Comparison of the effects of flooding vs. low-oxygen gas on pea (*Pisum sativum* L. cv. ‘Alaska’) primary roots

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Abstract: Flooding reduces soil oxygen necessary for root growth. In some mesophytes low levels of oxygen are mitigated by the formation of aerenchyma or expansion of intercellular spaces. But root immersion in water may have effects on roots in addition to reducing oxygen levels. At temperatures >15°C *Pisum sativum* primary roots develop cavities in the centers of their vascular cylinders in response to saturated or flooded conditions. In the present study we compared the response of flooded pea roots to their response to hypoxia without flooding by using an innovative system that allows separation of the gas environment of a root system from that of its shoot system. Seedlings were flooded after 4 d growth and compared to seedlings in unflooded medium and to seedlings in the gas-manipulation experiment. At 25°C in slightly moist vermiculite, roots 4 d after planting were exposed to a gas mixture with 10.5% oxygen and shoots to 20.5% oxygen and compared to normoxic controls. Oxygen levels in all containers were monitored, root growth was measured, and frequency and size of vascular cavities were determined for all treatments. Under flooding and low-oxygen gas, root growth was suppressed and vascular cavity frequency was strongly enhanced compared to controls. Significant differences in root growth responses were not seen between these differing hypoxic conditions, but low-oxygen gas caused larger cavities than flooding, which suggests flooding with water may have subtle effects different than simple hypoxia.

Keywords: aerenchyma, flooding, hypoxia, pea primary root, *Pisum sativum* L., vascular cavity

Introduction

The root system of a typical mesophytic plant normally relies mainly on the root-growing medium to convey the oxygen required for root growth and mineral absorption. This is because the tissues of such plants are often incapable of providing an internal path with rates of diffusion sufficient for the demand. The diffusion of oxygen is four orders of magnitude slower in water than in air (Ponnamperuma 1984, Kalita 1999, Drew et al. 2000). Furthermore, oxygen is less available in wet soil than in dry soil because water replaces air in some of the space among soil particles. Low oxygen availability (hypoxia) due to flooding can significantly reduce root growth and productivity of many plants.

On the other hand, some mesophytes have evolved variations in development and anatomy that do provide a path of enhanced diffusion through their organs, such as extensive intercellular spaces and aerenchyma (Armstrong 1970, Drew 1977, Drew et al. 1985, Justin and Armstrong 1987, Drew et al. 2000, Evans 2003). The ability of a plant to facultatively produce aerenchyma in response to flooding has been most thoroughly demonstrated and analyzed in *Zea mays*, and the longitudinal channels thus produced convey enough oxygen through the roots to enable them to maintain basic functions and even to continue growing (Drew 1977; Drew et al. 1979; Drew et al. 1985; Atwell et al. 1988; Gunawardena et al. 2001).

Lu et al. (1991) and Niki et al. (1995) reported that long, continuous cavities frequently develop in the centers of vascular cylinders of pea (*Pisum sativum*) primary roots grown in warm (>15°C), moist condi-
Cultivation methods were modified after Gladish and Niki (2000). For flooding treatments, tall, heat- and pressure-resistant polypropylene 1 L beakers (8.8 cm diameter at top, 21 cm height) were filled with coarse (2.5-7 mm particle diameter) vermiculite (GL grade, Nittai Corp., Ibaraki, Japan). The vermiculite was then moistened with 375 ml of distilled water (DW). The beaker tops were covered with aluminum foil and sterilized by autoclave at 120°C for 70 min. After autoclaving no free-standing water remained in the beakers. Vermiculite treated in this way poured freely from the beaker as though dry, so it will hereafter be referred to as “slightly moist vermiculite” to contrast it with flooded vermiculite. Under sterile conditions vermiculite was removed down to 7 cm from the top of the beaker to allow a small headspace and medium to cover the seeds. Pea seeds (*Pisum sativum* L. cv. Alaska) that had been surface-sterilized for 5 min in a solution of 1% sodium hypochlorite plus six drops of Tween 20 surfactant L⁻¹ were placed on the vermiculite surface (n = 22-25 per beaker). The seeds were then covered with 2 cm of the removed vermiculite, and the beakers were re-covered loosely with the original sterile foil and placed in a growth chamber at constant 25°C in continuous darkness. Four and 5 d after planting seven beakers each were sampled to determine primary root length and presence of vascular cavities to serve as pre- and post flooding controls (4 d and 5 d controls, respectively). After 4 d seven other beakers were flooded with DW to just below the planting level and sampled 1 d later to serve as the flooding treatment (4 d + 1 d flooding treatment).

