Ethylene is involved in vascular cavity formation in pea (*Pisum sativum*) primary roots

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Abstract: A lengthy cavity usually forms in the vascular cylinders of pea (*Pisum sativum*) primary roots in response to sudden flooding at 25°C. This is thought to be a form of aerenchyma. Ethylene has been shown to mediate inducible aerenchyma in maize, therefore the role of ethylene in the formation of cavities in pea roots was examined. Pea seedlings grown for 4 d in 2 L beakers in vermiculite moistened below field capacity – conditions that do not favor cavity formation – were flooded with solutions containing ethylene inhibitors (AOA, EGTA, and STS). Pea seeds were germinated and grown in suitable containers (0.8-1.0 L) for 4 d at 25°C in the dark in similar vermiculite. These were then exposed to various concentrations of ethylene for 1 d, or they were flooded and endogenous ethylene was measured periodically by gas chromatography. Observations of roots exposed to exogenous ethylene were made by light microscope. All three inhibitors of ethylene suppressed cavity formation in flooded roots. Exogenous ethylene exposure caused cavities to frequently form in a dose-dependent manner in unflooded roots and caused an increase in mean cortical cell size and number. Flooding increased the rate of ethylene release into the air space above the medium surface. These results indicate ethylene mediates vascular cavity formation and add to the evidence that vascular cavities are the result of programmed cell death and may function as a type of aerenchyma.

Keywords: aerenchyma, ethylene, flooding, pea (*Pisum sativum* L.), programmed cell death, vascular cavity

Abbreviations: AOA, aminooxyacetic acid; DI, deionized water; EGTA, ethyleneglycol-bis (beta-aminoethyl ether)-N,N,N',N'- tetraacetic acid; STS, silver thiosulfate

Introduction

Previous studies have shown that at temperatures above 15°C pea primary roots growing in vermiculite or other media saturated to "field-capacity" develop lengthy lysigenous cavities in the centers of their vascular cylinders as they grow (Lu et al. 1991, Niki et al. 1995). At first it was thought this was strictly a temperature-dependent response, but further study revealed that vascular cavities are caused by hypoxia at warm temperatures, and the phenomenon is possibly a type of flooding-induced aerenchyma (Gladish and Niki 2000, Niki and Gladish 2001). When pea seedlings grow in a medium such as vermiculite that is moist, but unflooded (e.g., 375 mL L^{-1} vermiculite) vascular cavities form in only about 20-25% of the primary roots. By contrast, in saturated or flooded media the frequency is 80-100% as long as the temperature is > 15°C (Lu et al. 1991, Gladish and Niki 2000, Niki and Gladish 2001). Similar results were obtained when the seedlings were grown in moist, but unflooded vermiculite in a hypoxic environment in the absence of flooding (Gladish and Niki 2000). When pea seedlings are grown at 25°C in moist, but unflooded vermiculite for 4-5 days and then are suddenly flooded, the primary roots rapidly form vascular cavities (Gladish and Niki 2000). Initial stages of formation can begin in as little as 3 h (unpublished data).

In suddenly flooded seedlings 4 d old or younger such cavities rapidly form from within 1 cm of the tip of the primary root to within 2-3 cm of the cotyledonary node, and the root continues to grow. In the primary roots of older seedlings, the maturation

of late metaxylem tracheary elements in the center of the vascular cylinder at the base of the root seems to prevent cavity formation, and the cavity only forms to within about 4 cm of the cotyledonary node. Such roots uniformly cease growing (Niki and Gladish 2001). Cells in the tip appear to undergo apoptosis-like programmed cell death (Gladish et al. 2006). The ability of the younger roots to continue to grow under flooded conditions suggests that the cavity provides a path for the diffusion of oxygen needed for growth. In this sense, vascular cavity formation appears to function similarly to the well-studied inducible cortical aerenchyma observed in roots of *Zea mays*. It is known that ethylene is part of the signal transduction pathway in the *Zea* aerenchyma induction system (Drew et al. 1979, He et al. 1996, Colmer 2003, Gunawardena et al. 2001) and the *Triticum* aerenchyma induction system (Huang et al. 1997).

