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DEVELOPMENT OF BIOCONTROL BACTERIA AS A NURSERY TREATMENT TO PROTECT YOUNG TREES FROM FUNGAL PATHOGENS

FINAL REPORT

N. Mesanza[1,2], B.D. Crawford[1], T.J.D. Coulson[1], E. Iturritxa[2], and C.L. Patten[1*]

[1]Biology Department, University of New Brunswick, Canada; [2]Production and Plant Protection, Neiker Tecnalia, Spain; *Principal Investigator

Background and objectives

Tree root and butt rot diseases are often caused by the fungi *Heterobasidion annosum* and *Armillaria mellea* and are characterized by chlorotic leaves, progressive thinning of the crown, slower leader growth, and rapid tree death (Edmonds et al., 2000). *Armillaria* is distributed worldwide (Wingfield et al., 2010) with a broad host range such as conifers, hardwoods, shrubs and some herbaceous plants (Williams et al., 1986), while *Heterobasidion* distribution is mainly limited to coniferous forests and plantations in the northern hemisphere (Korhonen et al., 1998, Woodward et al., 1998). Both fungi can colonize different hosts by direct contact between hyphae and roots, or by dispersal of spores (Rishbeth, 1959).

Currently, *Heterobasidion* and *Armillaria* infections are managed using silvicultural, chemical, and biological methods. Silvicultural practices include planting less susceptible tree species, stump removal, using proper planting and mixture schemes, and thinning when the spores are not dispersing. Chemical treatments are based on urea and borate, or soil fumigants such as methyl bromide and carbon disulphide, and biological control requires inoculating stumps with the fungi *Phlebiopsis gigantea* (Asiegbu et al., 2005) or *Trichoderma* after fumigation (Baumgartner et al., 2011). The application of these treatments is limited and often ineffective due to factors such as level of infection, environmental conditions and risks, cost, and legislation, among others. For example, the use of urea and borate causes temporal modifications in soil chemistry, and damages the ground vegetation and the structure of the fungal community (Asiegbu et al., 2005). The use of these chemicals is prohibited in many countries (Gonthier and Thor, 2013). The effective soil levels of fungal biocontrol agents may be difficult to attain (Shaw and Roth, 1978). While *Phlebiopsis gigantea* effectively inhibits the spread of *Heterobasidion* following colonization of stumps by basidiospores, it reduces fungal diversity and its use is approved in only a few countries (Gonthier and Thor, 2013).

Antagonistic bacteria are an attractive alternative treatment to protect trees against fungal pathogens. Biological control with bacteria has been proven effective against fungal pathogens of agronomic crops (Mark et al., 2006) and in fewer cases against forest fungal pathogens (Singh et al., 2008). Antagonism by bacteria is achieved by different mechanisms including antibiosis, competition for nutrients, parasitism, and induced resistance in the host (Whipps, 2001). The capacity of the bacteria to colonize the rhizosphere or the host seeds, and adapt to soil conditions are also factors (Mark et al., 2006). We have isolated bacteria from the roots of a healthy pine

