J. Amer. Soc. Hort. Sci. 131(1):164–173. 2006.

Roles of Intra-fruit Oxygen and Carbon Dioxide in Controlling Pepper (*Capsicum annuum* L.) Seed Development and Storage Reserve Deposition

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Additional index words. starch, carbohydrate, protein, hypoxia, internal atmosphere

Abstract. **Seeds developing within a locular space inside hollow fruit experience chronic exposure to a unique gaseous environment. Using two pepper cultivars, 'Triton' (sweet) and 'PI 140367' (hot), we investigated how the development of seeds is affected by the gases surrounding them. The atmospheric composition of the seed environment was characterized during development by analysis of samples withdrawn from the fruit locule with a gas-tight syringe. As seed weight plateaued during development, the seed environment reached its lowest O_2 concentration (19%) and highest CO_2 concentration (3%). We experimentally manipulated the seed environment by passing different humidified gas mixtures through the fruit locule at a rate of 60 to 90 mL·min^{-1}. A synthetic atmosphere containing 3% CO_2, 21% O_2, and 76% N_2 was used to represent a standard seed environment. Seeds developing inside locules supplied with this mixture had enhanced average seed weight, characterized by lower variation than in the no-flow controls due to fewer low-weight seeds. The importance of O_2 in the seed microenvironment was demonstrated by reduction in seed weight when the synthetic atmosphere contained only 15% O_2 and by complete arrest of embryo development when O_2 was omitted from the seed atmosphere. Removal of CO_2 from the synthetic atmosphere had no effect on seed weight, however, the CO_2-free treatment accelerated fruit ripening by 4 days in the hot pepper. In the sweet peppers, fruit wall starch and sucrose were reduced by the CO_2-free treatment. The results demonstrate that accretionary seed growth is being limited in pepper by O_2 availability and suggest that variation in seed quality is attributable to localized limitations in O_2 supply.**

In consideration of the developmental process that begins with a single fertilized egg cell and culminates in a germinable seed, nutritive and protective roles are generally ascribed to the maternal tissues in which these remarkable events take place. Recently Carman and Bishop (2004) suggested that in addition to minerals, metabolites, physical support, and maintenance of appropriate moisture, maternal tissues create a gaseous microenvironment necessary for embryogenesis to occur. Numerous authors have shown that a low concentration of O_2 outside a plant will adversely affect seed production, resulting in arrested embryo development and/or diminished seed weight (Kuang et al., 1998; Musgrave and Strain, 1988; Quebedeaux and Hardy, 1976; Ramonell et al., 2002; Sinclair et al., 1987), presumably by altering the seed microenvironment (Porterfield et al., 1999).

The idea that embryogenesis is affected by the atmosphere in the proximate tissues has implications for the origins of seeds as well as their future. Some authors have suggested that seed-bearing plants could not have appeared until the Earth's atmosphere had developed a sufficiently high-O_2 concentration (Quebedeaux and Hardy, 1976). Models of atmospheric O_2 over time show a peak of O_2 during the Carboniferous and Permian (Berner and Canfield, 1989), when seed ferns and gymnosperms developed and flourished. Modern angiosperms are thought to have arisen from ancestral open-carpel forms, coinciding with another period of high O_2 in the late Jurassic and Cretaceous. Looking to the future, when crop plants would be used as the central element of advanced life support systems for long duration space travel (National Research Council, 1997), our laboratory has been investigating how seeds develop when raised on orbital platforms, in a microgravity environment. The smaller seed size and wider variability in seed weights obtained in space have led us to hypothesize that the composition of the gaseous seed environment changes as a result of the cessation of buoyancy-driven convection in microgravity (Musgrave et al., 1997, 2000). The experiments described here were conducted to better understand the dynamic between metabolic gases in the seed environment and seed development.

Measuring the gaseous microenvironment of a developing seed is fraught with technical difficulties owing to size, access, and sampling problems. Goffman et al. (2004) have estimated CO_2 concentrations inside developing oil seeds to be 600–2000 times higher than in ambient air or inside leaves. Similarly, Vigeolas et al. (2003), using optical microsensors, demonstrated that low O_2 concentrations prevail in developing oilseed rape (*Brassica napus* L.) seeds. In cereals, the situation is similar. Van Dongen et al. (2004) found that wheat (*Triticum aestivum* L.) seeds are hypoxic (2.1% O_2) even when plants are growing under normoxic conditions, and Rolletschek et al. (2004) documented steep O_2 gradients within barley (*Hordeum vulgare* L.) seeds. Although these studies are more informative of the in situ conditions than prior work, which relied on emission studies of surface tissues of fruit or excised seeds (Banks and Nicholson, 2000; Bower et al.,

Received for publication 5 Apr. 2005. Accepted for publication 11 July 2005. Supported by NASA grants NAG10-329 and NAG2-1375.
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2000), they fail to capture the seed cohort behaving as a dynamic stand within maternal tissues, which themselves may be sources, sinks, or even competitors for the metabolic gases that make up the unique seed atmosphere. Here we describe long-duration in situ experiments in which the seed atmosphere was measured and subsequently controlled during development. The clustered arrays of seeds inside pepper fruit serve as cohorts for analyzing the developmental consequences of altering the internal atmosphere of the developing fruit.

Materials and Methods

Pepper cultivars used for these experiments were described previously (Blasiak and Musgrave, 2002). 'PI 140367' [from Iran; Plant Introduction number assigned in 1941 (Plant Genetic Resources Conservation Unit, Univ. of Georgia, Griffin)] produces small mildly hot peppers as individual pendulous fruit, while commercial cultivar Triton (NK Corp., Moscow, Russia) produces large sweet peppers in a capitate cluster of upright turbinate fruit. Fruit and locule volumes were determined by displacement; other characteristics of interest from the standpoint of defining the seed environment are listed in Table 1.

Growth and gas sampling. Plants were grown under greenhouse conditions in Amherst, Mass. (lat. 42.4°N) in experiments conducted sequentially over 2 years, under ambient light. Plants were grown in a soil-less peat-lite mix and fertilized weekly with a complete fertilizer. Gas sampling was accomplished during development by extracting 50–200 μL of locular gases with a gas-tight syringe, as described by Blasiak and Musgrave (2002). Gas samples were analyzed on a gas chromatograph (series 6890; Agilent Technologies, Wilmington, Del.) (Blasiak and Musgrave, 2002). Oxygen and CO_2 were measured simultaneously by injection onto a 1.83-m CTR1 column (Alltech, Deerfield, Ill.) at 35 °C, utilizing helium as the carrier gas at 29 mL·min^{-1}, linked to a thermal conductivity detector. Output from the detector was fed to a computer and integrated with EZChrom 6.8 software (Scientific Software, Pleasanton, Calif.). Ethylene was determined by injection of locular atmosphere samples onto a 1.83-m Porapak N column (Agilent Technologies) at 50 °C, utilizing nitrogen as the carrier gas at 50 mL·min^{-1}, linked to a flame ionization detector.