Consequently, it was hypothesized that ethylene is involved in the initiation of the vascular cavity response in pea primary roots. In the current study the role of ethylene in vascular cavities was examined by adding ethylene action and synthesis inhibitors to water used to flood pea seedling roots, by exposing seedling roots to exogenous ethylene, and by measuring the concentrations of ethylene in the air above flooded pea root systems.

Materials and Methods

Plant material and root growth

All experiments were performed using pea seedlings (*Pisum sativum* cv. 'Alaska') grown from seeds obtained from Gramco Co. (Hamilton OH, USA). Seeds were germinated and grown for each experiment as described below. Root growth was determined for the exogenous ethylene experiment after trials prior to sectioning by measuring root length using a standard ruler. For comparison to the exogenous ethylene experiment, seedlings were grown in two containers of each type for 4 d, 5 d, and 4 d plus 1 d flooded. No significant differences in growth ($p < 0.05$) were found between the two types of containers (canning jars and plastic beakers), so those data were pooled within treatments.

Ethylene inhibitors

For ethylene inhibitor studies, large beakers containing 2 L of vermiculite moistened with 750 mL of deionized water (DI) were covered with aluminum foil and autoclaved for 90 min. Pea seeds were sterilized for 5 min in a solution of 10% bleach with two drops of Tween 20 and rinsed three times in sterile water.

Under axenic conditions in a laminar flow hood, the seeds were planted in the vermiculite 2 cm beneath the surface (25-30 seeds each beaker). The foil was loosely replaced so as to maintain sterile conditions but allow air exchange. The beakers were placed in a growth chamber in darkness at 25°C for five days. Beakers were then flooded with DI (three beakers, positive controls) or inhibitor solutions (various treatments and concentrations, below) up to the cotyledons or left unflooded (three beakers, negative controls).

Treatment solutions included 1, 4, 8, and 10 mM silver thiosulphate (STS), an ethylene action inhibitor (Beyer 1976); 1, 5, and 10 mM aminooxyacetic acid (AOA), an ethylene synthesis inhibitor (Yu et al. 1979); and 20, 50, and 85 mM ethyleneglycol-bis(beta-aminoethyl ether)-N,N,N',N'- tetraacetic acid (EGTA), a Ca^{+2} chelator that interferes with the activity of the Ca^{+2}-dependent enzyme, 1-aminocyclopropane-1-carboxylic acid oxidase, which is necessary for ethylene synthesis (Petruzzelli et al. 2003). The beakers were then recovered with foil and placed back in the growth chamber. Some treatment concentrations were duplicated. 24 h later the seedlings were removed from the beakers, and each primary root was examined for the presence of vascular cavities at 1 cm and 3 cm from the tip by free-hand transverse sectioning. If a vascular cavity was found at either location the root was considered to have a cavity present.

Exogenous ethylene

For application of exogenous ethylene, 1 quart (960 mL) glass canning jars containing 0.8 L of vermiculite moistened with 300 mL DI or plastic beakers (modified with gas-exchange nipples) containing 900 mL of vermiculite moistened with 335 mL DI were covered with aluminum foil and autoclaved for 2 h. Pea seeds, sterilized as above, were placed in the vermiculite (n = 22-25 seeds each container) 2 cm beneath the surface. The foil was loosely replaced so as to maintain sterile conditions but allow air exchange, then the containers were placed in a growth chamber at 25°C.

After four days the foil covers were removed and replaced with neoprene stoppers perforated with two gas-exchange nipples (canning jars) or screw-on plastic caps (plastic beakers). The canning jars and plastic beakers were then taken to a specialized walk-in, controlled-temperature (25°C) chamber where they were perfused with air or air-ethylene mixtures at various concentrations at the rate of 25 mL min^{-1} for 24 h. Two of each type of container were exposed to each exogenous ethylene concentration (1, 5, 10, and 15 µL L^{-1}). A matching control set of four control containers that received air only accompanied

each trial. Then the seedlings were removed from the containers, and the primary roots were examined for the presence of vascular cavities by free-hand transverse sectioning at 1 cm and 3 cm from the tip. A root was considered to have a cavity if one was present in either location.

