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Biocontrol of the pathogen *Phytophthora parasitica* by arbuscular mycorrhizal fungi is a consequence of effects on infection loci

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The impact of colonization by the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* on tomato root necrosis caused by the soil-borne pathogen *Phytophthora parasitica* was investigated. Studies *in situ* permitted infection loci to be identified and the effects of the AMF on numbers to be elucidated. Effects were significant and, 7 and 16 days after inoculation with zoospores of the pathogen, roots of plants colonized by the AMF had 39% and 30%, respectively, fewer infection loci than those that were not. Concurrent studies of the rate of spread of necrosis within roots showed no changes caused by the AMF. At harvest, 26 days following inoculation with the pathogen, 61% of roots of noncolonized plants were necrotic compared with only 31% in AMF-colonized plants. It is concluded that effects on numbers of infection loci are one mechanism via which AMF achieve biocontrol of this pathogen in tomato. Measures of the effects of the AMF on root system architecture suggest that no significant changes occur and are thus not the reasons for the reduction in infection loci. The implications of these data for agricultural practice and biocontrol research are discussed.

Keywords: biological control, *Lycopersicon esculentum*, mycorrhiza, root architecture, sporulation

Introduction

The control of plant pathogens by applying biocontrol techniques has potential to reduce chemical inputs to agriculture and significantly enhance global sustainability. In recent years, several different organisms and micro-organisms that show natural antagonism to pathogens have been identified and much research is now ongoing, aimed at harnessing the potential that they suggest. One group of micro-organisms that shows particular promise is the arbuscular mycorrhizal fungi (AMF). This is an economically and ecologically important group of symbiotic fungi that colonize the roots of over 80% of plant species and are almost ubiquitous in natural and agricultural terrestrial ecosystems. Research has now demonstrated unequivocally the importance of AMF in controlling pathogens in natural systems and their potential for biocontrol of pathogens in agriculture. Important microbial pathogens such as *Phytophthora* (e.g. Mark & Cassells, 1996; Norman *et al.*, 1996; Trotta *et al.*, 1996; Cordier *et al.*, 1998), *Fusarium* (e.g. Zambolim & Schenck, 1983), *Aphanomyces* (Rosendahl, 1985) and *Sclerotium* (Krishna & Bagyaraj, 1983) have all been demonstrated to be bio-controlled by AMF and the area is the subject of several reviews (e.g. Hooker *et al.*, 1994; Azcon-Aguilar & Barea, 1996).

Moreover, the application of AMF to agricultural systems is particularly promising because not only do these fungi offer potential to control pathogens but they also provide other benefits including enhanced phosphorus nutrition and tolerance of metal toxicity and drought. However, potential benefits to agriculture are not currently optimized for two major reasons. Firstly, intensive agricultural practices, and in particular the application of large quantities of chemical fertilizers, can often lead to a reduction in colonization by AMF. Secondly, there has been little research into the mechanisms by which biocontrol by AMF is achieved; thus opportunities for identifying particularly effective AMF species and/or isolates or agricultural practices that favour their persistence or function are limited.

Several different mechanisms have been proposed to explain biocontrol by AMF including changes to nutrient status, biochemical changes in plant tissues,

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anatomical changes to cells, stress alleviation, microbial changes in the rhizosphere and changes to root system morphology (Hooker *et al.*, 1994). However, the likelihood is that many different mechanisms operate, with the relative importance dependent on plant/AMF genotype and environmental conditions. Research strategies should thus be to first identify the range of mechanisms involved and then scale the relative importance of each.

Here, research to identify mechanisms important in AMF biocontrol of the pathogen *Phytophthora parasitica* in tomato is reported. Previous research has demonstrated that biocontrol occurs and that enhanced nutrition, although possibly involved, does not explain all of the control achieved (Trotta *et al.*, 1996). There is also evidence from other studies using the same plant and pathogen species that biochemical changes take place in root cells (Pozo *et al.*, 1996) and that proliferation of the pathogen is greatly reduced in root systems colonized by AMF (Cordier *et al.*, 1996). More recently, both localized and induced systemic resistance (ISR) were also reported to be induced by AMF in a *Phytophthora parasitica*–tomato system and to be associated with a reduction in development of the pathogen (Cordier *et al.*, 1998).

