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Penetration of potato tuber lenticels by bacteria in relation to biological control of blackleg disease

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Summary

In a glasshouse experiment three potato cultivars were grown to maturity in wet or dry compost. The number of lenticels per tuber was greater with larger tubers but was unaffected by the cultivar or soil moisture regime. The frequency of lenticel penetration by a water soluble stain, safranin O, remained high as tubers developed to maturity in wet soil. In dry soil penetration declined markedly as the tubers matured. When tubers, harvested just before the onset of haulm senescence, were inoculated with *Erwinia carotovora* ssp. *atroseptica*, the frequency of lenticel rotting was less than the rate of stain penetration, indicating that additional factors were involved in the bacterial infection process. Inoculating mature tubers with bacteria and incubating them in anaerobic conditions frequently resulted in rotting of the lenticels. Comparable tubers incubated in aerobic conditions showed no rotting or invasion by bacteria. The results are discussed in relation to the biological control of blackleg.

Introduction

Potato blackleg caused by *Erwinia* spp. remains an important disease throughout the world, despite many attempts to control it. Breeding programmes have so far not produced blackleg-resistant commercial cultivars. Agronomic and certification (classification) procedures have had only limited success at reducing contamination on seed and there is no known chemical treatment. There have been a number of attempts at biological control by treating seed tubers before planting with formulations of antagonistic bacteria, mainly species of *Pseudomonas* and *Bacillus* (Xu & Gross, 1984; Scroth, 1985). Extensive experiments using bacteria isolated from the soil and plant environment were done in Edinburgh at the Scottish Agricultural College during 1987–90. In common with work elsewhere, variable and inconsistent levels of control were obtained, so that the methods could not be adopted in commercial practice (Chard et al., 1992). There may be several reasons for these results but they are not clearly understood. However, it is known that *Erwinia* bacteria infect tubers in the growing crop through the lenticels (Pérombelon, 1974).

Lenticels vary in their penetrability during the growing season and the availability of *Erwinia* also varies, but it is established that in general tubers are contaminated or

infected before harvest. The major source of the bacteria is the rotting mother tuber; rotting takes place mainly towards the end of the growing season, especially August and September in Scotland (Pérombelon, 1974). However, studies have shown that, at least in some fields, *Erwinia* spp. can be detected in the rhizosphere of weeds and various crop plants in late June and early July (McCarter-Zorner et al., 1985) and also in some fallow soils (Pérombelon & Hyman, 1989) so infection could be established earlier in the season, although most of the bacteria are *E. carotovora* ssp. *carotovora* (Pérombelon, 1992). Populations also vary with the growing conditions, and are small in dry and large in wet weather, or if soils have been irrigated (Elphinstone & Pérombelon, 1986). As antagonistic bacteria have usually been applied to seed tubers just before planting, their ineffectiveness could be due to their failure to penetrate the lenticels of the seed tubers and interact with the *Erwinia* in them. Possibly some penetration can occur, because a level of control can occasionally be achieved (Chard et al., 1992), but how the biocontrol agent and lenticels interact has not been investigated in any detail. Artschwager (1927) described the anatomy of potato lenticels, and Adams (1975a,b) considered both their structure and susceptibility to *Erwinia* and other pathogens, but it is not clear how lenticels on mature tubers behave when the tubers are subsequently planted, especially in relation to penetration.

This paper considers (1) how the number of lenticels of tubers of different sizes, of three cultivars grown in wet or dry soil conditions, varies; (2) how penetrability of the lenticels, assessed using the water soluble stain safranin O, changes during tuber development and maturation; (3) the incidence of lenticel rotting in fully developed tubers harvested before haulm senescence and inoculated with a suspension of *Erwinia carotovora* ssp. *atroseptica (Eca)*; (4) how lenticels of mature tubers incubated in aerobic or anaerobic conditions behave towards penetration by fluorescent tracer bacteria.