Inflow gas samples were periodically tested on a Carle model 211 Series S analytical gas chromatograph equipped with a series of 80/100 mesh custom-packed columns (Supelco, Bellefonte PA): 122 cm-long 8% NaCl modified alumina F1, 30.5 cm-long 8% NaCl modified alumina F1, and 61 cm-long molecular sieve 5a columns. The oven temperature was 80°C, and the retention time was 40 s.

Endogenous ethylene

To measure endogenous ethylene production, pea seedlings were grown for 4 d in foil-covered canning jars prepared with seedlings grown as described immediately above. Once in the walk-in chamber they were then flooded or not as treatment and positive controls, respectively (two jars each). Once the treatment jars were flooded, all jars were capped with neoprene stoppers perforated by a loop of tubing for sample extraction. Starting immediately, gas samples were extracted from the headspace of the sealed jars with a syringe at 1 h intervals. After this first 3 h measuring period, the headspace was flushed with air for an hour and additional samples were taken hourly for an additional 4 h. The jars were then flushed with air overnight. A third 4 h series was taken 18 h after flooding. A jar containing only moistened vermiculite but no seedlings was flooded and one without seedlings and not flooded were sampled during the first measuring period as negative controls.

Samples were run on a Carle model 211 Series S analytical gas chromatograph as described above.

Light microscopy

Segments of typical roots from the exogenous ethylene trial were prepared for light microscopy as per Niki and Gladish (2001). For evaluating the structural changes of tissues in roots, 2 mm segments were collected 1.5 cm from root tips, immediately immersed in 2.4% glutaraldehyde/0.3% paraformaldehyde in 0.02 M phosphate buffer (pH 7.2) and gently shaken overnight at room temperature. The segments were rinsed in buffer, dehydrated with an ethanol series (30, 40, 50, 65, 80, 90, 100, 100%; 20 min each step), and embedded in Historesin (Leica Instruments GmbH, Heidelberg, Germany). Three segments each from different roots were chosen at random from the air-treated and 10 nL L^{-1}

ethylene-treated groups. 2 μm thin-sections of the embedded segments were made on a Leica RM2065 ultramicrotome (Leica Instruments GmbH, Nussloch, Germany). Sections were stained with 0.025 % toluidine blue O. Sections were viewed with a Leica DMLB light microscope (Leica Microscopy and Systems GmbH, Wetzlar, Germany).

Digital photomicrographs of a cross section from each segment were modified for measurement analysis using Adobe PhotoShop v. 4.0 (Adobe Systems Inc. San Jose, CA USA). File size relationships were carefully maintained during modification. Measurements of whole root and vascular cylinder cross-sectional areas were taken using NIH Image v. 1.63 software (National Institutes of Health, Bethesda MD, USA). Likewise, wedge-shaped quarters of sections (one from each root) were used for sampling and estimating cortical cell cross-sectional areas.

Results

Ethylene inhibitors

At 25°C, when moistening of the medium was 375 mL L^{-1} of vermiculite, vascular cavities were very infrequent (Fig. 1-3).

By comparison to the flooded controls, which had very high frequencies of vascular cavities in the primary root populations (Fig. 1-3), AOA (ethylene synthesis inhibitor) reduced the frequency of vascular cavity formation in populations of flooded pea roots in a dose-dependent manner except at the highest
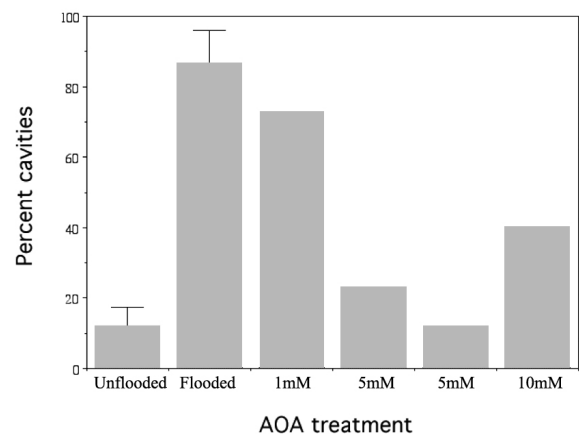

