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Nodule structure and nitrogenase activity of *Coriaria arborea* in response to varying pO_2

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Abstract

When excised root nodules of *Coriaria arborea* are assayed for nitrogenase activity at various pO_2 they show a broad optimum between 20 and 40 kPa O_2, with some evidence for adaptation. Continuous flow assays of nodulated root systems of intact plants indicate that Coriaria shows an acetylene induced decline in nitrogenase activity. When root systems were subject to step changes in pO_2 nitrogenase activity responded with a steep decline followed by a slower rise in activity both at lower and higher than ambient pO_2. Thus Coriaria nodules are able to adapt rapidly to oxygen levels well above and well below ambient. Measurement of nodule diffusion resistance showed that the adaptation is accompanied by rapid increase in resistance at above ambient pO_2 and decrease in resistance at below ambient pO_2. Plants grown with root systems at pO_2 from 5–40 kPa O_2 did not differ in growth or nodulation. The anatomy of Coriaria nodules shows they have a dense periderm which encircles the nodule and also closely invests the infected zone. The periderm is both thicker and more heavily suberised in nodules grown at high pO_2 than at low pO_2. Vacuum infiltration of India ink indicates that oxygen diffusion is entirely through the lenticel and via a small gap adjacent to the stele.

Introduction

Coriaria is a genus of actinorhizal plants found in disjunct distribution from Spain and Japan to New Zealand (Silvester, 1977). The plants are profusely nodulated and the distribution of *Frankia* in infected cells is somewhat unusual amongst actinorhizal plants. Firstly every cell within the infected zone is fully invaded by the bacterium and secondly the bacterial filamants line the periphery of the cell and send radiating hyphae towards the centre of each cell. The radiating hyphae have a terminal non-septate club-shaped structure called a vesicle (Newcomb and Pankhurst, 1982). This morphology is remarkably similar to nodules of the unrelated genus Datisca (Calvert *et al.*, 1979) but is quite different from nodules of other actinorhizal species which tend to have many uninfected cells in the bacterial zone, a centrifugal arrangement of filaments and in many cases the vesicles are spherical and septate.

In general it has been shown that actinorhizal nodules show optimum nitrogenase activity at atmospheric pO_2 when they are grown in air and are oxygen limited at below ambient and O_2 inhibited at above ambient pO_2 (Tjepkema, 1979). This is in strong contrast to most legume nodules in which nitrogenase appears to be oxygen limited over a wide range of pO_2 up to 80 kPa (Sheehy *et al.*, 1983).

The relationship between structure and nitrogenase activity at varying pO_2 levels has been studied for Myrica (Silvester *et al.*, 1988a) and Alnus (Silvester *et al.*, 1988b). In both cases the plants were shown to adapt both structure and optimum nitrogenase activity to growth pO_2 levels over a range from 5 to 40 kPa. Nodule structure in Myrica changed markedly with a large proliferation of nodule roots at low pO_2. In Alnus the major change in response to changed pO_2 was not in nodule morphology but rather in the morphology of the *Frankia* vesicles which appeared

much thicker walled at high pO_2. This change in vesicle structure has also been noted in *Frankia* grown in pure culture (Parsons *et al.*, 1987).

The current model of oxygen response in legume nodules predicts that a barrier to gas diffusion in the cortex strongly limits oxygen flow to the infected cells. Changes in ambient pO_2 have been shown to result in rapid changes in the diffusion resistance of this barrier thus controlling the rate of activity (Weisz and Sinclair, 1987b, Hunt *et al.*, 1987).

The situation in actinorhizal nodules is much less clear but the general understanding is that these nodules are much better ventilated than legume nodules. A combination of microelectrode oxygen analysis (Tjepkema, 1979) India ink infiltration (Tjepkema, 1983) and gas exchange analysis (Winship and Tjepkema, 1985, Silvester *et al.*, 1988a; 1988b) indicates that those actinorhizal nodules that have been studied are relatively well aerated and that the mechanism of oxygen regulation in these nodules may be fundamentally different to the legume nodules. As yet no general model of O_2 response in actinorhizal nodules has been proposed and this study was initiated to compare O_2 response in a nodule with a structure that differs quite markedly from the other plants that have been investigated.

Materials and methods

Seeds of *Coriaria arborea* Lindsay were collected from roadside plants in *Pinus radiata* forest south of Tokoroa, North Island, New Zealand and germinated in soil collected from beneath mature Coriaria plants. Nodulated plants werre transplanted from this soil after two to three months into aerated hydroponics solution and grown on quarter strength nitrogen-free solution (Smith *et al.*, 1983). When plants became established the liquid level was lowered and plants then maintained with only the lower roots in solution but the nodules and upper roots in a mist or aeroponic environment created by the breaking bubbles of the aerator. Plants were maintained in the greenhouse and transferred to the laboratory 5–10 days prior to experiments. Laboratory plants were kept in a simple growth chamber where they were illuminated with a high pressure mercury vapour lamp plus ambient daylight at photon flux net less than $200\,\mu E\ m^{-2}\ s^{-1}$ for 16 hours per day. The chamber maintained temperatures several degrees above laboratory temperatures in the range 22–28°C but was not otherwise controlled.

Plants were grown with root systems at various pO_2 by recycling gas mixtures through root chambers from large PVC gas bags (Silvester *et al.*, 1988a).

Assay procedures

Plants were assayed for nitrogenase activity using continuous flow acetylene reduction techniques. The basic methodology has previously been described (Silvester *et al.*, 1988a) but the present work introduced a number of refinements. Gas mixtures were made up in 20l plastic beach balloons using mass-flow meters and checked by gas chromatography. The mixtures were set up on a low volume manifold close to a two channel peristaltic pump which metered the gas through root cuvettes consisting of 60 ml plastic syringes. The cuvettes were maintained at 23°C in a water bath and plant illuminated with a mercury vapour lamp identical to the one used in the laboratory growth chamber.

Gas was normally pumped at 40 ml min^{-1} through a 20 ml cuvette giving a flushing rate of 30 s. Influent gas containing various oxygen levels plus 10 kPa C_2H_2 passed into the cuvette and the effluent gas was led via small bore tubing to an automatic switching valve that introduced 0.2 ml of gas into the gas chromatograph (Shimadzu GC-8A) every 60 s. The results were automatically analysed by an integrator (Shimadzu CR-3A) giving a read-out of ethylene concentration each 60 s.

Diffusion resistance was measured using the lag-phase method of Weisz and Sinclair (1988). Plants were exposed to acetylene and gas samples taken at 5 s intervals for 60–90 s. During this time C_2H_2 diffuses in and the product C_2H_4 diffuses out. The time taken to reach equilibrium C_2H_4 concentration is termed the lag-time and is a measure of the diffusivity of the system. It is essential for this technique that the physical lag-time of the system does not significantly interact with the lag-time of the nodules and for our experiments a flow rate of 150 ml min^{-1} and a cuvette volume of 10 ml were

used giving a cuvette flushing time of 4 s. To obtain samples at 5 s intervals, twelve or more syringes were inserted into the rubber exit tube of the cuvette, filled at the requisite times and stored by inserting into a rubber bung until analysed.