Materials and methods

Planting, cultivation and watering regimes. Three cultivars, Home Guard (first early), Maris Peer (second early) and Désirée (maincrop), were compared. Three tubers of each cultivar were planted in each of eight 30 cm diameter pots containing a peat-based compost. Planting was done in early September 1991 in a glasshouse at a temperature of about 20 °C; as soon as the plants emerged they were given supplementary lighting from mercury-sodium lamps for 16 h day^{-1} until haulm death. Four pots of each cultivar were assigned to a high moisture (wet) soil regime, maintained by standing pots in water-filled trays. The remaining four pots of each cultivar were maintained in a limited watering (dry) soil regime where pots were watered from above once or twice per week. The amount of water added depended on soil moisture readings and the growth stage of the plants, and varied between 300 ml and 3 l per pot in any one week. The two watering regimes began 8 weeks after planting and maintained soil moisture at approximately -60 HPa (wet) and between -400 and -600 HPa (dry) respectively. The soil moisture tension was recorded using

porous pot tensiometers (constructed by the Scottish Centre for Agricultural Engineering, Bush Estate, Roslin, Midlothian, Scotland). Plants were treated with a liquid fertilizer (Fisons Biofeed at the recommended rate) at 16 and 24 weeks from planting.

Tuber sampling. A sample of between four and ten tubers per cultivar from each soil moisture regime was harvested at 18, 24, 26, and 28 weeks after planting, except that, at the first harvest only one or two tubers were produced by Maris Peer and Désirée in dry conditions and none were found on Maris Peer plants in wet soil. Plants were showing the following stages: 1) week 18 - early tuber development with tubers typically < 0.25 cm in length; 2) week 24 - late tuber development; 3) week 26 - foliage senescent with haulm collapsing and tubers maturing; 4) week 28 - haulm dead and tubers fully mature. The surface areas of sampled tubers were calculated from their length (L), breadth (B) and width (W) using the formula $area=4\pi r^2$, where $r=(L+B+W)/6$. The numbers and distribution of lenticels on tubers were noted.

Lenticel penetration by safranin O stain. Lenticel penetrability was demonstrated by soaking washed tubers in a 1% w/v aqueous solution of safranin O for 30 min and washing off excess stain in running water. Penetration was observed with the naked eye by cutting across 15 lenticels on each tuber to a depth of approximately 1 mm. If the lenticel was penetrated a red spot of stain was clearly seen in the underlying tissue. For the first two samples all tubers of each cultivar were assessed, and for the latter two, five per cultivar from each soil condition were examined. In doubtful cases, free-hand lenticel sections were examined using a low power microscope. Evidence of barrier formation in lenticels was indicated by the browning of cell walls and the formation of a cambial phelloderm layer. Further representative lenticel sections were mounted in a 0.1% w/v solution of the fluorescent brightener Tinopal AN (Uvitex, Ciba Geigy) and examined under fluorescence microscopy as described below.

Lenticel infection of developing tubers by Erwinia carotovora ssp. atroseptica. Two lots of three tubers from the second sampling date of each cultivar from each soil moisture condition were inoculated with a cell suspension of *Eca*. The bacterial culture was grown on nutrient agar slopes incubated at 25 °C for 48 h and then suspended in sterile distilled water. Tubers were immersed in the suspension (1.0×10^8 cfu ml^{-1} as determined by plate counts) for 10 min and incubated at 20 °C for 7 days. The numbers of lenticels which showed symptoms of bacterial rot were then noted and after a further 7 days' incubation lenticel infection was again recorded.

Penetration of fluorescent Erwinia bacteria into lenticels of mature tubers incubated in aerobic and anaerobic conditions. Eca was grown on nutrient agar slopes containing 3% w/v Tinopal AN for 48 h at 25 °C and suspended in sterile distilled water to give a bacterial concentration of 2.5×10^{10} cfu ml^{-1}. The bacteria were not washed so as to disturb the cells and their flagella as little as possible. A sample of the suspension was

tested for pathogenicity on potato tuber slices to ensure that this had not been affected by the brightener, which was required at a high concentration in order to make the bacteria highly fluorescent. Mature tubers sampled from the wet soil regime 26 weeks after planting were allowed to stand in air for 2 days. Six tubers of Home Guard, six of Maris Peer and two of Désirée were then incubated in anaerobic conditions by placing them in a bacteriological anaerobic gas jar containing a BBL (Baltimore Biological Laboratories) Gas Pack which released a mixture of hydrogen and carbon dioxide. A comparable sample of tubers was placed in a second jar adjusted to allow free access of air. High humidity was maintained by including wet absorbent paper in each jar. After 2 weeks at room temperature the tubers were removed and inoculated with a fluorescent bacterial suspension (2.5×10^{10} cfu ml^{-1}) for 20 min, then incubated for a further 4 days as before in aerobic or anaerobic conditions. Tubers were then examined for rot symptoms and selected lenticels showing infection or no symptoms were studied by fluorescence microscopy, using a Leitz Orthoplan II microscope fitted with a 100 W mercury vapour lamp. Free-hand sections of lenticels mounted in 0.1% w/v glycerol were examined; penetration could be observed by bright fluorescence of the tissues. The residual Tinopal in the suspension liquid stained the potato cell walls, but the intensity of the fluorescence produced by the bacteria could easily be distinguished. It did, however, allow observation of any barrier formation assumed to be associated with suberin deposition.