Fig. 1. Inhibition of vascular cavity formation by AOA (aminooxyacetic acid) in flood water. AOA is an ethylene synthesis inhibitor. Seedlings were grown in moist, unflooded vermiculite for 5 d at 25°C, then roots systems were flooded for 24 h. Reported is the frequency of cavity occurrence in primary roots in each treatment population. 25-30 seedlings were grown for each treatment. N = 3 trials each for the controls.
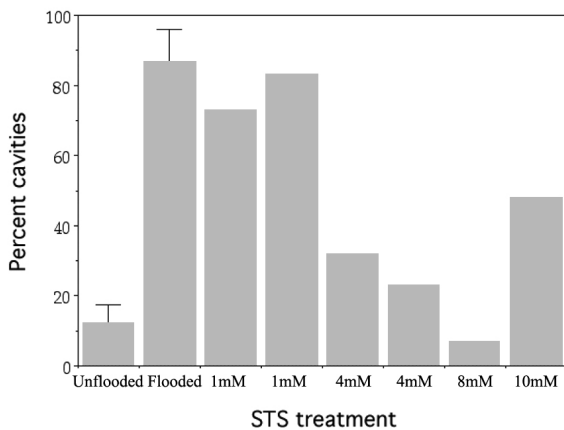
Fig. 2. Inhibition of vascular cavity formation by STS (silver thiosulphate) in flood water. STS inhibits ethylene action. Reported is the frequency of cavity occurrence in primary roots in each treatment population. N = 3 each for the controls (same as Fig. 1).

Fig. 3. Inhibition of vascular cavity formation by EGTA (ethyleneglycol-bis[beta-aminoethyl ether]-N,N,N',N'-tetra acetic acid) in flood water. EGTA is a Ca^{2+} chelator. Ca^{2+} is a cofactor for the enzyme 1-aminocyclopropane-1-carboxylic acid oxidase, which is necessary for ethylene synthesis. Reported is the frequency of cavity occurrence in primary roots in each treatment population. N = 3 each for the controls (same as Fig. 1).

concentration. 5 mM AOA reduced cavity frequency to a level comparable to roots growing in moist, unflooded vermiculite, the negative control condition (Fig. 1). Likewise, STS (inhibitor of ethylene action) did the same with 8 mM producing roughly the same frequency of cavity formation as the negative controls (Fig. 2). EGTA (1-aminocyclopropane-1-carboxylic acid oxidase inhibitor) reduced the frequency of vascular cavities (Fig. 3), but not to the same degree as AOA or STS probably because it works indirectly by chelating Ca^{2+} outside of cells.

Exogenous ethylene

In the same relatively limited moisture condition that normally suppresses cavity formation (negative control condition, above), exogenously-applied ethylene increased the frequency of cavity formation in a dose-dependent manner up to 10 μL L^{-1} concentration, though there was no significant difference between the controls and 1 μL L^{-1} ethylene (Fig. 4). Growth differences among control (plain air) groups were not significant ($p = 0.05$).