Nodule anatomy

Nodules for embedding and sectioning were harvested from aeroponically grown plants and immediately placed in 0.1 *M* PIPES buffer (pH 7.2) and the periderm cut from one side to facilitate infiltration. Nodule pieces were then fixed in 2.5% buffered gluteraldehyde for 1½ hours, rinsed for 30 min in buffer and post-fixed in 1% osmium tetroxide.

Serial dehydration of the tissue in acetone was completed over three hours and the acetone gradually replaced by Spurrs resin over the next 18 hours. Sections were cut at 0.75 μm using an LKB ultramicrotome and glass knives, stained with 0.05% toluidine blue in 1% sodium tetraborate and mounted in Depex.

Nodules for hand sectioning and staining were harvested from plants growing at various pO_2, sectioned and stained with Sudan Black B (0.3 g in 100 ml 70% ethanol) for 20 minutes followed by a rapid wash in 50% ethanol and mounted in glycerol.

The presence of suberised walls was confirmed by digestion of hand-cut sections in 50% chromic acid. This treatment effectively digests all cellulosic material leaving the suberised/cutinised walls intact (Johansen, 1940).

The pathway of gas diffusion was investigated using vacuum infiltration of India ink (Tjepkema, 1983). The ink, with 1.25% Triton ×-100 surfactant, was purified by centrifugation and filtration. Entire nodule clusters were evacuated to 760 mm, 380 mm and 190 mm Hg for five minutes and allowed to return to atmospheric pressure under the ink. Nodule lobes were then blotted dry and hand sectioned. Whole nodules were prepared for scanning electron microscope examination by critical point drying and coating with gold/palladium.

Results

Nitrogenase assay of excised Coriaria nodules was conducted in the traditional batch technique. Various pO_2 levels were flushed through the assay bottles for 3 minutes then C_2H_2 was added and acetylene reduction assayed at 5, 15, 30, 60 and 90 minutes after C_2H_2 addition. It was apparent that activity was changing quite dramatically during the assay. The results for rates over the periods five to 15 minutes and 60 to 90 minutes are shown in Figure 1. Under these conditions nitrogenase shows a broad optimum activity at between 20 and 40 kPa O_2. There is evidence that activity increases over the assay time at higher pO_2 levels and generally decreases at lower pO_2 levels. This form of assay in which nodules are excised from the roots and subject to the changing conditions inside small analysis bottles is not now recommended. All further work on nitrogenase was conducted using the flow-through system and it was established early that Coriaria nodules show an acetylene induced decline (Fig. 2). This shows a drop to about 50% of maximum rate followed by a small but steady decline which was not affected by switching the light off after 160 minutes.

The acetylene decline phenomenon was studied further by cycling the plant with air for varying lengths of time to determine whether nitrogenase recovers from the acetylene effect and what is the time course of that recovery. In this case a control plant, receiving acetylene in air continuously, was run in parallel with the experimental plant. The results (Fig. 3) show the experimental plant suffered a very significant decline that was not recovered during a 10 minute exposure to air, partially recovered in a 30 minute air period and showed near full recovery after a 60 minute exposure. It is significant that the plateau rates for the experimental plant were nearly constant at between five and six μmoles $C_2H_4\ g^{-1}\ min^{-1}$ except for the very last reading after five hours, while the control plant, continuously exposed to C_2H_2 showed a steady decline in rate.

Continuous flow techniques were used to check the effect of step changes in pO_2 on nitrogenase. In all cases it became evident that Coriaria is able to sustain a relatively constant rate of nitrogenase over a wide range of pO_2. One of the problems associated with this type of experimentation is the constant run-down in activity of plants when exposed to C_2H_2. This problem is overcome to a certain extent by running parallel plants as shown in the previous experiment. An example of the type of result is illustrated in Figure 4. Regardless of

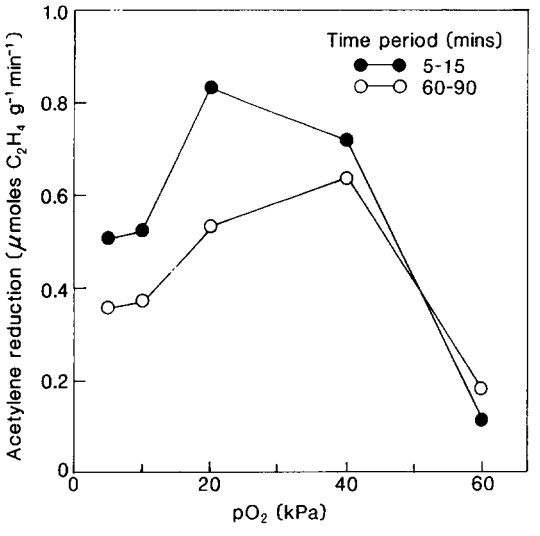


Fig. 1. Nitrogenase activity of excised *C. arborea* nodules at various pO_2. Nodules were excised from several plants, randomised and flushed with the different pO_2 gas mixtures prior to addition of C_2H_2. Nitrogenase was assayed at various times and the rates recorded for two time intervals 5–15 and 60–90 minutes after C_2H_2 addition are shown.

whether the oxygen level is raised or lowered, nitrogenase shows a very steep reduction followed by a slower recovery. In this case the changes running from 10 to 60 kPa O_2 indicated substantial recovery in most cases, with final activity showing little difference across the range of pO_2.

The ratio of nitrogenase activity for treated/control plants shows the extent to which the oxygen change has affected nitrogenase activity. The initial ratio of 1.57 being that prior to any exposure to oxygen is then followed by a range of values all of which are substantially higher than 1.57 (Fig. 4). The results show that Coriaria nodules recover quite rapidly from the effects of decreased or increased O_2 and that if they are exposed to relatively small increments in oxygen they recover and maintain a similar nitrogenase activity. There is some evidence that activity may even be enhanced at higher pO_2 as long as this is not given too rapidly. This result illustrates the very different response to oxygen that can be obtained by using continuous flow techniques (cf. Fig. 1).

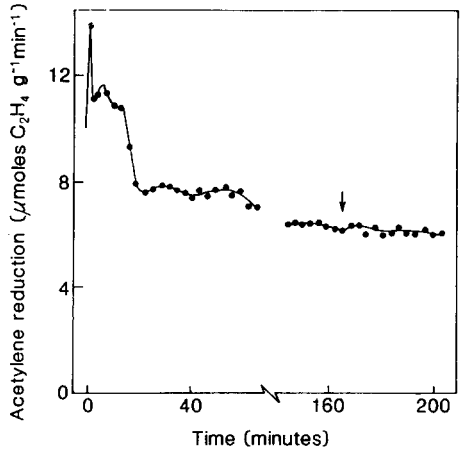


Fig. 2. Nitrogenase activity of the root system of an intact plant. Acetylene added at time zero and nitrogenase activity recorded at one minute intervals. Every third record of activity is recorded. Plant run under lamp until at 165 minutes the lamp was turned off.