In this paper, experiments that elucidate further the nature of biocontrol conferred by AMF are reported. Previous studies have all investigated events that take place once infection of the root by the pathogen has occurred. This study investigates infection events. It specifically tests the hypothesis that colonization by an AMF protects against the root pathogen *P. parasitica* in tomato by reducing the number of infection loci. Microcosms have been used that permit observation *in situ* and allow confounding effects of necrosis-linked changes in nutrient supply to be eliminated. Moreover, because (in studies with other species) AMF colonization has been linked to altered root system architecture (e.g. Hooker *et al.*, 1992; Berta *et al.*, 1995), and there is some evidence that these changes may be involved in biocontrol (Norman *et al.*, 1996), links between these two events are investigated.

Materials and methods

In these investigations microcosms were used that permitted the root system to grow within a sand substrate in the gap between two glass plates and allowed the roots to be visualized *in situ* (see Forbes *et al.*, 1996 for full specification). Nutrients were supplied continuously to eliminate localized changes occurring in the rhizosphere and also to prevent differences in nutrient supply confounding the data. Using these systems it was possible to identify *Phytophthora* infection points or loci within the root system and also measure the spread of necrosis. Moreover, root system architecture could be measured in the early stages of growth.

Each microcosm essentially consisted of a 'sandwich' constructed from two glass sheets between which the roots of the tomato plants could grow. The gap was 5 mm (this distance was selected as preliminary investigations of tomato roots had shown that none would reach this diameter under these growth conditions). Twenty microcosms were constructed and the gaps filled with (450 mL) 1- to 2-mm silica sand that had been washed and autoclaved (121°C for 60 min, twice). AMF inoculum, *Glomus mosseae* (BEG 12), was added to the sand in ten of the microcosms. This was achieved by distributing 5 g (dry weight) macerated root pieces of precolonized tomato plants within the sand before it was placed into the microcosms. The same quantities of noncolonized root pieces were mixed similarly into the sand of the remaining ten microcosms. Roots used as inocula were obtained from plants grown in a sterilized sand and soil medium. To ensure that contamination with other AMF or plant pathogens did not occur, plants were supplied with only sterile water and a 1/4-strength Hoaglands nutrient solution, also made up with sterile water. Roots were stained prior to use and examined for the presence of non-AMF fungi.

Seeds of tomato, *Lycopersicon esculentum* cv. Earlymech, were surface-sterilized by immersing in 5% sodium hypochlorite for 5 min and then rinsed in sterile de-ionized water. Five seeds were then sown into the sand near the centre of the upper edge of each microcosm. The twenty microcosms (ten inoculated with roots containing *Glomus mosseae* and ten with noncolonized root pieces) were maintained within a growth cabinet under conditions of 16 h light (24°C) and 8 h dark (22°C) and a humidity of 80%. Each microcosm was supplied with 1/4-strength Long Ashton nutrient solution (Hewitt, 1966), modified to contain 16 μM P, at a rate of 800 mL every 24 h, using a peristaltic pump via four tubes distributed equally along the open upper end of the microcosm, each with an internal diameter of 2 mm. Excess nutrient solution was removed via a drainage tube at the base of each microcosm. For the duration of the experiment the microcosms and associated tubing were covered completely with either black paint or black polythene, secured with Velcro, to prevent entrance of light whilst allowing temporary access for data collection. After germination, tomato seedlings were removed to leave one plant in each microcosm.

Twelve days after planting the complete root system formed by each tomato seedling (on both sides of the microcosm) was traced *in situ* onto separate transparent sheets. Roots of different orders (in order of development, i.e. 1^0, 2^0, 3^0, etc.) were recorded in different colours to facilitate automated processing (see below). This was repeated 20, 26 and 34 days after sowing (only data from the last three dates are shown). Measurements were not possible beyond this because the complexity of the root system made it impossible to determine root order accurately.