Modified locular atmosphere experiment design. How the seed atmosphere influences development was investigated by manipulating the composition of the gases surrounding the developing seeds without altering the exterior environment of the plant. A series of experiments were conducted in which we paired gas treatments to dissect the roles of individual gases in seed development. In the first experiment, designed to understand the role played by O_2 in the seed environment, the treatment gases were N_2 and a premixed gas comprised of 21% (mol/mol) O_2, balance N_2 (denoted "N_2/O_2"). The atmosphere surrounding the developing seeds in the N_2/O_2 treatment differs from untreated controls in a variety of factors: flow (as described below), CO_2 concentration, and O_2 concentration. In order to deconfound the effects of these factors in the seed microenvironment, we devised a synthetic atmosphere containing 3% CO_2, 21% O_2, and the balance N_2 to represent a standard seed environment. In the second experiment, treatment gases were N_2/O_2 (as in the previous experiment), and the synthetic seed atmosphere consisting of 3% CO_2, 21% O_2, and the balance nitrogen (denoted "$CO_2/O_2/N_2$"). The synthetic seed atmosphere, while not an absolute mirror of the dynamic atmosphere found within the fruit locule over the course of seed development, does approximate the gaseous components that the developing seeds would experience. Comparisons between these two treatments allowed us to assess the effect of the high CO_2 concentration on seed development. In the third experiment, these two treatment gases were again used in addition to a low-O_2 treatment consisting of 3% CO_2, 15% O_2, and the balance nitrogen (denoted "CO_2/low-O_2/N_2"). Oxygen effects were determined by comparing performance in the standard seed atmosphere with that obtained in a low O_2 (15%) synthetic atmosphere that also contained 3% CO_2. No-flow controls were provided in each of the experiments.

Modified locular atmosphere experiment protocol. Twenty-five days after anthesis (DAA), randomly selected pepper fruit of each cultivar were fitted with tygon tubes (0.8 mm i.d.; 2.3 mm o.d.) at the proximal end (≈2–3 cm below the calyx) and distal end (≈2–3 cm above apex). To insert tubes, a sterile rod was used to make a puncture smaller than the outer diameter of the tube. The tube was then inserted into the hole to a depth just exceeding the thickness of the pericarp, and the juncture of the tube and pericarp sealed with a generous application of stopcock grease. For fruit assigned to the gas treatments, the tubes at the proximal ends were connected to bottled high-purity treatment gases, which were bubbled through distilled water to hydrate them before being delivered to the pepper fruit. Flows through the fruit approximated 60–90 mL·min^{-1}. The distal tubes exiting the fruit were placed into Erlenmeyer flasks of water. All fruit were checked daily to ensure that a flow of gas was exiting the distal tubes as streams of bubbles in the outlet flasks. On the fruit designated for the no-flow control treatment, both the proximal and distal tubes were severed and sealed with silicone stopcock grease (denoted "control"). A diagram showing the basic set-up for these treatments is shown in Fig. 1A; 1B shows a 'Triton' pepper plant in one of the modified locular atmosphere experiments.

Treatments continued until harvest. Fruit were harvested when they had reached ripeness, as evidenced by complete red coloration of the pericarp. At harvest, fruit were measured and weighed. Fruit-wall thickness was measured at points around the midpoint of the pericarp and the results averaged. Seed was collected, weighed, and dried. Samples of pericarp from both the plumbed and unplumbed sides of the fruit were weighed and dried. Densities of similar samples, as well as the receptacle area were determined by displacement. Samples of both pericarp and seed were immediately frozen for biochemical analysis. Similar samples were placed in fixative and refrigerated for subsequent microscopy.

Biochemical analyses. Soluble carbohydrate and starch determinations (Stout et al., 2001) were performed on pericarp and seed tissue harvested as described above. Samples were freeze-dried prior to extraction. Comparable samples were analyzed for

Table 1. Characteristics of the seed environment inside the fruits of the two pepper cultivars used in this study, 'Triton' and 'PI 140367'. Data were determined from completely ripe fruits (n = 10).

	Mean value (SE)	
Characteristic	Triton	PI 140367
Pepper type	Sweet	Mildly hot
Fruit volume (mL)	57 (4.4)	10.4 (1.9)
Locule volume (mL)	17.5 (2.9)	4.6 (0.9)
Seeds (no./fruit)	95.0 (9.2)	85.5 (6.8)
Dry weight (mg/seed)	7.4 (0.17)	6.4 (0.42)

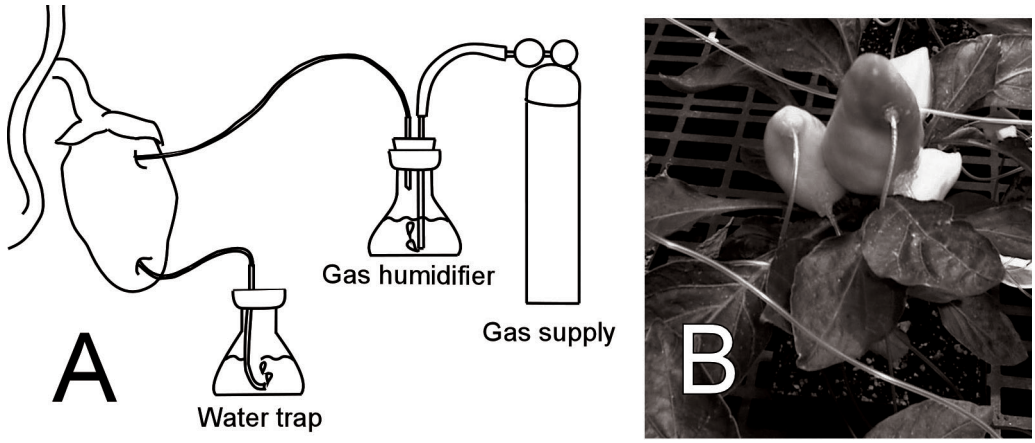

Fig. 1. A simplified diagram for plumbing of a single pepper is shown in panel A. In the actual experiment, the outlet from the gas humidifier was fed into a manifold from which individual lines were used to provide the gas to each pepper fruit. Panel B shows the actual experimental set up for the modified locular atmosphere experiments (a 'Triton' pepper is shown). Zonal ripening of the fruit was observed between gas ports in the N_2/O_2 gas treatment (right pepper), but not in the N_2 treatment (left pepper).

protein content according to the method of Mansfield and Briarty (1996) [Peterson's modification of the micro-Lowry method using a protein assay kit (P5656; Sigma-Aldrich Corp., St. Louis)].