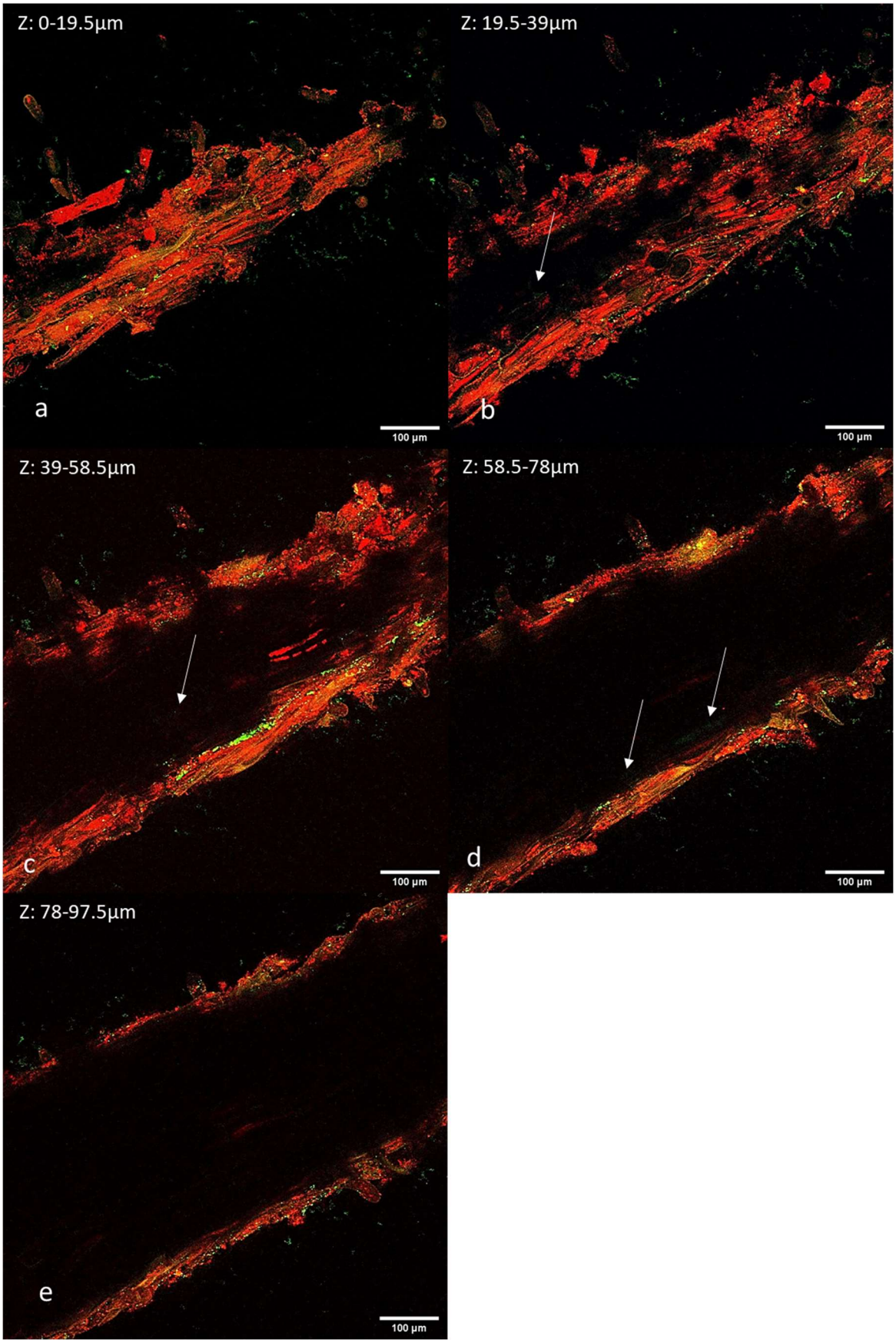
tree growing in a forest infected with *Heterobasidion* and *Armillaria*. Selected bacterial strains demonstrated an antagonistic effect against *H. annosum* and *A. mellea in vitro* and *in vivo* (Mesanza et al., 2016). *Erwinia billingiae* S31R1 and *Bacillus simplex* S11R41 inhibited the growth of the fungi *in vitro* by 68%-99%, and reduced mortality of one-year-old *P. radiata* trees from *A. mellea* infection from 36.5% to 11.6% and 7.1%, respectively. The objective of the research described here was to evaluate the suitability of biocontrol bacteria as a nursery treatment for seedlings to protect young trees from infection with fungal pathogens. Specifically, we assessed the ability of the bacteria to stably colonize young trees by determining their location and survival in tree seedling roots following inoculation.

Results

Initially, we aimed to determine the behaviour of biocontrol bacteria on roots of different tree species including *P. radiata*, *Abies balsamea* and *Picea glauca.* Seeds were surface sterilized and stratified for a period of 15 or 30 days, depending on the tree species. Germination percentage and times, and seedling growth rates varied greatly among the tree species, and only *P. radiata* seedlings developed sufficiently and in the necessary numbers to proceed with inoculation and assessment of biocontrol bacteria.