Fifty-five days after sowing AMF-inoculated plants were colonized, with arbuscles, vesicles and external

mycelium visible (determined by destructive sampling of two additional microcosms set up as above). Five of the AMF-colonized plants and 5 of the plants not colonized by AMF were inoculated with zoospores of *Phytophthora parasitica* (isolate 201 kindly provided by S. Gianinazzi, INRA-CNRS, Dijon, France). Zoospores were produced as follows. The pathogen was first cultured on clear V8 agar in the dark for 2–7 days. Ten pieces of mycelium (4-mm blocks) were then cut from the growing culture and placed in 9-cm Petri dishes, each containing 20 mL filtered soil extract (obtained by filtering 1 litre sterile de-ionized water through 200 g of soil, first through glass wool and then through Whatman no. 1 paper). The Petri dishes were placed in an incubator at 24°C for 2 days in darkness followed by 5 days in the light (light provided by fluorescent tubes within the incubator). Zoospores were then obtained by chilling the dishes (4°C) for 1 h and returning them to 24°C. Zoospores were released after 30 min. The soil extract containing the zoospores was filtered through Whatman no. 541 filter paper in order to eliminate fragments of mycelium. Approximately 7×10^{-4}–9×10^{4} zoospores mL^{-1} were obtained. Each plant was inoculated with 200 mL of this filtered soil extract. Inoculation was achieved by removing microcosms from the growth cabinets and sealing the drainage tube of each. The zoospore suspension was then poured into each microcosm to saturate the sand completely. The upper edges of the microcosms were then taped and they were placed horizontally for 3 h, being rotated onto the opposite side after 90 min. The zoospore suspension was then drained from each microcosm. Plants not inoculated with *Phytophthora* received 200 mL of the soil solution without zoospores. The plants were returned to the growth cabinets and allowed to grow for a further 26 days before harvest. The number of infection loci that formed on the root system was counted 7 and 16 days following inoculation. These were easily identified, using a stereo microscope, as necrotic areas on the surface of the root. After 16 days it became impossible to correctly distinguish separate points. Where location on the root made it discernible, the rate (percentage increase) of spread of necrosis along the root was measured between 7 and 10 days following inoculation with the pathogen (replicate measures in each microcosm ranged from 14 to 21).

At harvest (81 days after sowing, 26 days after inoculation with *Phytophthora*) root necrosis was measured using a grid-line intersect method (Kennedy *et al.*, 1986). Dry weights of stems, leaves and flowers plus tomato fruits were obtained by drying at 80°C to a constant weight and N, P and K analysis was performed using standard techniques. Roots were assessed for colonization by AMF using a staining procedure adapted from Koske & Gemma (1989) and a grid-line intersect method (Giovanetti & Mosse, 1980).

Root-system tracings on transparent sheets were analysed using image analysis (Quantimet 600, Leica), which provided data on root system length and numbers and lengths of individual root orders. Root lengths of harvested plants were measured similarly.

Data were analysed by ANOVA and Student's *t*-tests (GraphPad Prism, GraphPad Software, USA).

Table 1 Growth of AMF-colonized (+AMF) and noncolonized (–AMF) tomato following inoculation (+P) or not (–P) with zoospores of *Phytophthora parasitica*

Treatment	Leaves (g)	Stem (g)	Fruits/ Flowers (g)
–AMF, –P	5·86	3·85	1·92
–AMF, +P	4·67	3·03	1·59
+AMF, –P	5·25	3·51	1·96
+AMF, +P	4·92	3·32	2·44

Figures are means of determinations from five replicate microcosms at harvest, i.e. 81 days after sowing, 26 days after inoculation with *P. parasitica*. Figures within each column are not significantly different from each other ($P < 0·05$).

Results

As expected, because nutrient supply was maintained continuously (in a nutrient flow), there were no effects of root necrosis on shoot growth (Table 1). Nutrient (N, P and K) contents of roots and shoots were also similar (data not shown). In AMF-inoculated plants roots were 30% colonized.

No differences in root architecture were evident between AMF-colonized and noncolonized plants, with similar numbers of first to fourth order roots counted 20, 26 and 34 days following sowing (Fig. 1a–c). Although there were differences evident in the very low numbers of fifth order roots observed at days 26 and 34, these were not significant. Data for root length showed similar trends (not shown).

Seven and sixteen days following inoculation with *P. parasitica*, infection loci were counted. Numbers were reduced in AMF-colonized plants, with 39% and 30% fewer loci after 7 and 16 days, respectively (Table 2). There were no differences in the rate of spread of necrosis from these initial points of infection in AMF-colonized and noncolonized roots, with similar changes in the size of necrotic regions measured (Table 2).