Results

Soil moisture. Fig. 1 illustrates the soil moisture throughout the growing period. Differing rates of water application began on week eight after planting. Within 5 weeks wet and dry regimes became established and were maintained to the death of the foliage. The wet regime remained at -40 to -60 HPa, whereas the dry regime was rather more variable, varying from -370 to -500 HPa.

Plant growth. The cultivar Home Guard emerged 6 weeks after planting, cv. Maris Peer 4 days later and cv. Désirée a further 10 days later, reflecting the inherent earliness or lateness of each cultivar. There was no difference in emergence between the wet and dry regimes. Plants grew well in both regimes until late in the growing season, when plants in the dry conditions began senescing earlier. The growth of Home Guard and Maris Peer persisted longer than would have been expected in normal field conditions. Cultivars were finally harvested 10 days after the haulm on the late cultivar Désirée was dead. All tubers at harvest were normal in appearance, although those from the wet soil were often found to have proliferated lenticels, particularly in cvs Home Guard and Désirée. There were similar numbers of tubers on all treatments but, on the whole, tubers from the wet regime were larger.

Number and distribution of lenticels. There was no significant difference ($P=0.19$) in the mean number of lenticels per tuber found in the three cultivars, averaged for sampling time and tuber size. Numbers (± standard error) ranged from 122±6

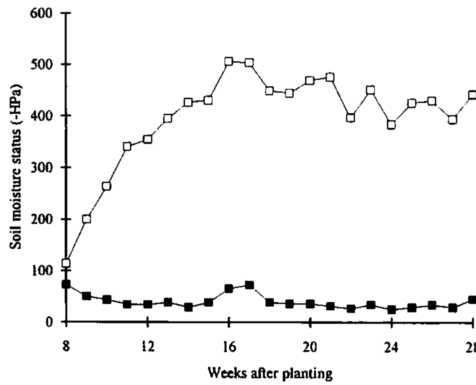

Fig. 1. Mean soil moisture status, measured at weekly intervals, in relation to wet (closed symbols) and dry (open symbols) watering regimes.

(minimum 36, maximum 274) for Home Guard, 105±6 (28–190) for Maris Peer and 116±8 (44–196) for Désirée. There was also no significant effect (P=0.47) of the watering regime on lenticel numbers, but significantly more lenticels were found on larger tubers (P<0.001) (Table 1). However, the number of lenticels per cm^2 on the tubers of less than 30 cm^2 surface area typically ranged from 10 to 40, compared with 2 to 10 in the larger tubers. In the smallest tubers, of less than 1 cm^2 surface area, more than 300 lenticels per cm^2 were recorded. Regression analyses confirmed that there was a strong negative relationship (r=0.95) between lenticel density and tuber surface area, that the main factor affecting lenticel density was tuber size, but that there was a gradual increase in lenticel number as the tuber swelled (c. one new lenticel for each extra cm^2). There were no clear differences in lenticel density associated with the cultivar or watering regime. Roughly equal proportions of lenticels were found in apical, mid- and basal portions of a tuber, and there was no evidence that distribution was affected by the cultivar, watering regime or tuber size.

Penetration of lenticels by safranin O. Lenticels showed penetration by safranin O as a red spot in the tissues below the surface (Fig. 2). Tubers of Home Guard at the first harvest showed about 25% of lenticels on tubers from both watering regimes

Table 1. Mean lenticel number per tuber in relation to tuber size and watering regime (pooled data for cultivar and sampling time ± standard error; df given in parentheses).

Tuber size (Surface area)	Watering regime		Overall mean
	Wet	Dry	
Small (<30cm^2)	96 ± 4 (51)	95 ± 7 (34)	96 ± 4 (86)
Large (>30cm^2)	153 ± 9 (32)	131 ± 9 (31)	142 ± 6 (64)