An extreme example of the above phenomenon was displayed by a plant grown at 5 kPa O_2 for four weeks and then assayed for nitrogenase over the range 1.7 to 52 kPa O_2 (Fig. 5). The plant had apparently accommodated to grow in a root environment of 5 kPa O_2 and thence over a period of two and a half hours showed remarkable adaptation to pO_2 levels up to 30 kPa. When the experiment was terminated the plant was showing significant adaptation to 52 kPa O_2.

The ability of Coriaria to adjust nitrogenase activity to a wide variety of pO_2 levels is similar to some of the phenomena described for legume nodules in which a change in the diffusion resistance of the nodule is invoked to explain the adaptability of the nodule. In the case of soybean nodules Weisz and Sinclair (1987a) showed that levels of nitrogenase were largely independent of external pO_2 and that plants adjust to changes in external pO_2 over a period of 4–8 hours by adjusting nodule diffusion resistance (Weisz and Sinclair, 1987b). In Coriaria the adaptation takes place in 5–30 minutes. Coriaria plants were tested for changes in nodule diffusion resistance by observing the time required for acetylene to diffuse in and ethylene out of nodules. This requires that nitrogenase activity be measured at 5 s intervals following exposure to C_2H_2. In these experiments a plant was exposed to acetylene for 70 s while 5 s samples were taken of the effluent gas then the plant was returned immediately to air at the same flow rate (150 ml min^{-1}). A delay of 15 minutes while these samples were analysed and after several runs in air the plant was either exposed to 40 kPa O_2 and similar measures of lag-phase made.

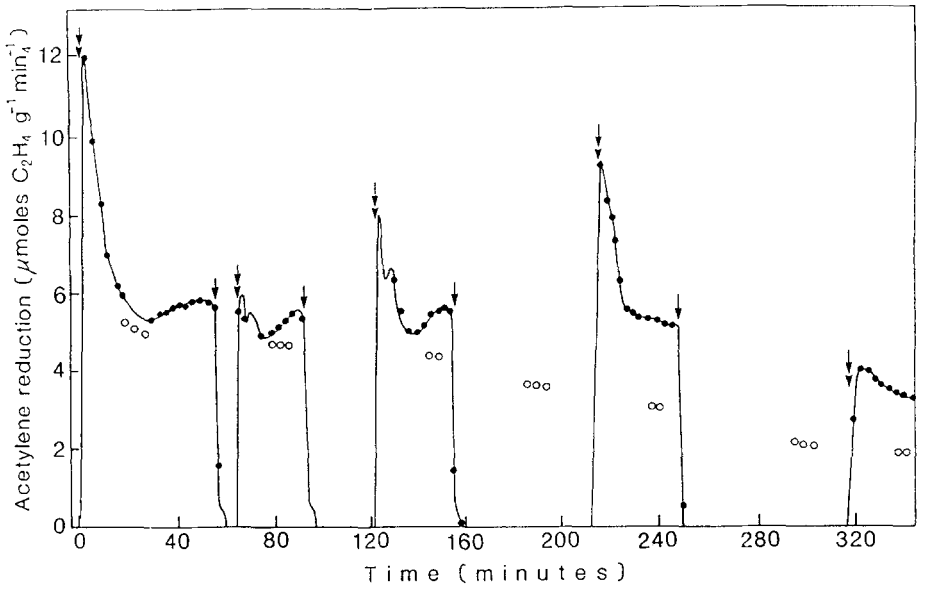


Fig. 3. Effect of various periods in air on nitrogenase activity in *C. arborea*. Two plants were run in flow through cuvettes with air/C_2H_2 as the flow gas. Double arrows indicate the start of acetylene gassing in the experimental plant. Single arrows indicate the point at which the plant was returned to air only in flow gas. The control plant (open circles) was run continuously in air/C_2H_2 and assayed periodically

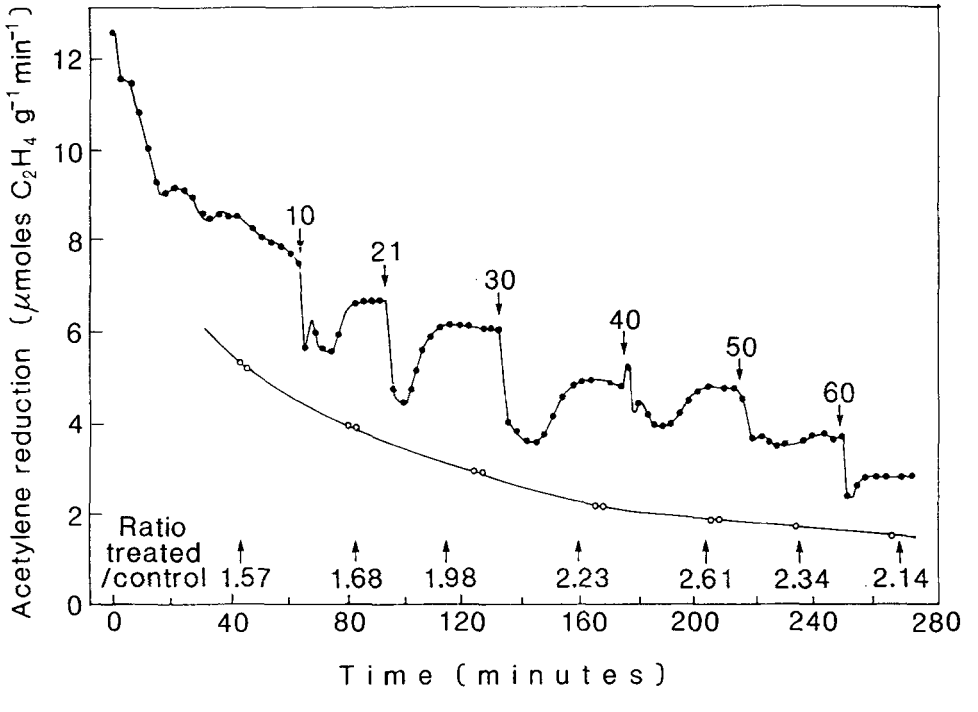


Fig. 4. Effect of varying pO_2 on nitrogenase activity in an intact *C. arborea* plant. Experimental plant (closed circles) was initially exposed to air/C_2H_2 (21 kPa O_2) and cycled with 10, 21, 30 kPa O_2 etc. at the times shown. A control plant (open circles, lower line) was continuously flushed with air/C_2H_2 and assayed periodically. The ratio of nitrogenase activity of treated plant to control plant is given for the various times indicated by the arrows.