Growth was suppressed significantly by each ethylene treatment compared to its control group ($p < 0.01$). Among the ethylene treated seedlings, root growth was most suppressed by 10 μL L^{-1} ethylene but least suppressed at the highest concentration ($p < 0.05$). Growth rate reduction due to exposure to exogenous ethylene was as great or greater than that caused by flooding. Growth was typically abolished by flooding and by 10 μL L^{-1} ethylene (by comparison to 4d and 5d controls, data not shown), but ethylene treatment resulted in increased root diameter, which suggests a change from longitudinal to radial growth. Though vascular cylinder diameter was increased significantly, radial diameter increase due to ethylene
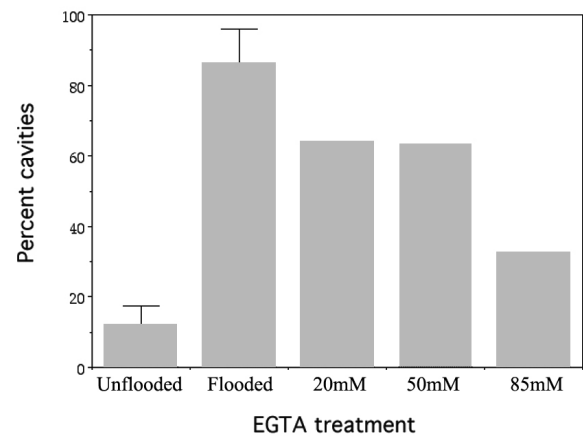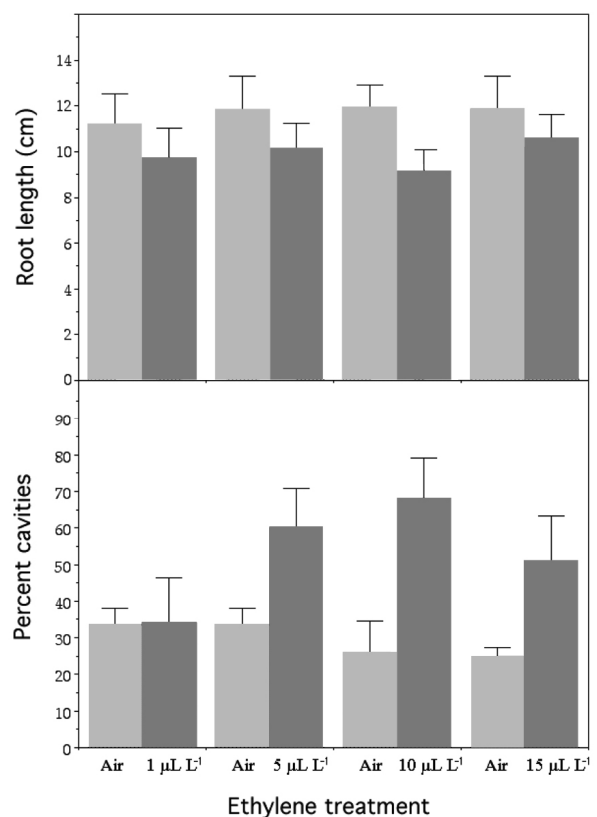

Fig. 4. Promotion of vascular cavity formation by exogenous ethylene. Under moisture conditions that minimize the frequency of vascular cavity formation at 25°C, 4 d-old seedlings growing in closed containers were continuously exposed for 24 h to flowing air or to airflow containing ethylene. Reported is the frequency of cavity occurrence in primary roots in each treatment population. N = 4 containers with 22-25 seedlings for each treatment.

exposure occurred mainly in the root cortex. This was due to an increase in mean cortical cell size (57%), cell number (27%), and an apparent increase in intercellular space size (Table 1; Fig. 5).

Table 1. Anatomical image analysis

	Total area (mm^2)	VC area (mm^2)	VC% of total area	Cortical cell area (μm^2)
Air	0.45 ± 0.13	0.04 ± 0.01	8.6 ± 0.5	430 ± 350
10 μL L^{-1} ethylene	0.86 ± 0.21	0.06 ± 0.01	7.1 ± 0.2	680 ± 700