**Growth tubes for controlled gas environment experiments**

Cultivation methods for seedlings in growth tubes were as described above for flooding treatments, except that the modified beakers (growth tubes) were used (Fig. 1). Before planting seeds, a petroleum jelly/paraffin impregnated tissue partition disk or an unmodified acryl ring (control) was placed on the vermiculite surface after vermiculite was removed down to 7 cm from the top of the beaker. Petroleum jelly/paraffin-impregnated tissue paper was used for a partition to divide the upper and lower parts of a growth tube (Fig. 1-2). Petroleum jelly and paraffin (both Wako Pure Chemical, Osaka, Japan) in a 5:1 ratio were melted at 52-54°C and mixed well. Kimwipe tissues (Kimberly-Clark, Roswell GA, USA) trimmed into appropriately-sized discs were immersed in the melted petroleum jelly/paraffin solution, and then allowed to cool on a flat screen. These impregnated tissue paper disks were attached onto an acryl ring with sealing tape (Fig. 2). The sealing tape also served to produce a tight fit when the

**Materials and Methods**

**Cultivation of plants for flooding treatment**

Cultivation methods were modified after Gladish and

Natural soils usually have very complex and sometimes heterogenous composition and structure (Ponnamperuma 1984; Kalita 1999). In *vivo* in the field when plant root systems become flooded they experience more than a simple reduction in oxygen availability. The choice of using solution culture to study the effects of hypoxia-induced aerenchyma is certainly understandable, given that waterlogging is the most common cause of hypoxia in a rhizosphere. The method allows investigators to minimize variables. It is not known, however, to what degree physiological and morphological changes caused by flooding are affected by the increase in water in the soil (or other medium) rather than the decrease in oxygen availability.

It is our opinion that the current understanding of these changes under hypoxia mentioned above has been limited by the inability to separate the effects of oxygen deprivation *per se* from possible effects associated with changes in the water amount and other factors that this causes in the rhizosphere, such as pH changes (Ponnamperuma 1984) or endogenous ethylene entrapment as a result of immersion (Drew et al. 1979). In this paper we present a technique that is promising for making changes in oxygen availability or for measuring changes of oxygen concentration in the rhizosphere without changing the water potential of the medium. This system can separate the aerial and subterranean environments around individual plants. We expected this method to permit the evaluation of the effects of low oxygen availability on primary root growth and vascular cavity formation in pea. We believed that we could create an oxygen environment for root and shoot systems more like that experienced in nature during flooding and mimic the hypoxia induced by flooding without actual immersion in water.
partition disk was placed into the growth tube. Surface-sterilized pea seeds (cv. Alaska; n = 22-25 per beaker) were laid on the partition disk, or directly on the vermiculite surface (control, acryl ring with no partition) and covered with 2 cm of the removed vermiculite as above. The foil cover was then loosely replaced on the growth tube, all the gas orifices were uncovered to allow gas exchange. The growth tube was then placed in a growth chamber in continuous darkness at 25°C. The loose-fitting foil cover and uncovered orifices allowed air exchange but maintained sterile conditions for the first 3 d, during which time seeds imbibed water and germinated.