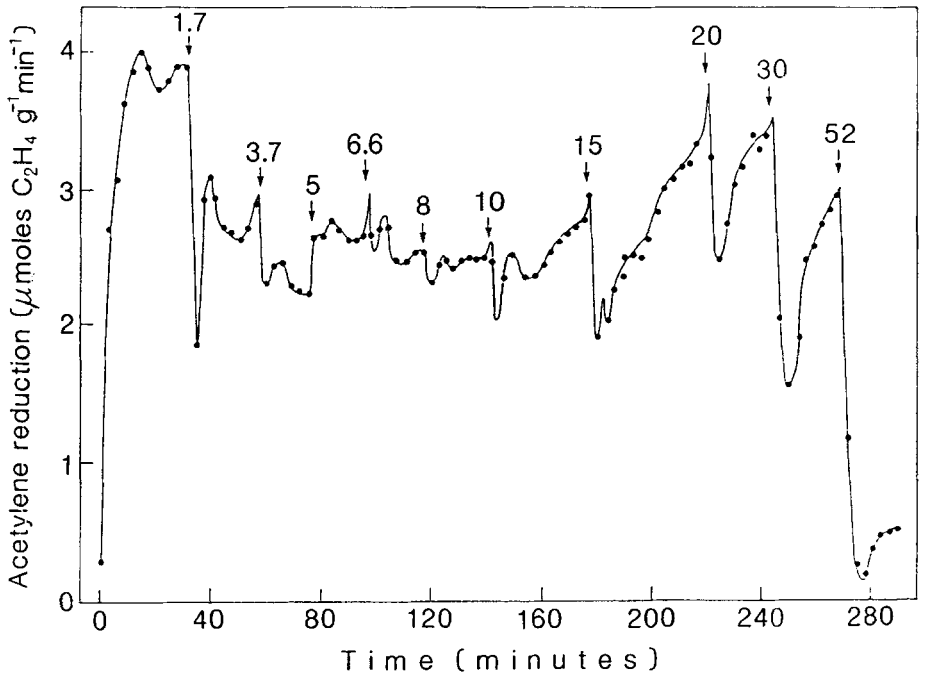


Fig. 5. Nitrogenase activity of a *C. arborea* plant grown with roots in 5 kPa O_2 then assayed at various pO_2 in flow-through cuvette. Initial pO_2 was 5 kPa followed by changes to pO_2 levels indicated at the arrows. Every third assay point is shown, the continuous line follows the complete record.

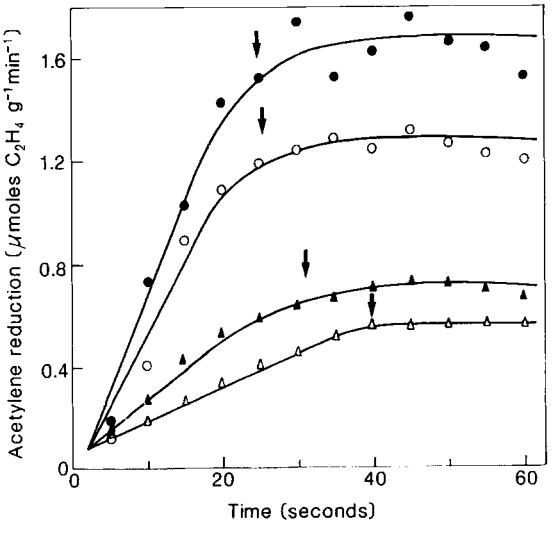


Fig. 6. Time taken for ethylene production to rise to equilibrium in *C. arborea* plant at different pO_2. Initial exposure in air (circles) showing two one minute exposures to air/C_2H_2 at time zero (closed circles) and 30 minutes later (open circles). The plant was then changed rapidly to 40 kPa O_2 (triangles) and exposed to 40 kPa O_2/C_2H_2 after two minutes (closed triangles) and after 30 minutes (open triangles). The arrows indicate the time required for ethylene production to achieve an equilibrium maximum rate in each case. Note the plant was exposed to C_2H_2 for one minute periods only and was not subject to acetylene induced decline. (see also Table 1).

Figure 6 illustrates the actual values for a plant tested in air and thence in 40 kPa O_2. In this case only the initial and 30 minute air values and initial and 30 minute 40 kPa O_2 curves are shown. This plant was tested three times in air and seven times in 40 kPa O_2 and the time constants which represent the half time to reach diffusional equilibrium (Weisz and Sinclair, 1988) are tabulated in Table 1 along with the maximum rates of acetylene reduction. It is quite clear from these results that Coriaria nodules show a dramatic change in diffusion resistance that is proportional to the increase in pO_2. Further to this the change is rapid and was already significantly increased two minutes after the change in pO_2 (Table 1).

In a similar experiment another plant was reduced from air to 5 kPa O_2 (Fig. 7) and shows a complementary reduction in diffusion resistance (Table 2). It will be seen that though there is only a small decrease in resistance (*i.e.*, decrease in time constant) this is consistent with the only small recovery in acetylene reduction after the lowered pO_2. Nevertheless the consistent change in resistance is accomplished within 15 minutes indicating a reversal of the phenomenon described above for the increase in pO_2.

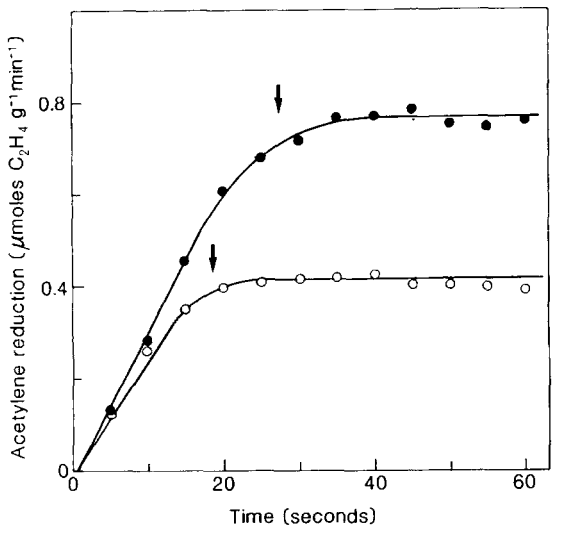


Fig. 7. Time taken for ethylene production to rise to equilibrium in *C. arborea* plant at different pO_2 (see caption Fig. 6). In this case a plant was exposed to air/C_2H_2 (closed circles) then quickly cycled to 5 kPa O_2 and a one minute assay shown for the plant after 30 minutes (open circles). The arrows indicate the time taken for ethylene production to rise to equilibrium maximum rate (see Table 2).

It is apparent that the ability of Coriaria nodules to recover from rapid changes in pO_2 is due in part, if not in full, to a change in the internal resistances to gas transport. This led to an investigation of the structures in the nodule which may be responsible for providing a diffusion barrier and an investigation of the pathway of gas transport.