To determine the colonization patterns of the biocontrol bacteria on *P. radiata* roots, *E. billingiae* S31R1 and *Bacillus simplex* S11R41 were transformed with plasmids encoding green fluorescent protein (GFP). Three-month-old *P. radiata* seedlings were watered with GFP-labeled *E. billingiae* or *B. simplex* suspensions, and roots were examined over a period of 31 days after inoculation (Mesanza et al., 2019). At each time point, fluorescent bacteria were visualized on the roots by confocal and/or epifluorescence microscopy. In general, although both bacterial strains were present all along the pine seedling roots, they were more abundant on the upper parts of the roots. However, the colonization patterns were different between the bacterial strains. *E. billingiae* colonized the intercellular spaces within the epidermal tissue (Fig. 1a-e) and appeared in clusters of high density (Fig. 1c). *B. simplex* was found mainly on the root surface and was distributed randomly (Fig. 1f-k). At later time points, clusters of *B. simplex* were found on the root surface between the primary and secondary roots.

We had initially planned to quantify the GFP-expressing bacteria on *P. radiata* roots using fluorescence-activated flow cytometry, however, preliminary tests indicated that the intensity of fluorescence from single GFP-labeled bacterial cells (especially *E. billingiae*) was low, the abundance of the bacteria (especially *B. simplex*) on roots was low, and the number of other, non-target root bacteria was high, rendering this method unsuitable. Therefore, bacteria were extracted from the roots, and GFP was quantified by sandwich enzyme-linked immunosorbent assay (ELISA) (Mesanza et al., 2019). Dilutions of the extracted bacteria were also plated on solid media containing appropriate antibiotics for quantification of viable, plasmid-containing cells. Both bacterial strains were detected on *P. radiata* roots over the duration of the 31-day experiment. Overall, *E. billingiae* colonized roots more rapidly and in higher numbers compared to *B. simplex*. After an initial increase in the population, the number of biocontrol bacteria decreased on roots. This may be due to depletion of nutrients or accumulation of secreted waste products, or the inability to detect fluorescent bacteria due to loss of the GFP expression and/or instability of the plasmid.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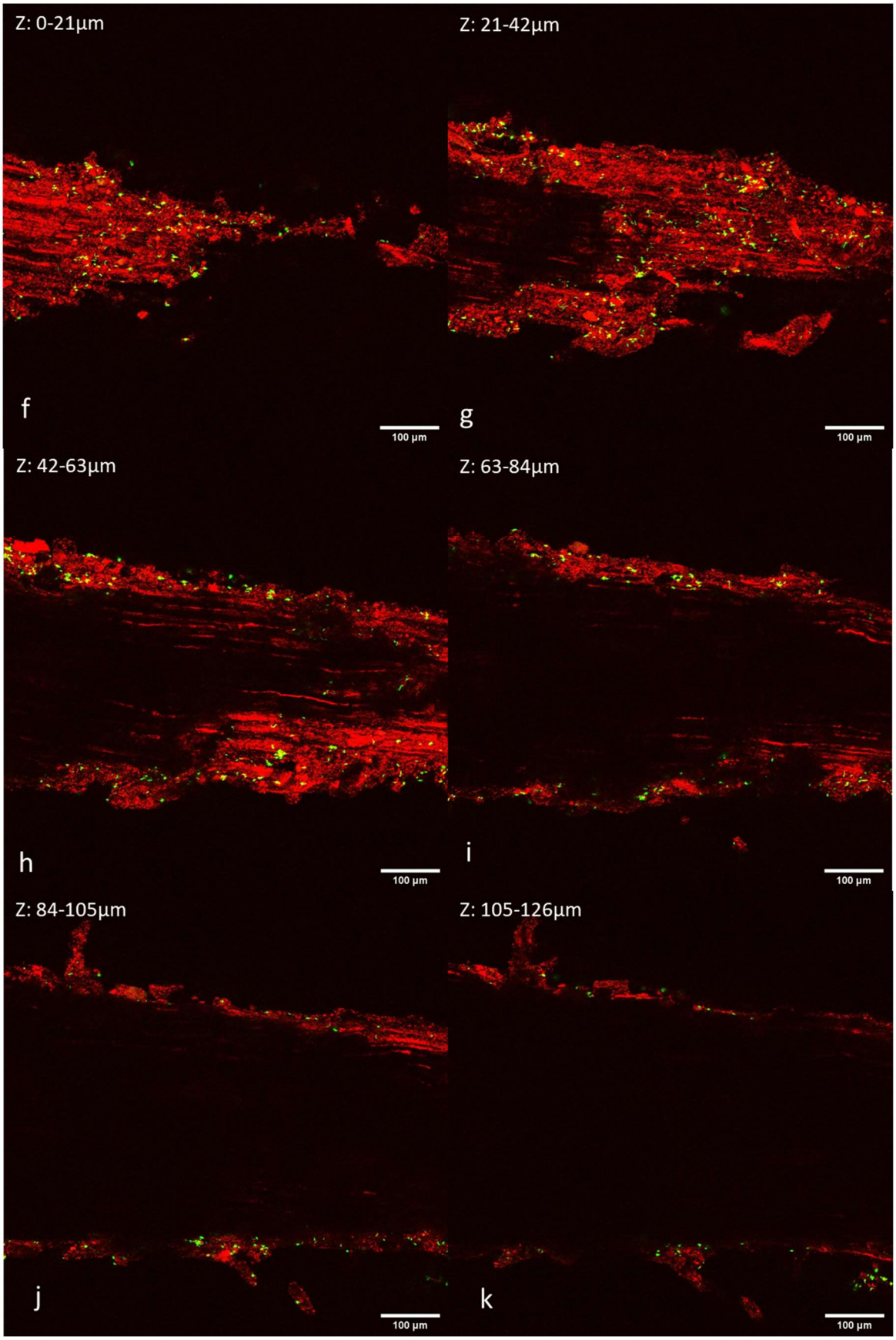
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Fig. 1. Colonization of *P. radiata* seedling roots by biocontrol bacteria. Bacterial strains appear as green fluorescence; plant tissue is shown as red autofluorescence. Each panel corresponds to an increment in depth of 19.5 µm in the z plane from the surface of *Pinus radiata* roots inoculated with *E. billingiae* (a-e) and 20 µm for roots inoculated with *B. simple*x (f-k). Green fluorescence from *E. billingiae* was detected in deeper layers (white arrows), while green fluorescence from *B. simplex* was only detected superficially. *E. billingiae* formed long clusters in deeper root layers (c).