Fixation and microscopy. Freshly harvested seeds from the experimental treatments were immediately fixed with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0, overnight for subsequent examination. Fixed seeds were then washed with 0.1 M sodium phosphate buffer. Embryos were dissected from fixed seeds and photographed using a dissecting microscope (Olympus Corp., Melville, N.Y.) and then further processed for cytochemical staining and light microscopic observation. Embryos were dehydrated with an ethanol series (30%, 50%, 70%, 90%, and 100% by volume ethanol) and then infiltrated with and embedded in L.R. White resin (Electron Microscopy Services, Fort Washington, Pa.). The embedded embryos were sectioned with a Leica microtome (Leica Mikrosystems Aktiengesellschaft, Vienna, Austria) and sections (thickness 1 μm) were stained with Periodic Acid Schiff's reagent (Carolina Biological Supply Co., Burlington, N.C.) for carbohydrates and Aniline Blue Black (Carolina Biological Supply) for protein. Stained sections were observed and photographed with an Olympus compound microscope. Seeds studied with transmission electron microscopy were fixed in a fixative of 2% glutaraldehyde and 1% formaldehyde, post fixed in 1% osmium tetroxide, and embedded in Spurrs resin (Electron Microscopy Services). Sections (65 nm thick) were stained with uranyl acetate and lead citrate and observed with a LEO 900 Transmission Electron Microscope (Carl Zeiss SMT, Thornwood, N.Y.). Whole embryos were also dissected from seeds and fixed with the same fixative as for transmission electron microscopy and observed with a LEO435VP scanning electron microscope (Carl Zeiss SMT).

Results

The gaseous environment surrounding developing pepper seeds differs from standard atmosphere in that it is high in CO_2 and low in O_2. Samples withdrawn from the fruit locule over time show that CO_2 ranges between 0.5% and 3% (5000–30,000 μmol·mol^{-1}) (Fig. 2, top) while O_2 ranges between 18% and 20% (Fig. 2, center), compared to the composition of standard atmosphere (400 μmol·mol^{-1} CO_2; 21% O_2) (Fig. 2, center). Locular CO_2 rose in both cultivars as the fruit developed, peaking as the

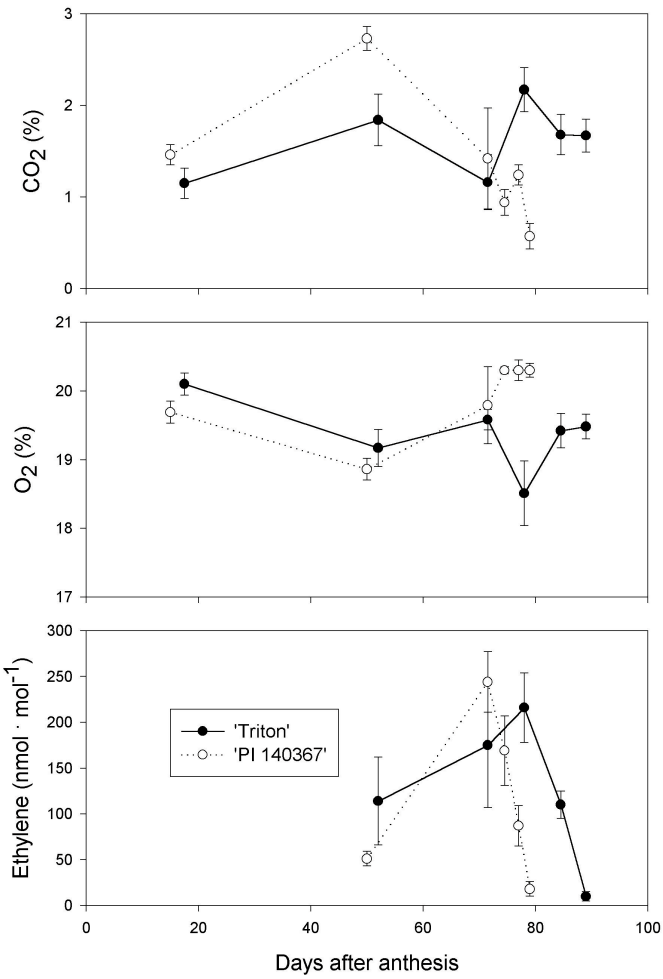

Fig. 2. Gas composition of the seed microenvironment during development in two pepper cultivars, 'Triton' and 'PI 140367', as measured by gas chromatography. Top panel, CO_2; middle panel, O_2; bottom panel, ethylene.

seeds attained their final dry weights (Fig. 3, center) ≈50 DAA, and subsequently declining. The 'Triton' pepper, with its larger locule (Table 1) showed a narrower range of CO_2 concentrations over the course of seed development than did the smaller hot pepper, 'PI 140367' (Fig. 2, top).

Pepper seeds attained their mature sizes quickly (Fig. 3, top panels), but accrued dry weight at a continuous rate until 40 to 60 DAA, at which time final seed weight is reached at the onset of pericarp ripening (Fig. 3, center; ripeness stage is denoted by symbols). In both cultivars, water content of the seeds declined in proportion to dry matter accumulation (Fig. 3, bottom), which resulted in increased seed storage reserves with no concomitant increase in seed size (Fig. 3, top). Ethylene concentration within the fruit locule has a relatively narrow peak at the dead ripe/post-ripe stage of fruit development (Fig. 2, bottom) after seeds have already attained their full size and full dry weight (Fig. 3, center panels). A second peak in locular CO_2 coincided with this fruit ripening event in both cultivars, and in 'Triton' this climacteric period coincided with the lowest O_2 concentration in the seed microenvironment (Fig. 2, center).

Steps in embryo development within the immature seeds are depicted in Fig. 4. Embryos at the cotyledon elongation stage (Fig. 4A) and curled cotyledon stage (Fig. 4B) were observed at 25 and 30 DAA, respectively, and embryos had fully developed by 35 DAA (Fig. 4C). Transmission electron micrographs of cotyledon cells show cytochemical changes (Fig. 4D–F) correlated with these stages of embryogenesis (Fig. 4A–C). Lipid droplets were scattered in the cytoplasm at 25 DAA (Fig. 4D) but were mostly arranged around vacuoles by 30 DAA (Fig. 4E). At later developmental stages, lipid droplets had crowded together to occupy a major portion of the cytoplasm (Fig. 4F). Deposition of protein began at 10 DAA along the membranes of vesicles (data not shown). As can be seen in Figs. 4E and 4F, relatively large protein bodies were observed in embryonic cells at 30 and 35 DAA, suggesting their formation by fusion of protein containing vesicles from earlier stages of embryogenesis. Starch grains were present in cotyledon cells of embryos at 20 and 25 DAA (Fig. 4D), but not at later stages. Lipid and protein are the major storage reserves in cotyledon cells at 35 DAA (Fig. 4F) and in mature embryos (data not shown).