Nodule structure

Plants were grown with root systems in sealed chambers and continuously gassed with recirculated gas mixtures containing 5, 10, 21, 40 kPa O_2. All plants grew extremely well and growth, as assessed by aerial growth, shoot length, dry weight, leaf colour *etc.*, was totally unaffected by the treatments. The ranging oxygen levels had no effect on the extent of nodulation or nodule size and during the growth period all plants formed new nodules. The only externally visible influence of oxygen on nodule morphology was on the degree of lenticel formation. Nodules from 5 kPa O_2 had extensive patches of lenticel scattered over the entire surface,

Table 1. Time constant (τ) (half time to reach equilibrium) of root nodules exposed to acetylene for one minute intervals. Three results in air and seven results for 40 kPa O_2. Time is the interval after first acetylene exposure at each oxygen level

pO_2 (kPa)	Time (mins)	Time constant (τ) (s)	Acetylene reduction (μmoles C_2H_4 $g^{-1}min^{-1}$)
21	0	11.5	1.64
21	15	14.4	1.41
21	30	13.4	1.26
40	2	17.1	0.72
40	30	27.3	0.56
40	45	30.0	0.59
40	60	30.0	0.65
40	75	26.8	0.67
40	150	27.8	0.75

Table 2. Time constant (τ) of root nodules exposed to acetylene for one minute intervals. Two results in air and five results for 5 kPa O_2. Time is the interval after first acetylene exposure at each oxygen level.

pO_2 (kPa)	Time (mins)	Time constant (τ) (s)	Acetylene reduction (μmoles C_2H_4 g^{-1} min^{-1})
21	0	14.2	0.95
21	15	15.0	0.76
5	2	14.5	0.36
5	15	10.4	0.43
5	30	10.0	0.41
5	45	10.5	0.41
5	60	10.2	0.40

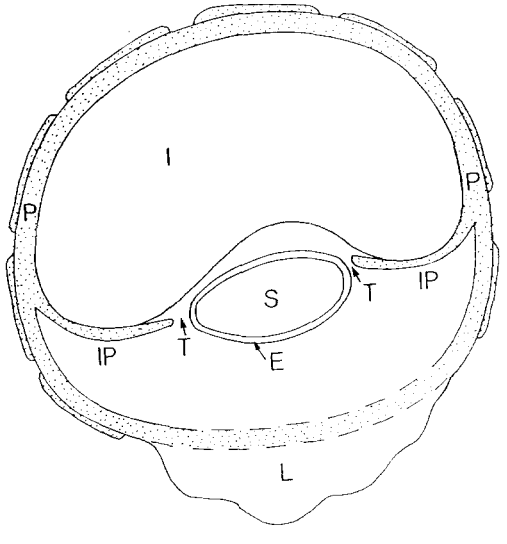


Fig. 8. Diagramatic representation of a mature *C. aborea* nodule. The infected zone (I) is a well defined semi-lunar area on one side of the nodule and displaces the stele (S) to other side. A dense periderm (P) closely invests the nodule and this peels off leaving the compact inner periderm. The periderm splits and follows closely under the infected tissue at (IP) leaving a small passage (T) between the periderm and endodermis (E). A prominent lenticel (L) occurs opposite the infected tissue and this is underlain by a weakly thickened periderm.

while nodules from 21 or 40 kPa O_2 showed a single elongate lenticel down one side of the nodule.

Thin sections and SEM preparations of Coriaria nodules reveal a number of important structural details that may help to explain the phsyiological responses described in the previous section. A general model of nodule structure (Fig. 8) shows the nodule contains a very compact semi-lunar area of infected tissue in which every cell is heavily infected. The stele is displaced to one side by the infected tissue and stands between the infected zone and the large single lenticel. The nodule is surrounded by a heavy periderm that lies immediately outside the infected cells: there is no cortex outside the *Frankia* zone.

All infected cells have a very similar morphology which is illustrated by a portion of a nodule grown at 10 kPa O_2 (Fig. 9a). Cells have a large central

