containing seeds within a 2% moisture content range were pooled. Values for the ethylene concentrations are the means ± SE (n).

Mustard		Canola	
Mean moisture content	Ethylene concentration	Mean moisture content	Ethylene concentration
%	$\mu l/L^{-1}$	%	$\mu l/L^{-1}$
62	2.88 ± 0.27 (20)	64	1.16 ± 0.27 (12)
46	13.62 ± 2.62 (10)	51	1.11 ± 0.14 (5)
44	6.31 ± 0.88 (5)	44	2.22 ± 0.29 (12)

cation to desiccation occurred at approximately 58% seed moisture content. Mustard seed exhibited a second peak in evolution at around 47% (Fig. 3) and then, below 40% seed moisture, it exhibited quite low rates of ethylene production more typical of the rates found in germinating seed (e.g. Saini et al., 1989). In canola, the transition from predesiccation to desiccation occurred at approximately 60% seed moisture. This was not associated with any change in the rate of ethylene evolution.

Mechanical wounding such as cutting or bruising typically results in enhanced ethylene production via the induction of ACC synthase (Kende and Boller, 1981; Konze and Kwiatkowski, 1981). In the present study, cutting isolated seed did not promote ethylene production. However, removal of the seed from the siliques may bring about a wounding response, so that the second wounding by cutting may have less effect. Nevertheless, dissected silique walls, "wounded" to facilitate seed removal, yielded only very low amounts of ethylene (Fig. 1). Although a number of tenable hypotheses can be offered to account for this lack of wound-induced ethylene, these results raise the possibility that the seed and the silique wall are unable to synthesize ACC synthase de novo, at least during the predesiccation phase of seed development examined here. In many tissues (Yang and Hoffman, 1984) the availability of the precursor ACC is the rate-determining factor in ethylene biosynthesis. Interspecies differences in the ethylene biosynthetic capacity of seed developing within siliques may be greatly influenced by factors such as the regulation of ACC synthase activity, the extent of ACC compartmentalization/conjugation, and the rate of ACC transport to the site of ACC oxidase activity. At the same time, ethylene production in the seed of the two species may also reflect differences in ACC oxidase synthesis or activity. Any such inherent differences should be considered in the context of the intact silique, where the high concentrations of accumulated gases (Tables I and II) raise the possibility of ethylene- or CO_2-mediated regulation of ethylene biosynthesis (Yang and Hoffman, 1984; Dilley et al., 1993).

Sampling of the gas space within siliques revealed an increase in internal ethylene concentrations as seed moisture contents dropped below 50% (Table II). In mustard, this was consistent with the general pattern of ethylene production exhibited by isolated seed in the continuous flow system. In canola, however, intact silique ethylene increased between 51 and 44% seed moisture content, despite the continuous decline in ethylene production observed in the isolated seed experiments. This apparent anomaly may be explained in terms of a decreased outward diffusion of ethylene as seed development progresses, as a result of thickening of the silique walls and an increase in stomatal and cuticular resistance to gas exchange in response to desiccation (Pate, 1984).

Clearly, the silique wall was of considerable importance in both species in modulating the concentration of ethylene surrounding the maturing seed. For example, internal ethylene levels were severalfold higher in mustard siliques containing seed at 46% moisture content than in the undesiccated green siliques, even though this seed (at around 70% moisture content) was capable of producing similar amounts of ethylene in the continuous flow system. Should these differences in ethylene in the presence of the silique wall involve ethylene-, O_2-, or CO_2-mediated changes in ACC synthase or ACC oxidase activity as a result of gas accumulation within the siliques, then a major factor determining internal ethylene concentrations in *Brassica* may be the change in the resistance of the silique walls to gaseous diffusion as maturation proceeds.

Whether solely related to higher levels of available ACC or in conjunction with elevated ACC oxidase activity, mustard seed exhibited a greater capacity for ethylene production than that of canola, both in the continuous flow system and in situ within maturing siliques. It is noteworthy that this interspecies difference in silique ethylene levels was greatest relatively late in seed maturation, during the desiccation phase in which net Chl degradation occurs (Johnson-Flanagan and Thiagarajah, 1990) (Table II).

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