penetrated by the stain. For other cultivars only Désirée in the wet regime produced sufficient tubers for reliable assessment, and here the penetration rate was less than 1%. At later sampling dates tubers grown in the wet regime had high penetration levels, with Maris Peer and Désirée usually showing 60% or more at all sampling times: Home Guard showed more than 50% at the second and third sampling but only 40% at the fourth (Fig. 3). In the dry regime penetration was substantial at the second sampling date, with Home Guard showing 30% and Maris Peer and Désirée showing more than 60%. At later times, however, lenticels of Maris Peer and Désirée showed no penetration, while Home Guard showed <20% and <2% at the third and fourth samplings respectively. The same general pattern of variation in penetrability with time and soil moisture regime was exhibited by large and small tubers (Fig. 4). Chi square analysis (Table 2) using the actual numbers of penetrated and non-penetrated lenticels confirmed that lenticel penetrability of tubers developed under the dry regime was significantly lower than that of tubers grown in wet soil. With regard to cultivar effects Home Guard showed less overall penetration, which may be related to its earlier maturity, whereas Maris Peer was associated with a high level of penetration, particularly in the wet (Fig. 3). The significant effect of tuber size on lenticel penetrability (Table 2) reflected the preponderance of larger tubers at later sampling times, where penetrability in dry conditions was greatly reduced, and the larger number of small tubers obtained at earlier sampling times when lenticel penetration was less affected by the watering regime. Microscopical examination and staining of lenticels with 0.1% Tinopal solution suggested that the lack of penetration was due to the formation of suberin barriers, but no specific biochemical test to identify the presence of suberin was used and no quantitative study was made.

Fig. 2. Tuber (cv. Désirée) soaked in safranin showing staining of cortex beneath lenticels, indicating penetration.

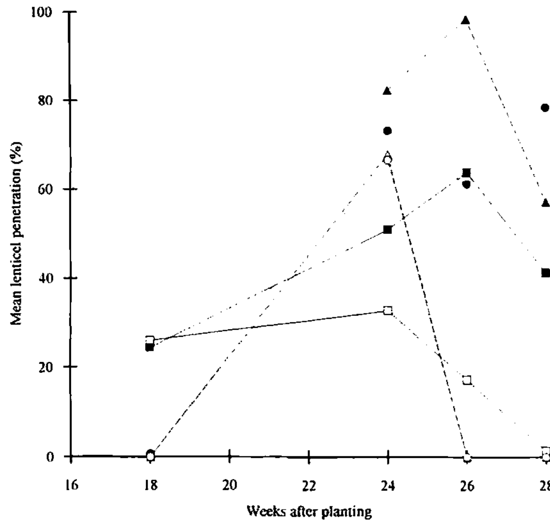

Fig. 3. Percentage penetration of lenticels by safranin in relation to cultivar, watering regime (wet, closed symbols; dry, open symbols) and time from planting (averaged for tuber size), Home Guard (squares); Maris Peer (triangles); Désirée (circles).

Infection of lenticels by Erwinia carotovora ssp. atroseptica. The average number of lenticels per tuber showing symptoms of bacterial infection following inoculation with *Eca* was low, irrespective of cultivar and watering regime. The frequency of tuber rots was always less than 5 per tuber; ranging between 0.3 to 2.0 for Home

Table 2. Chi squared analysis of lenticel penetration data.

Effect of watering regime	Observed number of lenticels (Expected number)		
	Penetrated	Non-penetrated	Significance level
Wet	666 (493)	609 (788)	
Dry	216 (389)	789 (616)	$P = < 0.001$
Effect of cultivar[1]			
Home Guard	272 (331)	448 (389)	
Maris Peer	329 (262)	241 (308)	
Desiree	178 (186)	227 (219)	$P = < 0.001$
Effect of tuber size			
Large	477 (505)	828 (800)	
Small	405 (377)	570 (589)	$P = < 0.016$

[1] (last three sampling times only)

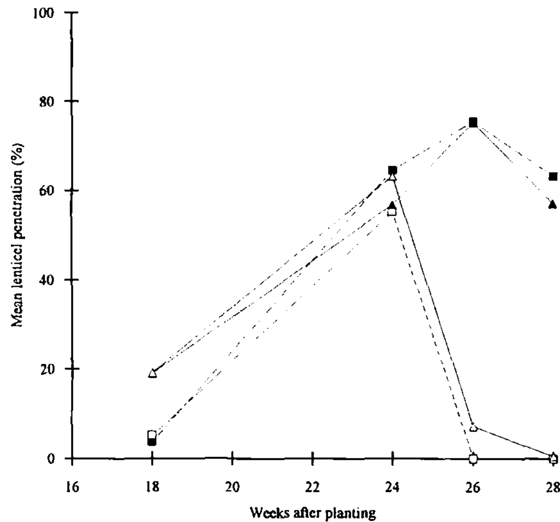

Fig. 4. Mean percentage penetration of lenticels by safranin in relation to tuber size (surface area: <30cm^2, small; >30cm^2, large) and watering regime (wet, closed symbols; dry, open symbols) and time from planting (averaged for all cultivars). Small tubers (squares); large tubers (triangles).