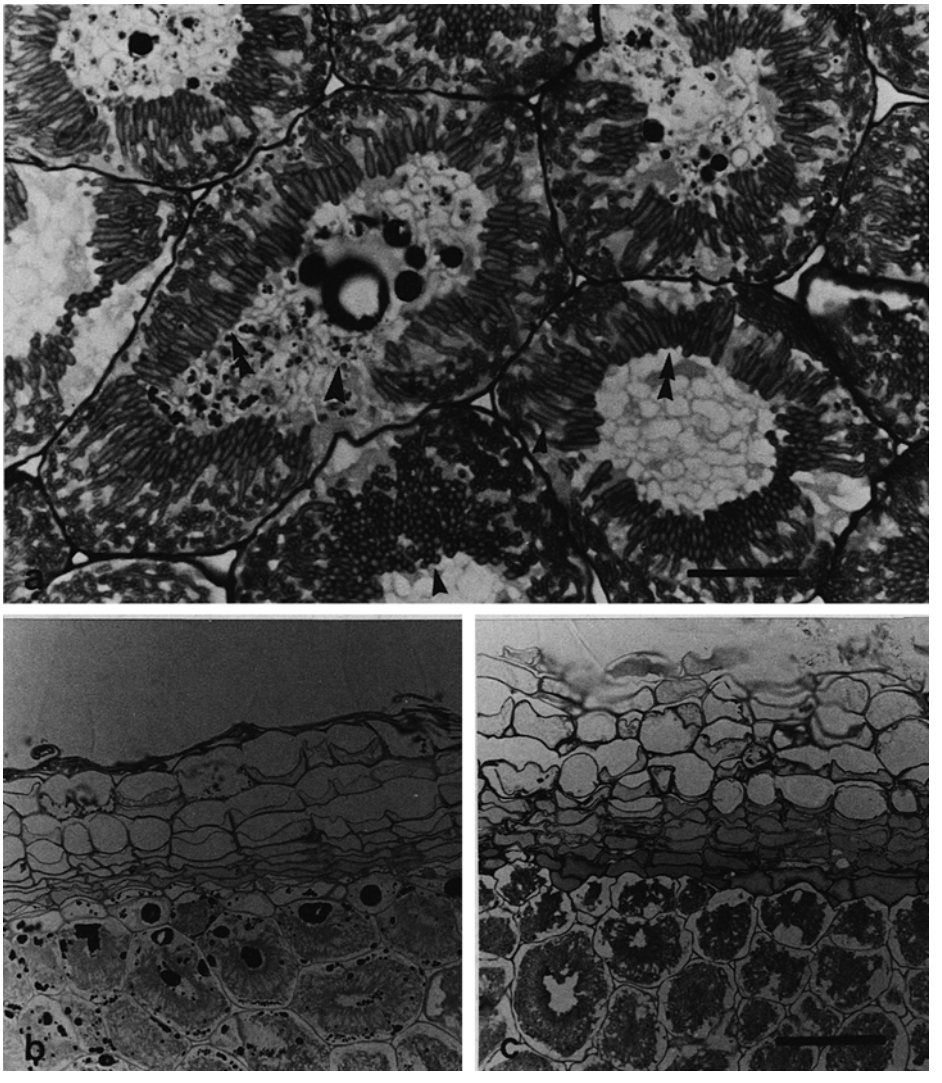


Fig. 9. Thin sections of *C. arborea* nodules grown at varying pO_2. Fig. 9a Transverse section of nodule grown at 10 kPa O_2 showing the anatomy of infected tissue typical of *C. arborea*. The large arrow indicates a central vacuole which often contains phenolic deposits. Small single arrows show the filamentous, branching hyphae of the *Frankia* which are often seen as small circles approximately 1 μm diameter when cut in section. The in-growing hyphae terminate in club-shaped, non-septate vesicles which are indicated by small double arrows. The vesicles are separated from the vegetative hyphae by a clearly visible septum. Fig. 9b and c Transverse sections of nodules grown at b. 5 kPa O_2 and c. 40 kPa O_2 showing the periderm outlying the infected tissue. At 5 kPa O_2 the periderm is thinner and the cells more loosely packed than at 40 kPa O_2. Bar equals 20 μm for 9a and 50 μm for 9b,c.

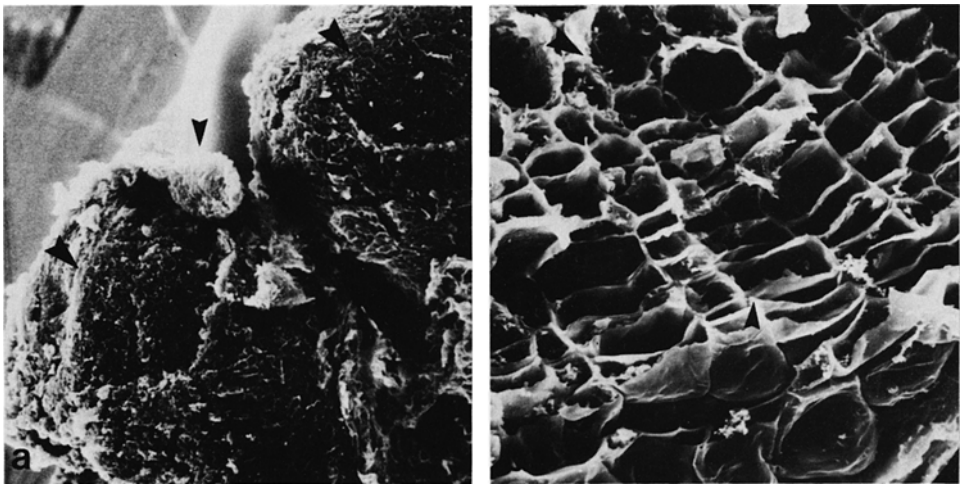


Fig. 10. Scanning electron micrographs of *C. arborea* nodules showing the distinct periderm. Fig. 10a External view of nodule grown at 21 kPa O_2. The small arrow indicates the lenticel which runs down one side of the nodule. Large arrows show areas where layers of the periderm have peeled away. Fig. 10b Internal view of a sectioned nodule. The small arrow indicate the tightly packed cells of the periderm adjacent to the infected tissue (large arrow).

vacuole and smaller parietal vacuoles which often contain phenolic deposits. The filamentous branching hyphae of the endophyte line the periphery of the cell and in newly infected tissue hyphae can be seen passing through cell walls. These outer hyphae are often seen as small circles of approximately 1 μm diameter when cut in section (Fig. 9a). Hyphae are oriented centripetally from this peripheral area and terminate in club shaped swellings or vesicles. The vesicles are clearly separate cells but are not subdivided within (Fig. 9a).

Varying growth pO_2 caused no change in internal anatomy of infected cells as revealed by light microscopy. The distribution of *Frankia* was always as described above in nodules grown at 5 to 40 kPa O_2. Similarly there was no apparent difference in cell wall thickness, or size and distribution of intercellular air spaces.

All nodules examined were surrounded by a dense periderm. In cross section this periderm consists of closely packed, thick-walled cells to the inside, exfoliating to the outside (Fig. 9b,c). In this case an obvious difference in periderm thickness is evident with the cells being less compact and possibly thinner walled at 5 kPa O_2 than at 40 kPa O_2 (Fig. 9b,c).

The peeling layers of periderm and the lenticel are evident in Figure 10a which shows the external structure of two air-grown nodules. The tight packing of the periderm adjacent to infected tissue is clearly illustrated in the SEM photograph of a section through the outer portion of an air-grown nodule (Fig. 10b).

However the most significant observation on nodule structure was revealed when hand-cut sections were stained with Sudan black B, a general lipid stain (Johansen, 1940) and an acknowledged suberin stain (O'Brien and Carr, 1970). The walls of the periderm were dark stained while the periderm generally showed up as an intense blue layer closely investing the infected zone (Fig. 11a). Of most interest is the split in the periderm that continues to invest the infected tissue to within two cells of the endodermis (Fig. 11a, 11b). It is assumed that the external and internal periderm are suberised and this was tested by chromic acid digestion of nodule sections. After 30 minutes in this treatment both the external and internal periderm survived along with the endodermis and xylem. Cortical cell walls, both infected and uninfected, disintegrated in chromic acid (Fig. 12a, 12b). It is most significant that in nodules grown at 40 kPa O_2 the periderm is suberised for virtually the total circumference, while nodules at 5 kPa O_2 show only a light suberisation through the lenticel area. Sections through newly infected tissue reveals a weakly suberised periderm and this along with the response to varying pO_2 strongly suggests the periderm is a major barrier to oxygen.