Striking changes in embryo development were observed when seeds developed in modified atmospheres (Fig. 5). A representative embryo from the no-flow control fruit is pictured in Fig. 5A. Embryos developing in the N_2/O_2 mix (Fig. 5C) exceeded no-flow controls in final size, while those developing in the N_2 atmosphere were arrested at an early developmental stage (Fig. 5E). Seeds containing these embryos had average dry weights of 5.4 mg (controls), 7.1 mg (N_2/O_2), and 1.8 mg (N_2). Cytochemical staining of this embryo tissue for protein and carbohydrate storage reserves is shown in the right panels of Fig. 5. Embryos developing in an O_2 and CO_2 free atmosphere were devoid of storage reserves (Fig. 5F; N_2 treatment). The embryos exposed to a carbon-dioxide free, normoxic (N_2/O_2) atmosphere amassed greater reserves of both protein and carbohydrate (Fig. 5D) than did embryos developing within their unaltered locular atmospheres (Fig. 5B).

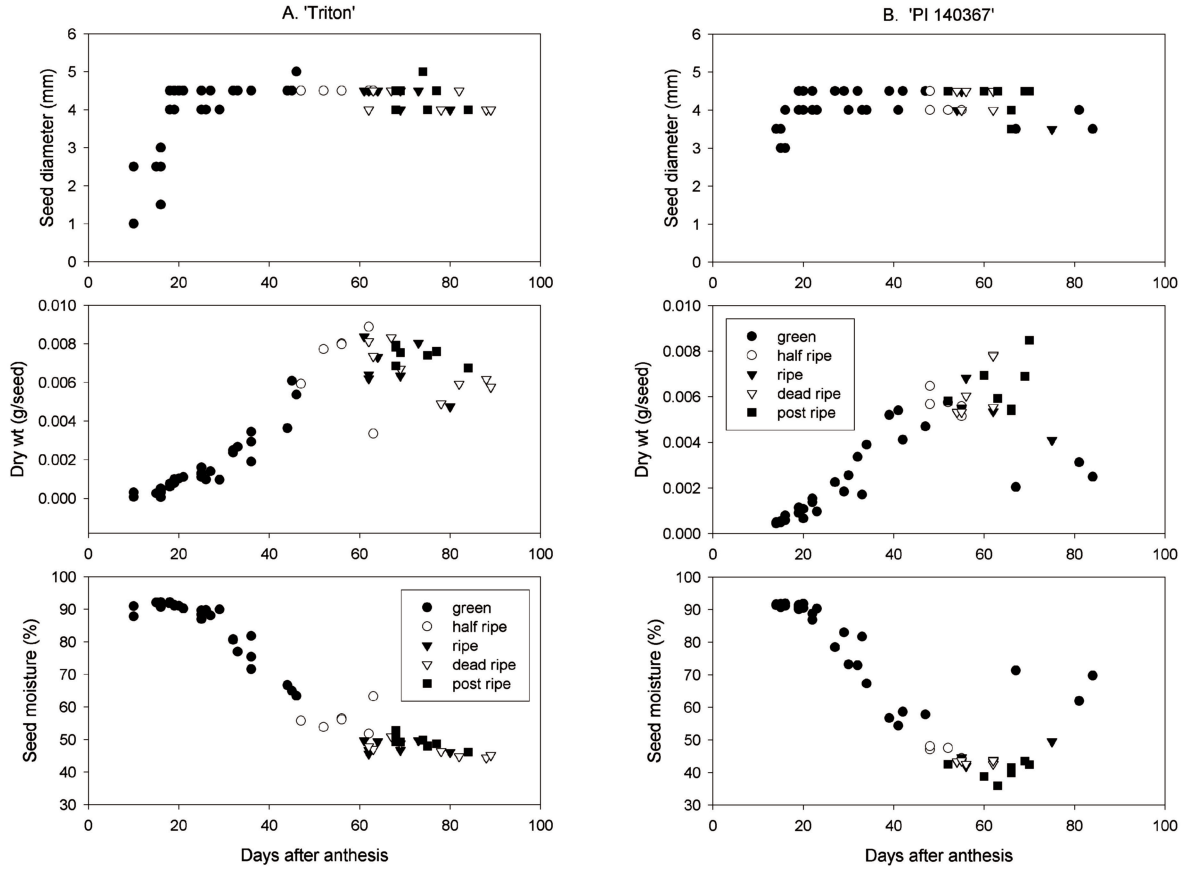

Fig. 3. Time course of seed development in 'Triton' pepper (**A**) and 'PI 140367' (**B**) in the context of fruit ripeness stages (refer to symbol key). Top panels show seed size; middle panels, seed dry weight; bottom panels, seed moisture.