Guard, 4.8 to 0.7 for Maris Peer and 1.8 to 0.8 for Désirée, in the wet and dry regimes respectively. The highest frequency obtained was still less than 5% of the total number of lenticels per tuber. Most lenticels appeared normal, although a proportion were proliferated in the case of Home Guard grown in wet soil.

Penetration of fluorescent Erwinia bacteria in lenticels of mature tubers incubated in aerobic or anaerobic conditions. Following inoculation, lenticels of tubers kept in anaerobic conditions were mostly proliferated and invariably showed visible symptoms of bacterial rotting, in contrast to those from aerobic conditions which appeared unaffected. Microscopical examination suggested that lenticels of tubers from aerobic conditions were suberized, whereas those in anaerobic conditions were not. The lack of an apparent suberin barrier allowed the spread of bacteria below the lenticel in normal (Fig. 5a) or proliferated lenticels (Fig. 5b). On the other hand, in lenticels from tubers incubated in aerobic conditions the barrier prevented bacterial penetration (Fig. 5c).

Discussion

In developing a strategy for biological control of blackleg the importance of establishing interaction between the biocontrol agent and the blackleg organisms at a

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Fig 5 (a)

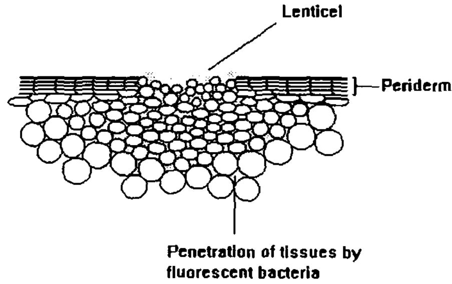

Fig 5 (b)

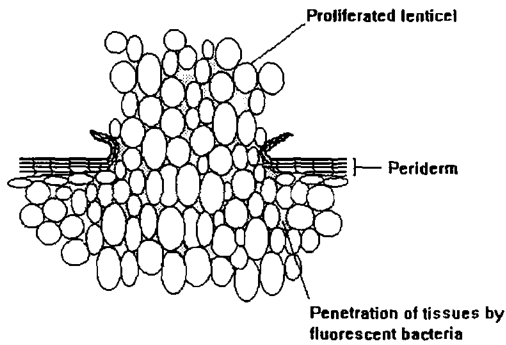

Fig 5 (c)

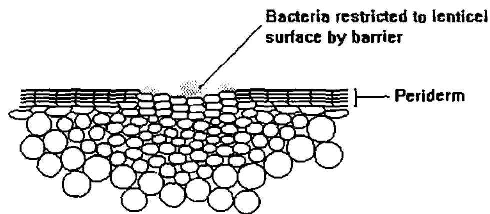

Fig. 5. Diagram showing varying degrees of penetration of lenticels by fluorescent *Erwinia* bacteria in relation to barrier formation and lenticel condition.
(a) Lenticel showing penetration by *Erwinia* in the absence of barrier formation.
(b) Proliferated lenticel lacking barrier showing penetration by *Erwinia*.
(c) Lenticel showing bacteria mainly confined to lenticel surface by barrier.

critical time in the infection cycle is recognized. An important site of initiation of blackleg infection is the tuber lenticel which might, therefore, be considered as a site for introduction of a bacterial antagonist. However, to ensure lenticel colonization by the antagonist is successful, a greater understanding of lenticel behaviour and characteristics is necessary. Weber & Bartel (1986) recorded differences between cultivars in the number and size of lenticels, but in the present experiment the numbers formed on three cultivars were similar, with no obvious differences in lenticel size. The average number of lenticels quoted by Burton (1965) for mature tubers of cv. Golden Wonder was 110, while the number of lenticels per cm^2 varied from one to three compared with two to 40 recorded in this experiment with three cultivars. There is substantial variation in lenticel numbers for individual tubers within a cultivar. Burton (1966) found that lenticel numbers in Golden Wonder varied from 70 to 150 and in our work with Home Guard, for example, the numbers on individual tubers ranged from 36 to 270. This variation is largely related to the variation in tuber size, and in each of our three cultivars more lenticels were recorded on larger tubers, although the number per unit area declined with increased tuber size. Using a range of other cultivars, Meinl (1966) reported that the total number of lenticels increased with tuber size and could also be influenced by growing conditions, e.g. high phosphate application gave increased numbers. In our experiments different soil moisture regimes established at about the time of tuber initiation were found to have no effect on lenticel numbers. However, this does not exclude a possible effect of soil moisture at an earlier phase of growth when stomata are developing on stolons.