The gas pathway into nodules was traced

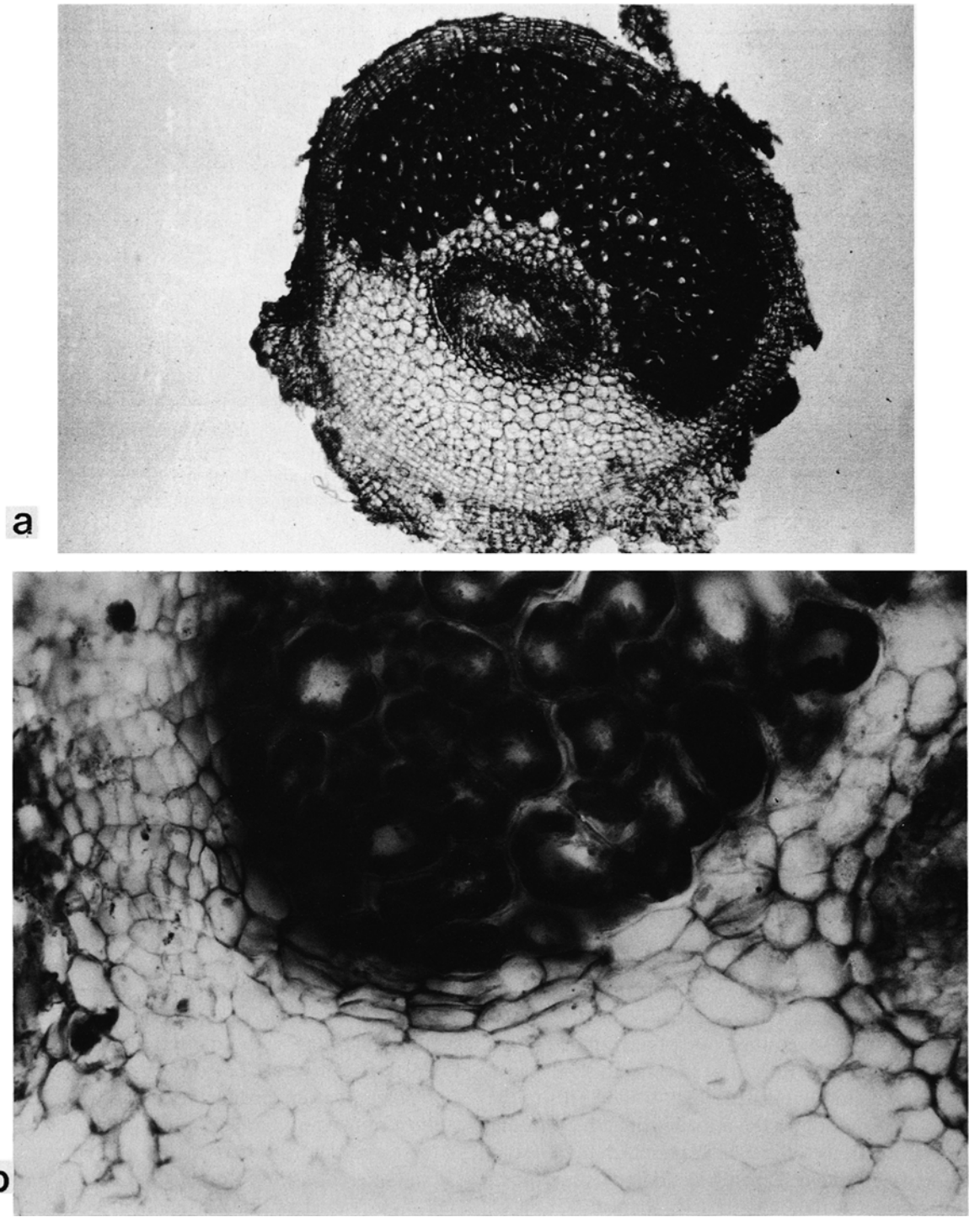


Fig. 11. Hand-cut sections of *C. arborea* nodules stained with Sudan black B. Fig. 11a shows a complete section of a nodule grown at atmospheric level of oxygen. The blue stained periderm extends around the infected tissue but only partially through the lenticel area. The internal periderm (refer Fig. 8) has both thickened walls and blue staining and extends to within two cells of the endodermis. Fig. 11b is a higher magnification view of the internal periderm area.

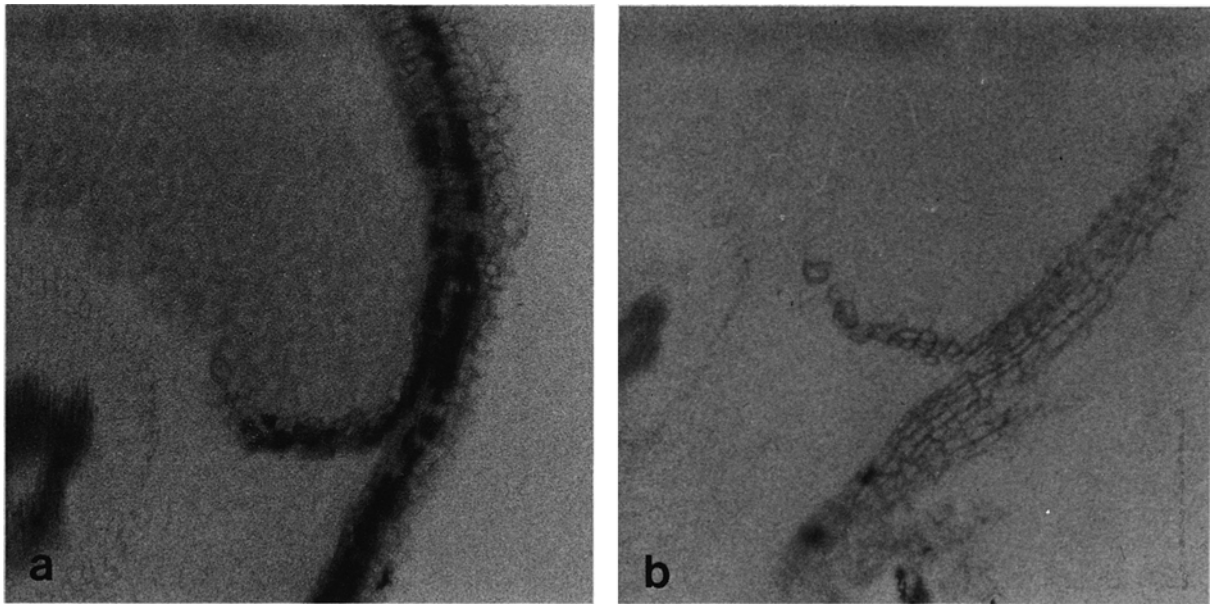


Fig. 12. Results of digestion of hand-cut *C. arborea* nodule sections in 50% chromic acid. Fig. 12a is a relatively thick section in which the periderm is well preserved while cortical cells show signs of wall breakdown. Thinner sections (Fig. 12b) show an almost complete loss of cortical walls while the internal and external periderm remain intact.