Apparatus for delivery and monitoring of air and low-oxygen gas mixes

Three d after sowing, the foil was then replaced with a plastic screw cap, and the growth tube was connected to the gas supply and DO sensor system while inside a growth chamber. Compressed normoxic air or a low-oxygen gas mixture was bubbled through water in “flow indicators” and delivered to growth tubes at regulated flow rates (100 ml min⁻¹) at 101 kPa (Fig. 3). Low oxygen gas mixtures were generated by mixing air and nitrogen gas supplied by compressed gas tanks (Fig.3). Oxygen concentration was detected and monitored by using Clark-type dissolved oxygen (DO) sensors (Horiba model OM-14, Kyoto, Japan) and the sensors were calibrated with flowing normoxic air (100 ml min⁻¹) just before experiments began. The analog signals from the DO sensors were converted to a digital signal by using a digital multimeter (Keithley Instruments model DMM 2000-SCAN, Cleveland OH, USA) monitored automatically by a personal computer. Sensor units were constructed for precisely measuring temperature and fine changes of oxygen concentration in gases in the system (Fig. 1B). The DO sensors were inserted into custom-manufactured aluminum cylinders (fabricated at Takushoku University College of Engineering, Hachioji, Japan) with joining holes to accept vinyl tubing from the growth tube or reference gas sources. These cylinders were fitted with flow-limiting “release” holes to allow regulated exit of gases while maintaining a small back-pressure in the system. The outflow gas from the orifices of the upper and lower parts of the growth tube was connected to sensor units via vinyl tubing. Normoxic air from a compressed-air tank was supplied to the upper and lower inflow orifices (Fig. 1) for the first 1 d. To ensure system stability, gases were run through the gas delivery system bypassing the growth tube for the first 0.5 h of a trial before gases were introduced to a growth tube. Percent oxygen was quantified as described above at least five times for each partition possibility trial (no petroleum jelly/paraffin impregnated tissue disc partition, no...
developing seedlings; tissue partition present, no seedlings; tissue partition present, seedlings present). A trial was conducted with 12 seeds rather than 25 to test the effect of root penetration of the barrier on its separation abilities.

After 3 d in the growth chamber and 1 d of normoxic air exposure (top and bottom), some growth tubes (n = 7 tubes) were sampled for root length measurement and the presence of vascular cavities (4 d controls). For other growth tubes (n = 7 tubes) the lower, root-bearing zones were then exposed to a low oxygen air/nitrogen mixture (10% oxygen, 10.1 kPa partial pressure) while the upper inflow orifices continued to receive normoxic air for 1 d (3 d + 1 d air + 1 d gas treatment). Some growth tubes (n = 7 tubes) were continuously provided normoxic air via the upper and lower inflow orifices for 1 d (5 d controls). At the end of the gas flow treatment the primary roots were sampled for root length measurement and the presence of VC.

Evaluation of root growth and cavity formation after flooding or low oxygen gas

After treatments, primary root lengths were measured and freehand sections were taken variously 1, 2, 3 and/or 5 cm from the root tip of primary roots from each seedling to determine if vascular cavities were present. A beaker or growth tube was also prepared for each treatment category for the purpose of measuring VC cross-sectional area by image analysis using Photoshop Extended CS5-JPL (Adobe Systems, Singapore). 15-16 roots were randomly selected from each beaker and free-hand sectioned at the same locations as above. These sections were stained with 0.025% toluidine blue O. The first ten from each growth tube or beaker with a VC were digitally photographed and analyzed. If there were less than ten but more than two with VC in the sample group, they were all analyzed.

Results

Oxygen levels in beakers of slightly moist vermiculite loosely covered with aluminum foil were 8.4-8.9 mg L⁻¹ (20.9% V/V) in gas phase with or without young seedlings. When such beakers were flooded with water, the oxygen levels very rapidly dropped to about 3.5 mg L⁻¹ in liquid phase. In flooded beakers with developing root systems the oxygen levels subsequently slowly decreased to about 2.0 mg L⁻¹ within 18 h (Fig.4).

The gas delivery apparatus (Fig. 3) successfully maintained constant flows of gases through the upper and lower parts of the growth tubes at the desired oxygen levels for the duration of all trials except when a partition was not present in the growth tube. Low-oxygen level in the lower (root-zone) compart-
ment of the growth tube was readily and dependably controlled by initially adjusting the flow ratio of air and nitrogen gas (Fig. 5). With no partition tissue (acryl ring only), air introduced into the upper part of the growth tube and a low-oxygen gas mixture introduced into the lower part of a growth tube predictably became mixed (Fig. 5A). When the upper and lower parts were partitioned by petroleum jelly/paraffin-impregnated tissue paper partitions, oxygen concentrations of outflow gases from the upper and lower parts typically were shifted by less than 0.5% (Fig. 5B). When roots perforated a partition (Fig. 2), outflow gases from the upper and lower parts were not shifted by more than 1.5% oxygen, the lower compartment being more strongly affected than the upper (Fig. 5C). On the other hand, this shows that, while separation was otherwise good, perforation of the tissue barrier by roots did slightly increase transfer of oxygen from the upper to the lower part of a growth tube. Reducing the number of seeds by half did not significantly alter this result (data not shown).