At harvest (26 days following inoculation with *P. parasitica*), necrosis of roots in noncolonized plants was extensive, amounting to 61% of the root system (Table 3). However, in AMF-colonized plants the figure was only 31%. The length of root systems was also changed by the presence of the AMF, with the length of colonized root systems increased by 50% (Table 3).

Discussion

These data provide further evidence for biocontrol of the tomato root pathogen *P. parasitica* by the AMF *Glomus mosseae*. However, although reductions in root necrosis were relatively large they are not without

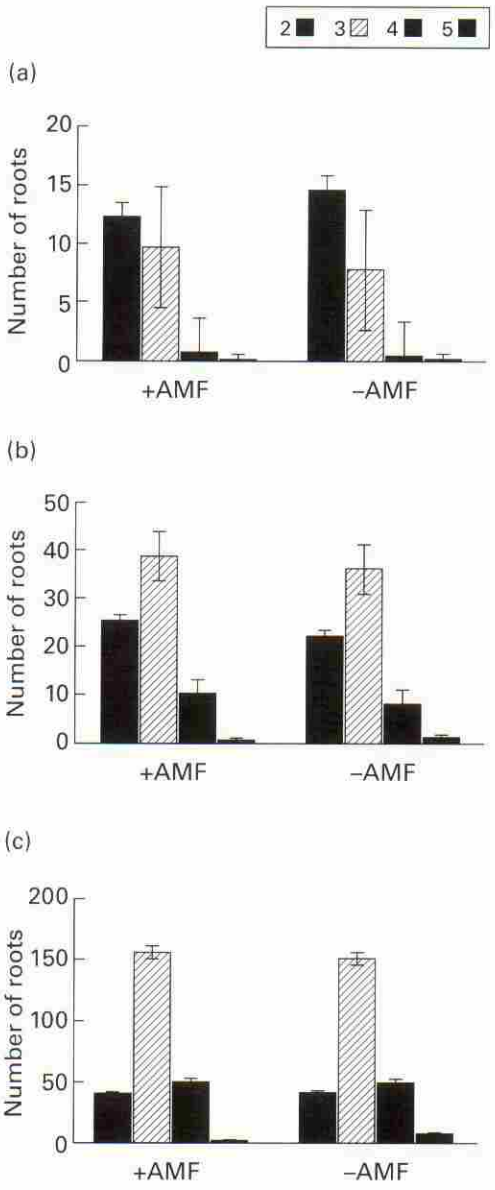

Figure 1 Effects of colonization by AMF on root system architecture of tomato (a) 20 days (b) 26 days (c) 34 days following sowing. Columns show numbers of second order roots (2), third order roots (3), fourth order roots (4), and fifth order roots (5). Figures are mean determinations from ten microcosms.

precedent, with similar levels of AMF-induced reduction in necrosis caused by *Phytophthora* observed previously. For example, Norman *et al.* (1996) reported a similar reduction in root necrosis caused by *P. fragariae* in micropropagated strawberry and there is also a recent report by Cordier *et al.* (1998) of a 79% reduction in necrosis caused by *P. parasitica* in tomato in the presence of the same isolate of AMF as was used here, *Glomus mosseae* (BEG 12).

The extent of the biocontrol achieved clearly suggests possible practical benefits of AMF as protective agents against disease caused by *P. parasitica* in tomato.

Table 2 Number of infection loci and rate of necrosis spread in AMF-colonized (+AMF) and noncolonized (–AMF) tomato following inoculation with zoospores of *Phytophthora parasitica*

Treatment	Number of infection loci per plant		Rate of necrosis spread
	7 days	16 days	(% increase)
–AMF, +P	122 a	510 a	38 a
+AMF, +P	74 b	360 b	34 a

Figures are means of determinations from five replicate microcosms, 7 and 16 days after inoculation with *P. parasitica*, i.e. 62 and 71 days after sowing. Figures within the same column followed by the same letter are not significantly different from each other ($P < 0.05$). No infection loci were present in noninoculated roots.