Anatomical data obtained by digital image-analysis of cross sections taken 1.5 cm from the tips of pea primary roots exposed to flowing 10 μL L^{-1} ethylene or air for 24 h after 4 d of growth at 25°C. Data are means ± standard deviation from three roots. All differences were significant ($p < 0.05$). Cortical cell area is the mean area of individual cells. VC = vascular cylinder.
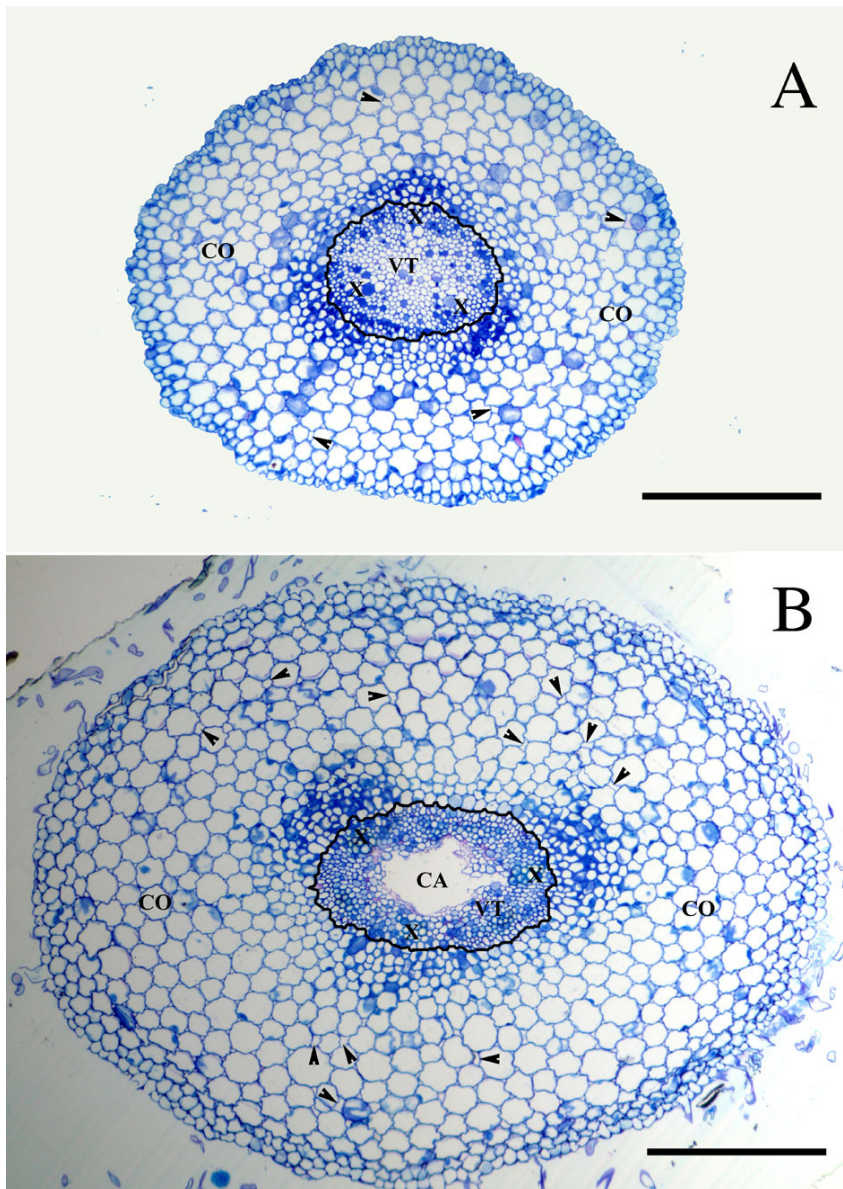

Fig. 5. Cross sections of pea primary roots taken 1.5 cm from the tip. Vascular cylinders are outlined in black. CA, vascular cavity; CO, cortex; VT, vascular tissue; X, xylem poles; Arrowheads, examples of large intercellular spaces; Bar = 300 μm. A. A typical root grown for 4 d in a canning jar in conditions under which vascular cavities infrequently form (moist, unflooded vermiculite at 25°C). The jar was then closed and exposed to flowing air for 24 h. B. A typical root grown for 4 d as "A". The jar was then closed and exposed to flowing air containing 10 μL ethylene/L air for 24 h.

Endogenous ethylene

During the first 3 h of the experiment, flooding caused the pea seedlings to release ethylene into the container headspace at a rate that was three to four-fold greater than seedlings in the unflooded containers. The rate of ethylene production was greatest in the period 1-2 h following flooding (Fig. 6).

The small amount of ethylene found 15 min after the start of the experiment in the absence of plants in the unflooded negative control jars was slightly less than the initial concentrations from containers with unflooded seedlings. This negative control indicated that perhaps there was about 8 nL ethylene per L of air in the post-harvest laboratory at the start of the experiment, presumably from ripening fruits elsewhere in the facility or from small leaks in the gas distribution system. Alternatively, this residual (artifactual) ethylene may have been associated with the vermiculite or with the water used to moisten and flood it. The 5 nL L^{-1} difference in ethylene concentration between the unflooded and flooded negative control jars is probably attributable to the difference in the gas headspace of the two controls, and this idea favors the latter (2nd) explanation for the background ethylene that was measured. The 5-7 nL L^{-1} higher ethylene concentration in the jars with flooded seedlings at the start of the experiment was probably due to this artifactual ethylene in the more restricted headspace caused by flooding. 1 h of flushing with air (which came from outside the laboratory) between the first and second runs and the overnight flushing between the second and third runs left the headspace gas in all containers with seedlings with 3 nL L^{-1} or less ethylene concentration. The approximately two-fold higher ethylene rates of concentration increase in the flooded jars measured in the second and third runs may have been mainly due to the difference in gas space between flooded and unflooded jars, though the differences after 3 h were greater than 5 nL L^{-1} in both cases (Fig. 6).