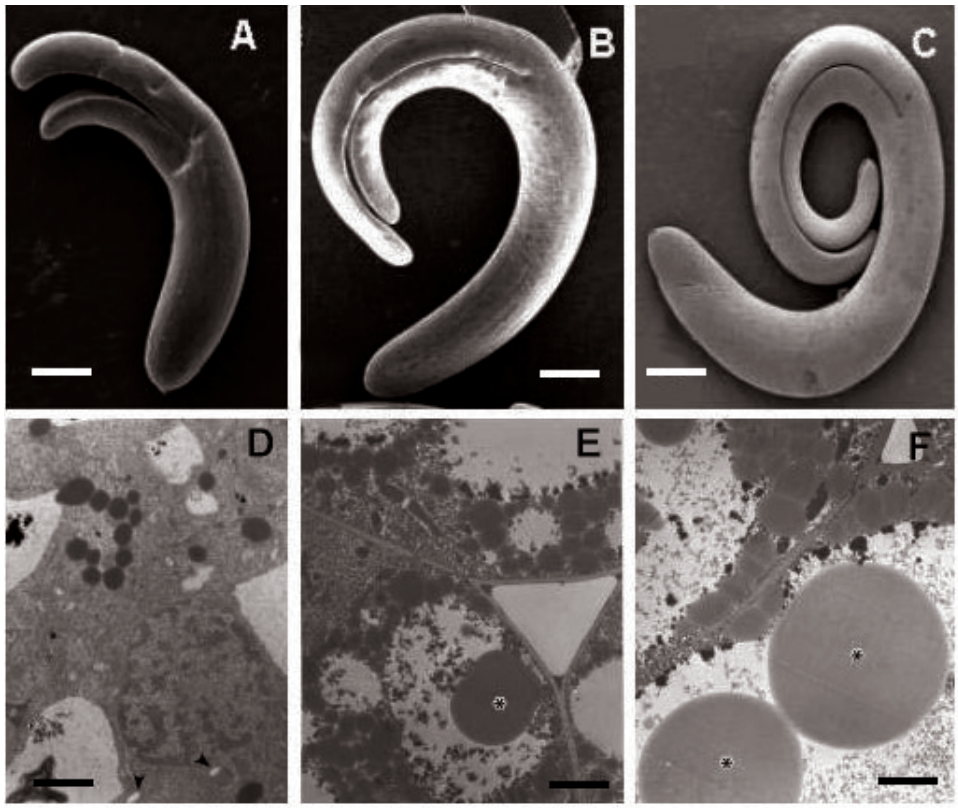

Fig. 4. Scanning electron micrographs showing embryo stages (**A–C**) and transmission electron micrographs (**D–F**) showing the ultrastructure of cotyledon cells during normal development in 'Triton' pepper; A = embryo at 25 d after anthesis (DAA), B = 30 DAA, and C = 35 DAA. Scale bars in A–C represent 200 μm. At 25 DAA, starch grains (arrowheads) and lipid droplets (dark granules) were present (D) in cotyledon cells of embryos. At 30 DAA, globular protein bodies (asterisks) were present in vacuoles (E) and were relatively large by 35 DAA (F). Lipid droplets/oleosomes (gray or dark granules) show a dynamic progression in their distribution during development (D–F). Scale bars in D, E, and F represent 2.5 μm.

The stimulation of seed growth by the N_2/O_2 atmosphere, measured in terms of seed dry weight, occurred in both pepper cultivars and was independent of changes in fruit weight that accompanied the flowing gas treatment. As shown in Fig. 6, 'Triton' pepper seeds had a 16% increase in seed dry weight when developing in the N_2/O_2 atmosphere compared to the control (averaged across the three experiments) and 'PI 140367' seed weight increased by 10%. Fruit weight increased in the N_2/O_2 treatment in 'Triton' pepper but did not significantly differ from the control in 'PI 140367'.

In the subsequent experiments, use of the standard seed atmosphere (3% CO_2, 21% O_2, and the balance N_2) allowed us to investigate differential effects of flow, CO_2 concentration, and O_2 concentration on seed growth and development. Comparing seed development in this atmosphere with no flow control showed that in both cultivars, average seed weight was substantially increased by the flowing atmosphere (Fig. 7). Interestingly, in both cultivars, this increase was not accompanied by any significant increase in maximum seed size, but was the result of a decreased proportion of low-weight seeds. Decreasing the high levels of locular CO_2 by flowing a CO_2-free atmosphere over the developing seeds had no effect on their growth, but flowing a low-O_2 atmosphere over the developing seeds decreased their final weights even though the seeds of developing peppers are normally exposed to partial pressures of O_2 below atmospheric standard (Table 2).

As in initial experiments with the N_2/O_2 atmosphere, in which fruit weight of cultivar 'PI 140367' was not affected by treatment (Fig. 6, lower panel), carbohydrates in the pericarp were also unaffected by the internal atmosphere treatments (Fig. 8, lower panel). In contrast, pericarp carbohydrates in the 'Triton' pepper varied greatly among treatments, demonstrating significant effects of flow, CO_2 concentration and O_2 concentration (Fig. 8, top panel). The N_2/O_2 flowing atmosphere caused pericarp carbohydrate profiles that most closely resembled the control.

Flow significantly increased the seed soluble carbohydrate and decreased protein in 'PI 140367' (Table 3). In 'PI 140367', flow delayed the time to onset of ripening and prolonged the ripening period. Removing CO_2 from the flowing gas resulted in a ripening sequence comparable to the control, but this effect was not observed in the 'Triton' pepper (Table 4).

Discussion

During the 3-month period between anthesis and fruit maturity, pepper seeds are developing within a gas-filled locule with limited continuity to the outside atmosphere (Blasiak and Musgrave, 2002). Their rapid expansion in the more slowly developing fruit results in a tightly packed cohort of individuals having continuity at their bases with the vascular system of the parent plant, but whose surfaces are exposed, to varying degrees, to maternal tissues, to their sibs, and to the locular atmosphere. There is limited capability for gas movement across the peduncle of 'Triton' pepper (Blasiak and Musgrave, 2002), and although some degree of gas movement is possible through the peduncle