In conclusion, the biocontrol bacterial strains assessed in this study may provide *P. radiata* roots with stable protection against pathogenic fungi. Although the population of fluorescent bacteria appeared to decrease over time, formation and localization of dense microcolonies in specific plant tissues suggest that both of the bacterial strains have the ability to colonize roots of *P. radiata* seedlings. *B. simplex* is primarily an exophyte while *E. billingiae* may be an endophyte. Typically, endophytic bacterial populations are more stable in the roots due to greater protection from environmental stresses, reduced competition with external competitors, and direct access to nutrients within plant tissues.

Significance

In the temperate forest, root and butt rot fungi are considered the greatest causes of economic losses (Garbelotto, 2004). The timber volume losses caused by *Heterobasidion* infection are due to tree decay, diameter growth reduction, wind throw and stand susceptibility to storm damages (Garbelotto and Gonthier, 2013). The infected trees are also more susceptible to pest infestations (Goheen and Otrosina, 1998). *H. annosum* was first reported in Ontario in 1955, is now well-established in southern Quebec, and is spreading north and east. It is also a significant problem in many other regions of Canada, the United States, and Europe. Economic losses in the forest industry due to *Heterobasidion* infection often reach more than a billion dollars annually. *Armillaria* usually causes the highest mortality in the early stages of plantation development, e.g. between 20-50% in the first six years of *P. radiata* stands (Hood and Sandberg, 1993). Once the plantation is in a medium-late stage of infection, volume losses are caused by lethal infections and chronic infections that reduce radial/length growth (Bloomberg and Morrison, 1989, Mallet and Volney, 1999). The best defence against these fungal infections is prevention.

Our overall goal is to develop a treatment to protect tree seedlings against *Heterobasidion* and *Armillaria* by applying beneficial rhizobacteria in the nurseries. Early application in the nursery is advantageous for several reasons. The volume of bacteria needed is lower and can be applied under controlled conditions. The bacteria have time to colonize and adapt to the rhizosphere conditions, and consequently, the seedlings are protected before they are in contact with the fungi. In combination with other management techniques, nursery treatment with biocontrol bacteria is expected to reduce economic losses due to lethal or chronic tree infections with fungal pathogens.

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