Discussion

The results of the present study reconfirm that flooding induces the formation of lengthy lysigenous cavities in the centers of vascular cylinders of primary roots of 'Alaska' pea seedlings if they are not already present (Gladish and Niki 2000; Niki and Gladish 2001). On the other hand, inhibition of ethylene synthesis or ethylene action by a variety of ethylene antagonists will significantly suppress this effect. For most of these, there seems to be a concentration maximum threshold above which inhibition becomes impaired. Application of exogenous ethylene causes vascular cavities to form in roots that otherwise would
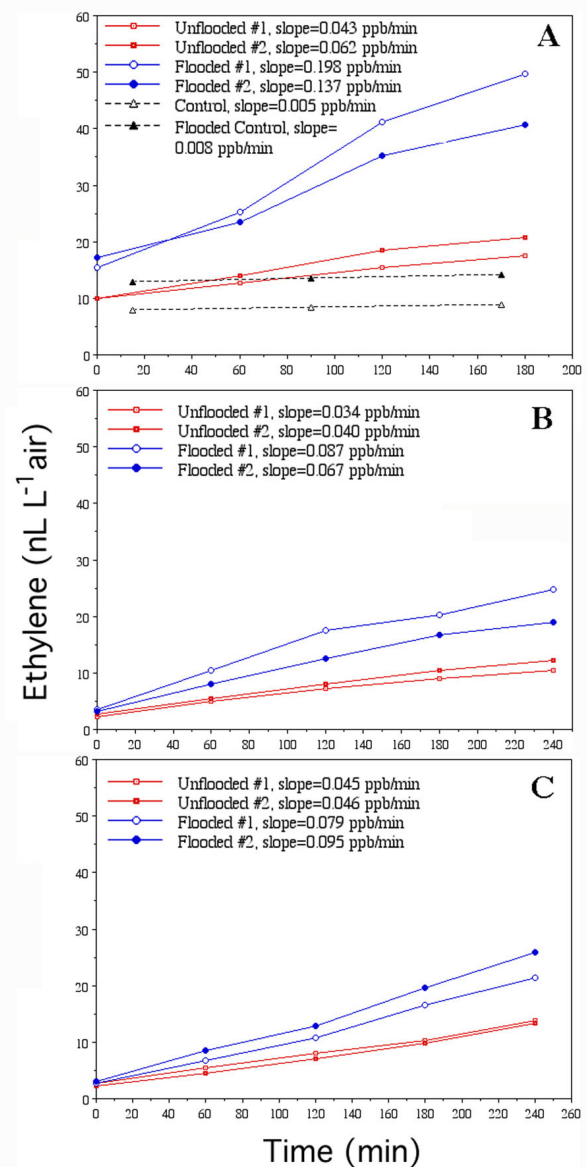

Fig. 6. Production of endogenous ethylene following flooding. 4 d-old seedlings growing in jars under moisture conditions that minimize the frequency of vascular cavity formation at 25°C were flooded or not, the jars sealed, and headspace air samples were periodically collected. A. Run #1, the first three hours following flooding. B. Run #2 followed run #1 after 1 h flushing the jars with air. C. Run #3, 18 h after flooding.

not have them. The results also show that young 'Alaska' pea seedlings increase their ethylene production when flooded. These results are consistent with the hypothesis that ethylene is an essential component in the signal transduction pathway that connects flooding to the formation of vascular cavities in pea primary roots.