Root growth (length) for controls at 4 d was slightly less in growth tubes with partitions compared to unmodified beakers and at 5 d it was slightly more, but the differences were not significant ($t$-test, $p \leq 0.1$; Fig. 6). Differences in mean growth after 5 d between flooding-treated and low oxygen gas-treated primary roots were not statistically significant ($t$-test, $p \leq 0.1$; Fig. 6). Exposing roots to flooding in beakers or to half of ambient oxygen level in a growth tube with a partition resulted in significant reductions in growth rates compared to controls for each treatment group ($t$-test, $p \leq 0.01$; Fig. 6).

Roots exposed to normal air in a growth tube grew the same amount in 5 d as roots in unmodified,
unflooded beakers, but roots in normal air in a growth tube had significantly more frequent VC formation (Fig. 7). These VC also tended to be away from the tip. Primary roots grown in a beaker under control conditions either had no VC or a narrow one 3 cm or more from the tip (Fig. 7; Table 1). Primary roots grown in a growth tube with a partition with normoxic air-flow during the fifth day either had no VC or a moderately wide one.

VC typically were most common 1-2 cm from root tips under flooding or low oxygen air (Fig. 7). The frequency of VC 1 cm from the tip was very much higher than 4d or 5d controls regardless of the cause of hypoxia ($t$-test $p \leq 0.01$; Fig. 7) Flooding and 10% oxygen gas mixture were highly, and equally, effective at inducing vascular cavities near the tip (1 cm from tip), but low-oxygen air was significantly more effective at triggering VC formation in older primary vascular tissues of the roots (2-3 cm from the tip) than flooding ($t$-test, $p \leq 0.05$, Fig. 7). Hypoxic conditions, such as during flooding or exposure to low-oxygen gas, induced greater frequency of cavity formation and usually larger cavities in comparison to those few that formed in the controls (Fig. 7, Table 1).

Discussion

The results show that the apparatus used in this study for creating a hypoxic rhizosphere in the absence of waterlogging was stable and reliable (Fig. 5), and in several ways produced comparable results with respect to growth and the formation of vascular cavities (VC) as flooding (Figs. 6-7). The study by
Gladish and Niki (2000), which also used pea (Pisum sativum) primary roots and involved manipulation of the rhizosphere oxygen levels, had as a shortcoming that the shoot systems were exposed to the same atmosphere as the root systems. Since developing cotyledons and epicotyls in darkness are doubtless stressed by hypoxia too, and because it is not known how their response to hypoxia may affect a developing root system, new methods were required to address this problem. Furthermore, the results showed that a partition between the upper and lower parts of a growth tube (for the shoot system and root system respectively) was necessary if management of desired differences in oxygen levels were to be accomplished (Fig. 5A-B). The results also showed that penetration of the barrier by roots allowed only a small increase in the mixing of gases between the two compartments (Fig. 5C).

Gladish and Niki (2000) reported that pea roots in well-aerated, circulating water always make cavities unless the temperature is cool (10°C), and pea roots in warmer (25°C) but slightly moist vermiculite make cavities much less frequently (ca. 25% of the population. The difference in absolute water potential between our “slightly moist” vermiculite, which suppresses the frequency of VC formation, and vermiculite saturated to “field capacity”, which favors frequent VC formation (Lu et al., 1991; Gladish and Niki 2000) is slight (Tadashi Hirasawa, pers. comm.). Nevertheless, the water amount in the medium, independent of oxygen level, does seem to have a positive influence on VC formation (Gladish and Niki 2000). Consistent with this observation, Ober and Sharp (1996) found by direct measurement using an oxygen microelectrode that intracellular oxygen was less in the centers of maize roots growing in an aerated solution culture than roots growing in wet vermiculite. Since, from the outer limit of the water boundary layer on the epidermis inward, the roots in their experiment were the same (i.e., all cells were immersed in an aqueous continuum, as is typical in a plant tissue), this suggests that what matters for root internal oxygen status in the absence of pre-existing aerenchyma is the nature of the oxygen-bearing medium beyond the boundary layer on the root. Such evidence causes us to argue that factors enhancing the formation of VC in roots are more complex than relative oxygen availability alone can explain.