The number of lenticels per tuber, or the number per unit area of tuber surface, does not appear to be a feature in determining bacterial infection. Thus, cvs Home Guard, Désirée and Maris Peer show differences in their susceptibility to blackleg (Anon., 1993), but no significant difference in lenticel numbers. However, the penetrability of lenticels may vary considerably with a number of factors, and this may provide a much better index of their susceptibility to colonization or infection by micro-organisms. In the present work the penetration of lenticels by a water-soluble dye was found to vary with the stage of tuber development and soil moisture regime, and the interaction between these two factors. *Streptomyces scabies* was reported by Fellows (1926) to infect tubers through stomata and newly formed, unsuberized lenticels, and Lapwood & Hering (1968) observed a brief period of lenticel susceptibility on different internodes of the tuber as they develop. This would suggest that lenticel penetrability was at a maximum during the very early stage of tuber formation. In the present work penetration of safranin O was only moderate or unexpectedly low at the first sampling date, 18 weeks after planting, but it was not possible to cover the full range of cultivars and treatments at this time because of the inadequate numbers of tubers available for sampling. In dry soil the penetration level was at a maximum (about 30–60%) during the active growth phase, and declined rapidly as haulms senesced. In wet soils, penetration rates reached a higher level (50–80%) during the active growth phase, but remained high at later harvest times up to haulm death. This behaviour pattern was found in all three cultivars, although

differences in the actual level of penetration at any particular time could vary between cultivars. Adams (1975b) did similar experiments using three cultivars grown in the field with a wet regime (irrigated to field capacity) and a dry regime (not irrigated), and found that early in the season, 10 weeks after planting, tubers showed the greatest penetration of the dye fluorescein, where 33% and 30% of lenticels were penetrated in the wet and dry regimes respectively. Three weeks later the number penetrated was halved and at haulm senescence 12% was recorded for both moisture regimes. Part of the difference in the two sets of results may be due to the fact that Adams grew plants in the field in summer and used cvs Majestic, Pentland Crown and King Edward. Adams also found some differences between cultivars, although differences in developmental stages had considerable effects.

Variation in penetrability by a water soluble stain may be attributable, at least in part, to the extent of lenticel suberization. Thus, the suberization of lenticels as tubers mature is associated with less penetration, but exposure to wet conditions may cause lenticels to proliferate, resulting in a rupture of the suberin barrier. Diriwachter & Parberry (1991) found lenticel infection by *Spongospora subterranea* to be encouraged by wet soils which not only favoured the activity of the fungus but were considered to delay suberization of lenticels and induce proliferation. However, in the present study the frequency of lenticel rots was much less than that of stain penetration, suggesting that penetration of lenticels by a water-soluble dye may not be a good indicator of their susceptibility to infection. The low rates of penetration when India ink was used to assess penetration (unpublished work) would suggest that a suspension of fine particles would be a more appropriate material than a solution of stain to assess visually penetrability, though more tests are necessary. Silvester & Harris (1989) used vacuum infiltration of India ink as a means of indicating oxygen diffusion through lenticels of nodules of *Coriaria arborea*. The use of tracer fluorescent bacteria proved to be an effective direct method of assessing penetrability and also afforded scope to observe the presence of any barrier formation.

In our study the apparently suberized cell walls were an important barrier to penetration by bacteria. This is illustrated by the extensive penetration by fluorescent bacteria that occurred in mature tubers held in anaerobic conditions, where barrier formation in proliferated lenticels was inhibited. Very few lenticels in mature tubers held in aerobic conditions and showing (even when proliferated) well developed layers of phelloderm cells showed penetration by bacteria or fine particle suspensions. This indicates that lenticels on newly planted seed tubers, even in wet soils, are poor entry sites for bacteria.

As explained earlier, most biocontrol agents have been applied to tubers just before planting, whereas blackleg bacteria enter lenticels during the previous growing season and remain latent until the following year. It follows that applications of biocontrol formulations just before planting, even if the lenticels proliferate in the soil, does not allow good penetration of lenticels and hence gives the agent little chance of interacting with the resident *Erwinia* population to suppress its growth. It is possible that the appropriate time to apply the agent is to the growing crop early in the previous season.

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