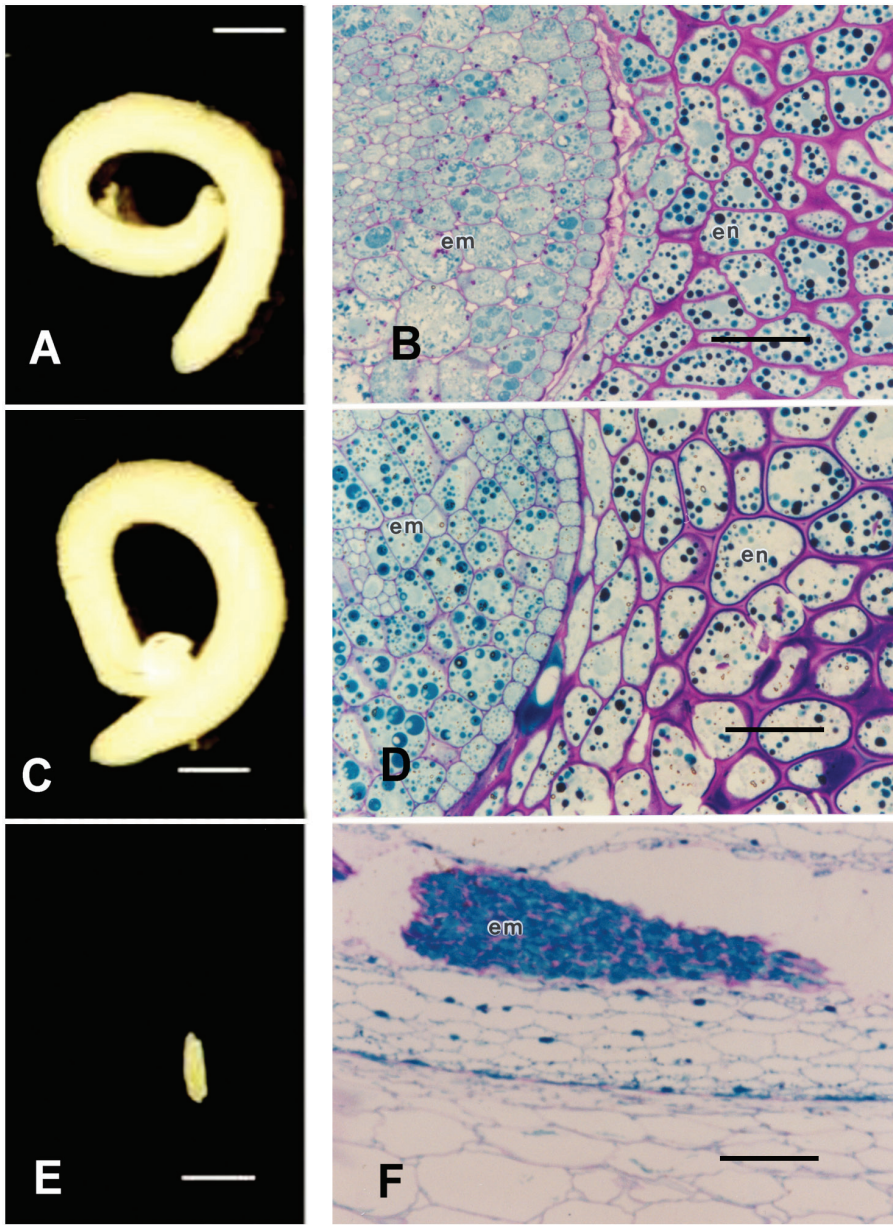

Fig. 5. 'Triton' pepper embryos dissected from mature seeds produced in a modified locular atmosphere experiment. A representative embryo from the control (no flow) treatment is shown in A. Panel C shows an embryo produced in the N_2/O_2 treatment (21% O2, balance nitrogen) while panel E is the embryo from a seed produced with nitrogen (N_2) flowing through the fruit locule. Scale bars = 667 μm for the left panels. The panels on the right side of the figure show cytochemical staining of storage reserves in sections from these embryos. Periodic acid Schiff's reagent staining shows carbohydrate (magenta) and aniline blue black staining shows protein (blue) in the embryo (em) and endosperm (en) tissues. Panel B, the control (no flow), shows a few starch grains remaining in the embryo cells, while the majority of the storage substances are protein. Protein bodies are larger and more darkly stained in panel D, from the N_2/O_2 treatment (21% O_2, balance N_2). No starch grains are present. Note the thicker cell walls in the endosperm layer. In panel F, the embryo produced in the N_2 treatment has not developed, and the endosperm tissue is devoid of storage reserves. Scale bars = 50 μm for the right panels.

of 'PI 140367', (Blasiak and Musgrave, 2002), the cessation of embryo development in the presence of a locular atmosphere of N_2 indicates that supply of metabolic gases to the developing seeds through the maternal tissues is insufficient to maintain seed growth (Figs. 5 E and F).

The seed, to a fundamental degree, is a rapidly metabolizing tissue developing within an atmosphere of declining O_2 and increasing CO_2 (Fig. 2). CO_2 is very high in the locule, 15–100 times higher than ambient atmosphere, but this does not seem to have any detrimental effect on seed development (Table 2). This, perhaps, is not surprising since other rapidly growing plant tissues, such as roots, are routinely exposed to similar high levels of CO_2 (Schneider and Musgrave, 1992). The relatively modest decrease in O_2 within the locule, however, does decrease seed growth in both pepper cultivars (Table 2), with the greater effect in the 'Triton' pepper, possibly because the 'PI 140367' pepper seeds are receiving some increment of O_2 through the placental tissues.

Changing proportions of seed-storage reserves reflect developmental stages in embryogenesis (Fig. 4). That the differing proportions of CO_2 and O_2 are not affecting a developmental sequence within the pepper embryos is supported by the discovery that altering these components has no discernible effect on storage reserves in the mature seed. Reducing O_2 in the seed atmosphere retards growth, but does not alter the developmental sequence of the seed storage reserves. Development of both seed and fruit of 'PI 140367' are affected by flow of gas through the locule. Seed soluble carbohydrates are increased and accumulation of protein is reduced (Table 3), a pattern indicative of a delay in seed maturation in other species (Musgrave et al., 2005). Fruit ripening is delayed by flow in 'PI 140367' and the pace of ripening is slowed (Table 4). These data suggest that a gaseous component, possibly ethylene, is being removed in the exhaust stream of the treatment gas. The fact that removing CO_2, a competitive inhibitor of ethylene, from the flowing gas eliminates the delay in fruit ripening further suggests a possible role for that gas in fruit ripening. 'Triton' pepper does not exhibit this effect, possibly because its locular gases equilibrate with the external atmosphere through the pericarp rather than the placental tissues, although whether this would indicate the presence of a symplast avenue for growth factor movement or a different system of developmental regulation has not been explored. The striking difference in response between these two cultivars does, however, brings home the hazards of assuming that closely related plants having superficially similar growth forms will respond similarly under experimental conditions.

Perhaps the most important result in these experiments was the effect of increased gas exchange on the developing seeds. Average seed weight increased with flow, but maximum seed weight did not (Fig. 7). There was, however, a marked decrease in the number of small seeds, indicating that a greater proportion of the seeds were able to reach a maximum weight (Fig. 7). This supports the proposition that developing pepper seeds are having their growth limited, in situ, at O_2 partial pressures reduced only incrementally from standard atmosphere, and at levels that other researchers have demonstrated to have