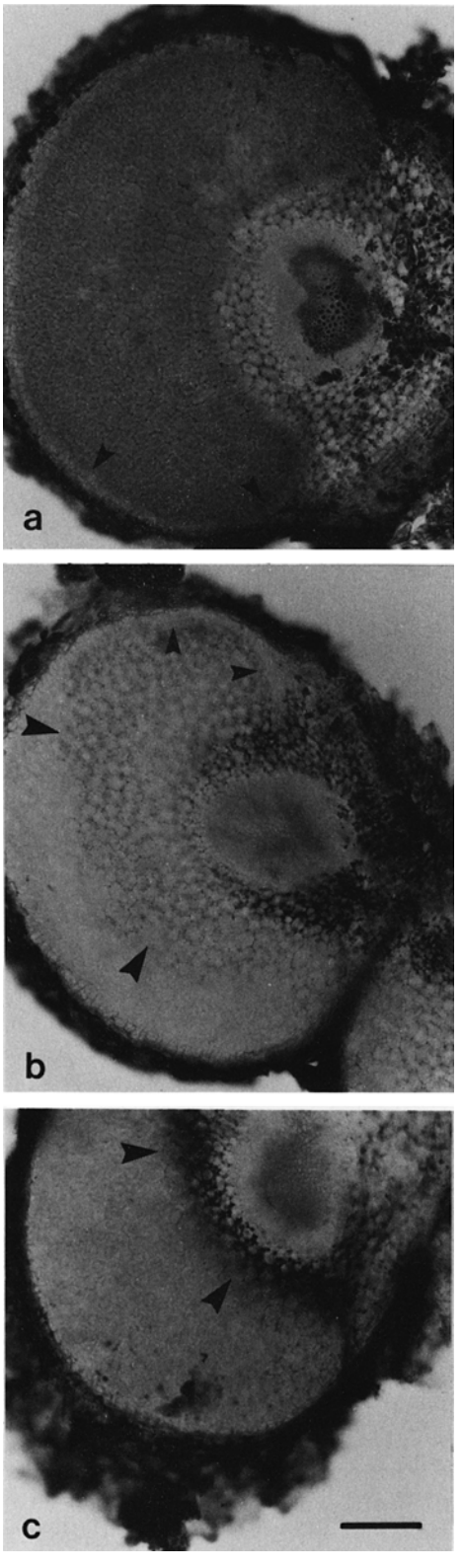
vacuum infiltration of India ink. Nodules were infiltrated at one, one half and one quarter of full vacuum in an attempt to sequentially follow the pathway of gas transport. Hand-cut sections of nodules treated in this way (Fig. 13) show that the periderm is an absolute barrier to infiltration and that gas diffusion is via the lenticel and thence through the narrow space adjacent to the stele. Under full evacuation India ink penetrated completely through the nodule and outlined the extensive intercellular network of gas passages in the infected tissue.

Discussion

The acetylene induced decline of nitrogenase, which is well documented for a variety of legume nodules (Minchin *et al.*, 1983) is thought to be the result of an increase in nodule diffusion resistance thus reducing oxygen diffusion to respiratory sites (Sheehy *et al.*, 1983). Effects on actinorhizal nodules are highly variable with nodules such as Alnus, Myrica and Casuarina showing a variable decline and then a return to almost full activity (Tjepkema *et al.*, 1988, Silvester *et al.*, 1988a, 1988b). Datisca is the only genus of actinorhizal plants so far reported that shows a typical legume type decline (Tjepkema *et al.*, 1988). Our results confirm a similar result for Coriaria in which the decline is consistently 30 to 50% of the maximum rate. Thus the structural similarity and uniqueness of Coriaria and Datisca is confirmed in a similar physiological response.

The similarities of Datisca and Coriaria are extended into the degree of ventilation of the nodule and lack of haemoglobin. Tjepkema (pers. comm.) has shown that both nodule types are well ventilated and that they both lack haemoglobin. We confirm that Coriaria is well ventilated with intercellular air spaces extending from the lenticel right through the infected tissue. We have also tested extensively for haemoglobin and although nodules are often deep red in colour we have obtained no trace of haemoglobin; the red colour is anthocyanin.

Coriaria plants are able to adapt and grow well at root oxygen levels from 5 to 40 kPa and this seems to be a general property of all actinorhizal plants. However it is the short term adaptation to varying pO_2 that sets Coriaria apart. Myrica plants show very significant nodule morphology changes



to varying pO_2 and very little ability to adapt to short term changes in pO_2 (Silvester *et al.*, 1988a). Alnus on the other hand shows some changes in nodule morphology and significant changes in *Frankia* vesicle morphology in response to growth pO_2 and is capable of some short term adaptation to changing ambient pO_2 (Silvester *et al.*, 1988b). Coriaria however represents the extreme case. Nodule response to growth pO_2 is minimal, but the nodules appear to have almost infinite capacity to rapidly adapt to changing ambient pO_2. There seems little doubt that this capacity results from an ability of the nodules to change diffusion resistance.

The change in nodule diffusion resistance to ambient pO_2 was first proposed for clover nodules (Sheehy *et al.*, 1983) and recent work on soybean nodules (Hunt *et al.*, 1987, Weisz and Sinclair, 1987a, b) confirms the presence of a varying diffusion boundary. In the case of legume nodules the infected tissue is enclosed by a layer of tightly packed cells in the inner cortex that limits oxygen diffusion.

Coriaria nodules are bounded by a very tight and apparently well suberised periderm that appears to be quite impermeable. The evidence from India ink infiltration and the fact that infected cells lie immediately against this layer suggests it is a major if not a complete barrier to gas diffusion. The lenticel is obviously very permeable to gas but may respond in the way described by Pankhurst and Sprent (1975) as a moisture sensitive gas barrier. We believe a most likely site for gas control is the narrow gap between the endodermis and the internal periderm. This layer is consistently only two cell layers wide and represents a diffusion boundary in its own right. A change in turgor of these cells could affect quite dramatic changes in the diffusion of gases to the infected area.

The physiological responses of Coriaria nodules to both elevated and depressed oxygen are

Fig. 13. Freehand transverse sections of *C. arborea* nodules vacuum infiltrated with India ink. Fig. 12a Nodule evacuated to 760 mm Hg shows infiltration of ink across the entire infected zone but no penetration of ink into the periderm as indicated by the small arrows. Fig. 12b Nodule evacuated to 380 mm Hg. The ink does not penetrate the periderm, represented by small arrows, but instead enters via the lenticel and infiltrates to approximately half way across the infected zone as indicated by large arrow. Fig. 12c Nodule evacuated to 190 mm Hg. The arrow shows the infiltration of ink only a small distance into the infected tissue. Bar equals 200 μm.

dramatic. A drop in oxygen results in an immediate drop in nitrogenase activity and this is consistent with a relatively well ventilated nodule and with a metabolic system very closely linked to and limited by its energy supply. This drop is normally followed by a partial or complete return of activity consistent with the change in diffusion resistance we have observed. The rapid drop and recovery that we see on an increase in pO_2 is more problematical. The instantaneous drop in nitrogenase activity on increasing pO_2 we interpret as an acetylene induced transient. This has been previously described for Myrica and Alnus (Silvester *et al.*, 1988a, b) and appears to be a switch off of activity similar to a conformational change of nitrogenase. In the case of Coriaria we believe this is followed by a change in diffusion resistance which reaches full equilibrium after approximately 15 minutes.

While a reasonably consistent and coherent model for oxygen protection and oxygen tolerance is now emerging for legume nodules it appears that every genus of actinorhizal plants has a different anatomy and a different response to varying pO_2. One unifying mechanism appears to be the possibility that many nodules, both legume and non-legume have a process of altering diffusion resistance in response to water deficit and oxygen. However it is now evident that these changes must occur in different types of tissue and much more integrated anatomy and physiology will need to be studied to elucidate the phenomenon.

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