In the present study, root growth repression due to exposure to low-oxygen gas (approx. 10-11% oxygen) was similar to that caused by flooding. Over time, though, the respiratory demand of the roots further reduced oxygen availability under flooding (Fig. 4). Given that measured oxygen levels in flooded beakers were eventually less than one-fourth the levels in unflooded ones, but inhibition of growth was not so different than exposing them to low-oxygen air, this shows that VC may serve as aerenchyma that compensates during flooding. On the other hand, because the oxygen availability declined with time under flooding but did not under low-oxygen gas, one would predict that the frequency of VC formation would be greater under flooding than in low-oxygen gas. But this was not the case (Fig. 7). This result suggests that water-induced hypoxia has a somewhat different influence (or magnitude) than low-oxygen gas in VC formation. It is noteworthy that there was a difference in cavity frequency between the 5 d beaker controls and the 5 d growth tube controls. We think this was probably not because of a relative humidity difference in the rhizosphere air spaces because gases were always bubbled through water before being delivered to the growth tube, but a physiological effect from the flowing of the gases through the rhizosphere, by comparison to still air in the standard beakers, cannot be ruled out.

When pea seeds were germinated and grown in control conditions, cavity formation did occasionally occur as reported previously (Gladish and Niki, 2000; Niki and Gladish, 2001). But these cavities were

**Table 1** Cross-sectional areas (µm²) of pea root vascular cavities due to exposure of roots to flooding with water, exposure to low-oxygen concentration gas, and controls.

<table>
<thead>
<tr>
<th>Distance from root tip</th>
<th>5 d unflooded control</th>
<th>4 d+1 d flooded</th>
<th>4 d +1 d air control</th>
<th>4 d +1 d 10% O₂ gas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1cm</td>
<td>no cavities</td>
<td>6981±3372a</td>
<td>(2 or fewer)</td>
<td>11,523±2789a</td>
</tr>
<tr>
<td>2cm</td>
<td>no cavities</td>
<td>7506±3742b</td>
<td>no cavities</td>
<td>10,652±4276b</td>
</tr>
<tr>
<td>3cm</td>
<td>(2 or fewer)</td>
<td>7618±4740c</td>
<td>5765±2311 (3)</td>
<td>4972±3332c</td>
</tr>
<tr>
<td>5cm</td>
<td>2272±1100d (5)</td>
<td>(2 or fewer)</td>
<td>5883±3148d (6)</td>
<td>(2 or fewer)</td>
</tr>
</tbody>
</table>

15-16 normal-looking roots were sampled at random from 23-24 roots per grown per beaker or growth tube. Ten roots with VC were selected at random from among those for image analysis, unless there were fewer than ten with VC, indicated by (n). 

n < 3 was considered too few to provide a reliable value. Shown are means ± SD; units are square micrometers. Pairs indicated by a, b, c, d were significantly different from each other (p ≤ 0.10).
relatively small. In contrast, in hypoxic conditions, such as during flooding or exposure to low-oxygen gas, cavity formation frequency was much greater (Fig. 7) and the size of cavities was usually larger in comparison to those few that formed in the controls (Table 1).

Although the present study did produce some data that suggest that water saturation is a separate factor from hypoxia in the induction of vascular cavities, these results were somewhat equivocal. Low-oxygen air alone caused more frequent cavity formation in the more mature primary tissues than hypoxia caused by flooding (Fig. 7). Since one would predict the reverse to be the case if there were to be differences at all, this surprising result begs further inquiry. The root tip plays an essential role in root growth and requires more oxygen than other tissues of a root (Drew et al. 1994). In the present study the frequency of VC was greater near the tip when roots were suddenly flooded or exposed to low-oxygen gas, and when they occurred in older 5 d controls they were more common farther back (Fig. 7). We speculate that in 5 d controls cavities may have formed near the tip as with chronic VC at an earlier root developmental stage, perhaps beginning around 4 d (Fig. 7), but they did not always continue to develop over time, as previously reported (Lu et al., 1991).

In other respects the growth tube gas system produced results nearly identical to flooding intact root systems. We look forward to using this experimental system to further explore these questions and to evaluate other species that are known to respond to hypoxia somewhat differently than pea, e.g. maize and rice.

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References


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

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. Mitsuo Takahashi is an oxygen chemist interested in morphological and physiological changes in plant roots resulting from hypoxia.