However, the most interesting aspect of the data is what they indicate of mechanisms. In any host–pathogen interaction, the extent of disease will depend upon three factors: (i) the number of infection loci that form on the root, (ii) the rate of pathogen spread within the root, and (iii) time. Clearly, a biocontrol agent can influence only (i) and (ii) and so these are presently useful starting points for any study into mechanisms. In this study the effects of the AMF on both of these parameters were measured to test the hypothesis that effects on the number of infection loci were, at least in part, responsible for biocontrol of *P. parasitica* by AMF.

These data clearly show that the number of infection loci is reduced in root systems colonized by the AMF (Table 2) and thus provide the first evidence for this mode of action. They also show that colonization by the AMF had no measurable effect on the spread of necrosis. This is an important finding as it suggests that the factor primarily responsible for determining the degree of necrosis was, in this case, the number of infection loci formed, i.e. an infection-related process. Further evidence to support this hypothesis can be derived from the data in Tables 2 and 3. Here, the ratio of the number of loci present on the roots of AMF-colonized plants to that on the roots of noncolonized plants 16 days following inoculation (Table 2) can be calculated as 0·71, similar to the ratio of 0·76 between the corresponding lengths of necrotic roots present in the same plants at harvest (Table 3).

It is, of course, possible that the experimental system could have influenced the outcome and it cannot be

Table 3 Length and necrosis of AMF-colonized (+AMF) and noncolonized (–AMF) tomato roots following inoculation with zoospores of *Phytophthora parasitica*

Treatment	Total root length (m)	Necrotic length (m)	Necrosis (%)
–AMF	29·6	18·2	61
+AMF	45·0	13·8	31

Figures are means of determinations from five replicate microcosms at harvest, i.e. 81 days after sowing, 26 days after inoculation with *P. parasitica*. Figures within the same column are significantly different from each other ($P < 0.05$). No necrosis was present in uninoculated roots.

concluded that infection-related processes would be as important in other circumstances. For example, by design the nutrient flow system employed minimized effects of nutrient limitation due to root damage and maximized opportunities for secondary infection. However, the aim of these investigations was not to replicate field conditions or to scale the relative importance of different mechanisms, but to identify the mechanisms themselves. Nevertheless, it is possible to draw some parallels. The microcosm system would, for example, be similar to the environment found in free-draining soils with a high water content or in horticultural rock-wool-based growth systems, so would demonstrate likely events and outcomes under these conditions.

It is clear that the effect of AMF on numbers of infection loci is a mechanism through which biocontrol of *P. parasitica* can occur. This finding will have important implications for both future mechanism-led research and also for research into biocontrol applications. For the latter, these data suggest, for example, that the timing of inoculation with AMF and the dynamics of colonization are likely to be very important, with the protective effect dependent on reducing primary and secondary infection by the pathogen at the root epidermis, rather than retarding development *in planta*.

Precisely how the AMF interacts with *P. parasitica* and affects zoospore encystment and/or penetration is not evident from the present data. It is hypothesized that, if changes in number of infection loci occurred, they could be due to altered root development induced by AMF. However, no significant changes were measured, with both noncolonized and AMF-colonized root systems having a similar architecture (Fig. 1a–c). Although unusual, this is not unique. Most plants studied to date have exhibited increased branching of roots in response to colonization by AMF (e.g. Hooker *et al.*, 1992; Berta *et al.*, 1995; reviewed by Atkinson *et al.*, 1994), but reductions in branching and even interactions with temperature have also been reported (Forbes *et al.*, 1996). The factors and events involved in these effects on number of loci clearly deserve investigation. Interestingly, it was observed in a separate study that root exudates from AMF-colonized strawberry plants reduce sporulation of *P. fragariae* (Norman & Hooker, 2000). If similar changes and events occur in the association between AMF, tomato and *P. parasitica*, they could explain the present results. However, further investigations are needed to test this hypothesis. Other factors that could explain these observations include AMF-induced changes to root exudation resulting in changed zoospore chemotaxis, and direct effects of AMF on epidermal cells resulting in altered susceptibility to infection. Effects on surface electrical charge and thus changes to electrotaxis of zoospores are also a possibility. However, it should be recognized that the original AMF inoculum was not derived from axenic culture and the microcosms were not maintained as a closed system, although precautions were taken to reduce contamination by other micro-organisms. Accordingly it is possible that the changes measured may have resulted from changes induced by AMF on the population dynamics of other, antagonistic, micro-organisms within the microcosm. The involvement of these processes should be studied further.