It has previously been shown that ethylene is required for the formation of cortical aerenchyma in maize (He et al. 1996, Saab and Sachs 1996, Drew et

al. 2000, Gunawardena et al. 2001) and several other species (Jackson 1985, Satler and Kende 1985, Huang et al. 1997). Hypoxia is known to increase ethylene production (Atwell et al 1988), and it is well known that flooding induces hypoxia (Ponnamperuma 1984, Drew 1997, Colmer 2003) with consequent aerenchyma induction in some non-wetland plants (Drew 1977; Drew et al. 1979; Drew et al. 1985; Jackson 1985, Drew et al. 1994; Colmer 2003; Visser and Voesenek 2004). Considering that when vascular cavities form to their full extent in pea primary roots exposed to flooded conditions such roots are able to continue to grow, but that they cannot continue to grow if the cavity does not extend to the maximum extent (Niki and Gladish 2001), the above results are further evidence, albeit circumstantial, that vascular cavities in pea primary roots constitute a form of inducible aerenchyma, as proposed by Gladish and Niki (2000). But this response is apparently only effective as aerenchyma for 4 d-old or younger seedlings (Niki and Gladish 2001) or perhaps for situations when inundation does not immerse the entire root.

We think it is noteworthy that the increase in cortical cell size that occurred when roots were exposed to exogenous ethylene was accompanied by an apparent increase in intercellular space. Although this was a qualitative judgement on our part based on observations at the light microscope level, Justin and Armstrong (1987) described *Pisum sativum* as having dense, hexagonal cortical cell packing with consequent very low porosity (0.6%), but they reported a six-fold porosity increase with flooding. This may well have been due to the presence of increased endogenous ethylene caused by the flooding, such as we report above, leading to increased intercellular space. It is unlikely that the increased porosity they reported was due to vascular cavities, as these have rarely been observed at 15°C (the temperature they used) and are only found in primary roots of *Pisum sativum* and other cool-season legumes (Lu et al. 1991, Rost et al. 1991, and unpublished data), which they did not evaluate in their study. We speculate that the increased size of intercellular space we observed was simply a function of increased cell size. This idea is consistent with the conclusions of Justin and Armstrong (1987) regarding the relationship between cell packing and porosity, but contradicts their suggestion that hexagonally packed roots are physiologically immune to ethylene, though this too may be temperature-sensitive. On the other hand, the use of lysigeny in the vascular cylinders of the primary roots of *Pisum sativum*, rather than the cortex, is consistent with their observation that plants with hexagonal packing of root cortical cells rarely make cortical aerenchyma.

Studies have shown that cells that collapse to form induced aerenchyma in response to flooding, hypoxia, or ethylene exposure have characteristics associated with programmed cell death (He at al. 1996, Drew 1997, Drew et al. 2000, Schussler and Longstreth 2000, Gunawardena et al. 2001, Evans 2003). In fact, the role of ethylene in programmed cell death in plants appears to be rather broad. Recently, ethylene has been reported as being an important part of the programmed cell death signal transduction pathway for many processes, for example, endosperm development (Young et al. 1997, Young and Gallie 1999), fruit ripening (Adams-Phillips et al. 2004), leaf senescence (Jing et al. 2002, Lim et al. 2007), flower petal senescence (Yamada et al. 2007), metal toxicity response (Yakimova et al. 2007), hybrid lethality syndrome (Masuda et al 2007), and the hypersensitive response to pathogens (Pennell and Lamb 1997, Kunkel and Brooks 2002, Bouchez et al. 2007, Zhang et al. 2007). Based on the present study, previous studies (Niki and Gladish, 2001) and preliminary data (unpublished), we propose that vascular cavities are another, and somewhat unusual, type of aerenchyma and an example of programmed cell death in a relatively under-studied group of plants. As far as we know vascular cavities have only been observed in the primary roots of cool-season or cold-tolerant, herbaceous legumes, namely *Cicer arietinum*, *Lens culinaris*, *Lupinus latifolius*, *Pisum sativum*, *Vicia fava* (Rost et al. 1991), *Phaseolus coccineus*, and a few varieties of *Glycine max* (unpublished results). Valuable information may be obtained about these important, adaptive processes by further study of these species.

In conclusion, the evidence shows that ethylene biosynthesis is a key element in the signal transduction pathway between flooding and the process of vascular cavity formation and that it is involved in other anatomical responses that enhance survival in hypoxic conditions, such as flooding.

Acknowledgements

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Dr. Daniel K. Gladish has studied the effects of temperature and hypoxia on root development, and he is currently studying the role of programmed cell death in root development.

Dr. Teruo Niki is interested in morpho-logical and physiological changes of plant roots resulting from low oxygen conditions.