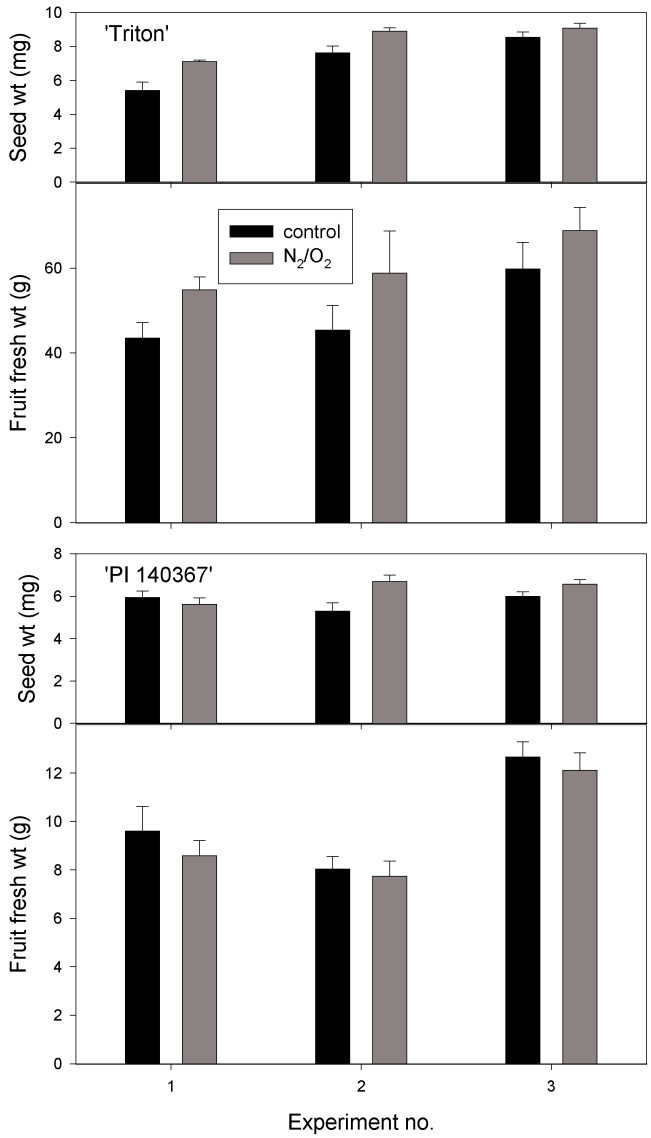

Fig. 6. Effect of the N_2/O_2 flowing atmosphere (21% O_2, balance N_2) on seed weight and fruit weight in 'Triton' and 'PI 140367' pepper over three separate experiments, compared to the no flow controls. Mean values and SE are shown. For Expt. 1, n = 5; for Expt. 2, n = 8; for Expt. 3, n = 10.

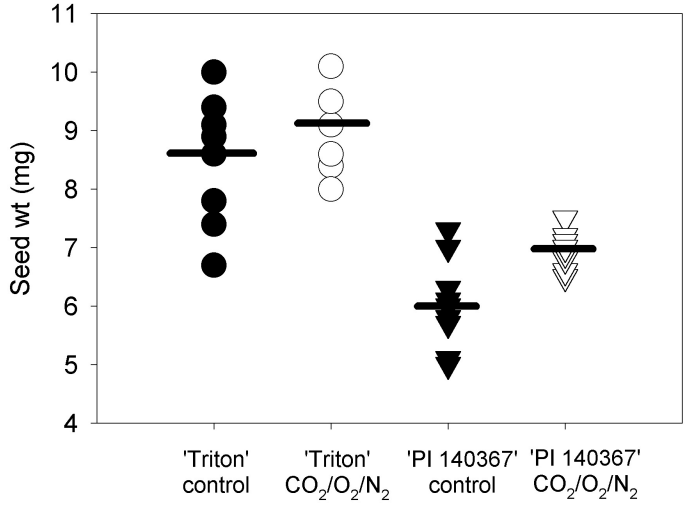

Fig. 7. Comparison of mature weights when seeds developed in a flowing standard seed atmosphere containing 3% CO_2, 21% O_2, balance N_2 ($CO_2/O_2/N_2$), or in the no-flow control. Data points from one representative experiment are shown for two pepper cultivars, 'Triton' and 'PI 140367'. The horizontal lines mark the means of the data. n = 8.

no effect on metabolizing tissue (Armstrong and Gaymard, 1976). Oxygen supply does not match embryo demand within the fruit.

Aschan and Pfanz (2003) reviewed nonfoliar photosynthesis in plants and concluded that internal CO_2 recycling contributed significantly to carbon acquisition. For example, *Brassica* L. species seeds obtain over 99% of their dry matter from photosynthesis that takes place within the seed pod as opposed to carbon translocated in from the leaves (Sharma and Ghildiyal, 1992; Sheoran et al., 1991). Intra-pod photosynthesis controls biosynthetic fluxes in enclosed seeds both by increasing internal O_2 and by supplementing the energy supplies needed to drive biosynthetic processes (Borisjuk et al., 2004; Ruuska et al., 2004). Blasiak and Musgrave (2002) demonstrated the role of light in enhancing locular O_2 concentrations in four pepper cultivars, including the two used in the present study. The important role of fruit photosynthesis in maintaining sufficient O_2 for successful seed development is emphasized by our demonstration of reduced seed weight when locular O_2 is limited (Table 2). In contrast to the effect of internal fruit atmosphere on seeds, we found that fruit wall carbohydrates were unaffected by the experimental gas treatments in 'PI 140367'. In 'Triton' pepper, however, the treatment without CO_2 had 80% less sucrose than in pericarps of the fruit receiving the standard seed atmosphere. Similarly, in 'Triton', the flowing standard atmosphere gave twice the pericarp sucrose than in the no-flow controls (Fig. 8). Both results demonstrate a role for internal CO_2 recycling in carbohydrate metabolism in cultivars in which the primary diffusion route for the internal atmosphere is through the pericarp.

Prior studies on the role of atmospheric composition on seed development have treated the whole plant (Kuang et al., 1998; Musgrave and Strain, 1988; Ramonell et al., 2002), the plant shoot (Quebedeaux and Hardy, 1976) or the fruit (Sinclair et al., 1987) with altered gas mixtures. In this study, we demonstrate that seed growth in two cultivars of pepper is inhibited by the normal locular atmosphere. The effect of increased locular O_2 on seed growth was positive in both cultivars of pepper. The pepper is an ideal system for directly studying the role of metabolic gases in seed development because the seeds themselves can be exposed to the test atmosphere. Confounding effects caused by exposure of other tissues are minimized using this system, and the uncertainty regarding whether the intervening tissues have modified the treatment gas is avoided. Direct manipulation of locular atmosphere is possible, leaving the remainder of the plant comparatively unaffected, and the prominent placement of the seeds in the absence of encasing maternal tissue allows ready measurement of the atmosphere in which the seeds are actually developing.