To conclude, although a mechanism by which AMF protects against infection by *P. parasitica* has been identified, it is not presented as a universal phenomenon. There is already sufficient evidence from studies with tomato and *P. parasitica* alone (Cordier *et al.*, 1996; Pozo *et al.*, 1996; Trotta *et al.*, 1996; Cordier *et al.*, 1998) to suggest that a range of different mechanisms exists. Alternatively, it is hypothesized that effective 'biocontrol' by AMF is likely to be a consequence of several, possibly interacting, mechanisms. The challenge for research in the future must be to increase understanding of the range of mechanisms involved and determine the plant, pathogen, AMF and environmental factors that together dictate the scale and timing of their expression.

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An efficient baiting assay for quantification of *Phytophthora cinnamomi* in soil

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A quantitative baiting assay system for *Phytophthora cinnamomi* with greater sensitivity than the orthodox serial dilution end-point method was developed. A system of efficiently handling large numbers of subsamples using racks of tubes is described, and the factors determining baiting efficiency with blue lupin (*Lupinus angustifolius*) radicles were studied. Significantly greater baiting efficiency was obtained when the subsample size was decreased. The optimum temperature was found to be ≈ 25°C for baiting and 20–25°C for bait incubation. Air-drying of soil for two days killed *P. cinnamomi* propagules. Escape of zoospores from test soil was reduced by the presence of overlying material. Reduction was greatest when the overlying material was of fine particle size and of greater depth. This confirmed the need in assay work to keep soil subsample size small and avoid destruction of the soil structure in preparation. The peak of zoospore release from naturally infested soil occurred on the first day. Infection on radicles decreased as a function of distance from radicle tip.

Keywords: air drying, baiting assay systems, *Phytophthora cinnamomi*, premoistening, soil particle size, temperature

Introduction

As part of a larger programme of research to develop biological control of *Phytophthora* spp., an avocado orchard site that was infested with *Phytophthora cinnamomi*, and which was eventually to be replanted with avocado, was used to test biocontrol treatments. In order to assess these treatments, it was necessary to quantify the level of *P. cinnamomi* present in the soil over time. The orthodox serial dilution end-point technique (Tsao, 1960) was considered appropriate as an initial guide to infestation levels, but neither well suited to quantifying low levels of inoculum nor sufficiently accurate. This work aimed to develop a more sensitive and statistically more robust technique.

A variety of techniques have been used to estimate numbers of propagules of a pathogen in soil. Historically, the most significant and most widely used have been the soil dilution plate method and the serial dilution end-point method. The principles and application of the soil dilution plate method are described by Johnson & Curl (1972). Tsao (1983), in discussing the method, stated that it is effective when the *Phytophthora* inoculum density is relatively high (> 10 propagules per g of dried soil). Naturally infested soils often have low levels of *Phytophthora* and hence require low dilutions, and it was not deemed an efficient method unless the antimicrobial agents in the selective medium were highly effective (Tsao, 1983). Lower dilutions normally result in much greater interference by commensal organisms.

The serial dilution end-point method of Tsao (1960) is commonly used to estimate the disease potential of *Phytophthora* spp. in soil. The soil is diluted with sterilized soil in a series. Soil samples are saturated with distilled water and a susceptible host is incubated at a suitable temperature in the water above the soil as a bait. Typically, three seedlings in three replicate pots are used for each weight, in a series from 50 g down to 1/256 of this. The disease potential index is the reciprocal of the highest dilution giving a positive result. Weste & Ruppin (1977), in isolating *P. cinnamomi* from forest soils in Australia, used a modified method, omitting the sterile dilutant soil.

A variety of seedlings, plant parts and fruit have been used as baits for *Phytophthora* spp. (Tsao, 1983; Erwin & Ribeiro, 1996). For *P. cinnamomi*, *Eucalyptus* seedlings, conifer needles and lupins are among the baits commonly used.

Surface disinfection of plant material with ethanol or sodium hypochlorite to reduce surface contamination prior to plating is normal practice. However, disinfection may reduce isolation success (Montgomerie &

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