We have demonstrated that seed growth in pepper is O_2 limited under normal terrestrial conditions. A decreased O_2 concentration is maintained in metabolizing plant tissues surrounded by standard atmosphere primarily due to diffusion resistance imposed by the surrounding tissues (Porterfield et al., 1999). In microgravity, lack of buoyancy-driven convection leads to a thickening of the boundary layer around objects, resulting in decreased diffusion efficiency (Musgrave et al., 1997). Other researchers have noted symptoms of hypoxia in plant tissues grown in microgravity,

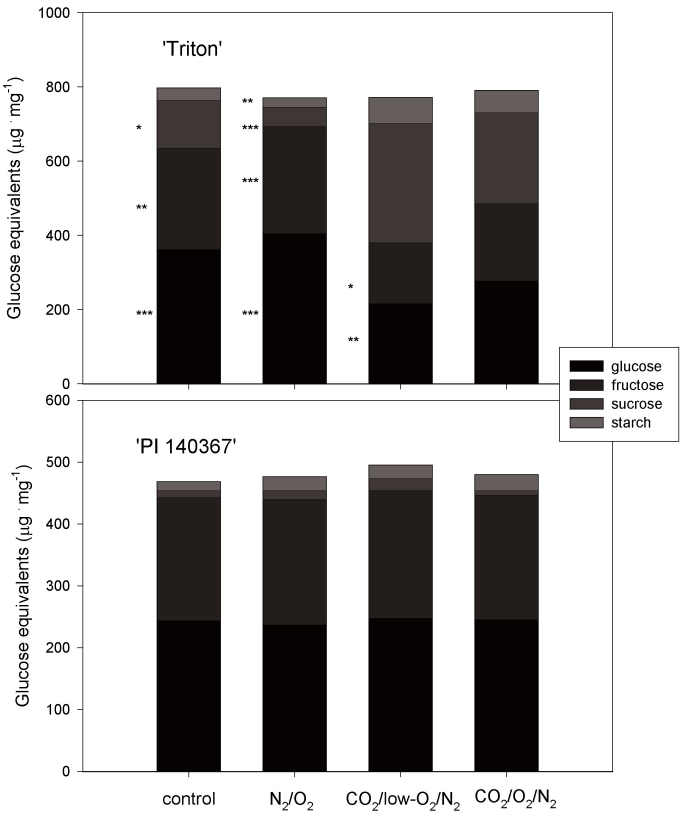

Fig. 8. Carbohydrates in the pericarp tissue of 'Triton' and 'PI 140367' pepper cultivars. Four treatments are shown: the no flow control; N_2/O_2 (21% O_2, balance N_2); CO_2/low-O_2/N_2 (3% CO_2, 15% O_2, and the balance N_2); and $CO_2/O_2/N_2$ (3% CO_2, 21% O_2, and the balance N_2). Bars marked by asterisks indicate that they are significantly different from the $CO_2/O_2/N_2$ treatment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. n = 10.

particularly in tissues that cannot be mechanically aerated (Krikorian, 1996, and references therein). Seeds, encased as they are in maternal tissues, may be expected to be especially susceptible to disruptions in their natural O_2 supply occasioned by changes in diffusion parameters, especially since they are demonstrably O_2 stressed even under earth-normal conditions. The discovery of differing pathways of gaseous diffusion at the cultivar level (Blasiak and Musgrave, 2002), as well as the suggestion in the current work that tissue responses to altered atmospheres varies with cultivar, suggests that progress to alleviate the stresses potentially imposed by extra-terrestrial conditions should be pursued at both the engineering and the horticultural levels.

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Table 2. Effect of intra-fruit CO_2 and O_2 on seed weight in two pepper cultivars, Triton and PI 140367. Results from a flowing standard atmosphere ($CO_2/O_2/N_2$) are compared with a flowing atmosphere without CO_2 (N_2/O_2) or a flowing atmosphere with lower (15%) oxygen (CO_2/low-O_2/N_2).

	Gas treatment				
	$CO_2/O_2/N_2$	N_2/O_2	CO_2/low- O_2/N_2	n	*P*
	Dry wt (mg/seed)				
CO_2 effect					
Triton	8.90	8.99	---	18	ns
PI 140367	6.83	6.62	---	18	ns
O_2 effect					
Triton	9.09	---	7.84	10	0.0003
PI 140367	6.96	---	6.31	10	0.0007

Table 3. Effect of intra-fruit CO_2 and O_2 on seed storage reserves in two pepper cultivars, Triton and PI 140367. Results with a flowing standard atmosphere ($CO_2/O_2/N_2$) are compared with no-flow controls, a flowing atmosphere without CO_2 (N_2/O_2), or a flowing atmosphere with lower (15%) O_2 (CO_2/low-O_2/N_2). Starch and soluble carbohydrates are expressed as glucose equivalents. *P* values were calculated for the results of each paired treatment parameter.

	Gas treatment					
	None	$CO_2/O_2/N_2$	N_2/O_2	CO_2/low-O_2/N_2	n	*P*
			Seed storage reserves (mg·g^{-1} dry wt)			
Flow effect						
Triton						
Soluble carbohydrate	49.8	47.9	---	---	8	NS
Starch	14.7	15.0	---	---	3	NS
Protein	12.6	12.2	---	---	8	NS
PI 140367						
Soluble carbohydrate	34.3	40.4	---	---	10	0.03
Starch	13.6	14.6	---	---	3	NS
Protein	14.5	13	---	---	10	0.01
CO_2 effect						
Triton						
Soluble carbohydrate	---	47.9	54.7	---	7	NS
Starch	---	15.0	13.5	---	3	NS
Protein	---	12.2	12.8	---	7	NS
PI 140367						
Soluble carbohydrate	---	40.4	39.1	---	10	NS
Starch	---	14.6	13.5	---	3	NS
Protein	---	13	14	---	10	NS
O_2 effect						
Triton						
Soluble carbohydrate	---	47.9	---	46.0	7	NS
Starch	---	15.0	---	13.5	3	NS
Protein	---	12.2	---	11.9	8	NS
PI 140367						
Soluble carbohydrate	---	40.4	---	41.7	10	NS
Starch	---	14.6	---	13.7	3	NS
Protein	---	13	---	12.9	10	NS

Table 4. Effect of locular CO_2 and O_2 on rate of ripening in two pepper cultivars, Triton and PI 140367. Results with flowing standard atmosphere ($CO_2/O_2/N_2$) are compared with the no-flow control, a flowing atmosphere without CO_2 (N_2/O_2), or a flowing atmosphere with lower (15%) O_2 (CO_2/low-O_2/N_2).

	Gas treatment					
	None	$CO_2/O_2/N_2$	N_2/O_2	CO_2/low-O_2/N_2	n	*P*
			Time to first color (d)			
Triton	21.8	22.2	21.4	24.9		
Flow	x[z]	x			10	NS
CO_2		x	x		10	NS
O_2		x		x	10	NS
PI 140367	26.9	29.2	25.8	29.5		
Flow	x	x			10	0.008
CO_2		x	x		10	0.00004
O_2		x		x	10	NS
			Time from first color to ripeness (d)			
Triton	14.1	14.6	14.3	15.0		
Flow	x	x			10	NS
CO_2		x	x		10	NS
O_2		x		x	10	NS
PI 140367	2.5	6.4	4.9	5.9		
Flow	x	x			10	0.001
CO_2		x	x		10	NS
O_2		x		x	10	NS

[z]x denotes treatments included in each paired statistical comparison.

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