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Bursaphelenchus xylophilus and associated bacteria under oxidative stress conditions

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Plant pathogens have evolved a machinery of antioxidant enzymes and detoxyfying systems to reduce the plant oxidative burst impact upon invasion, and allow their successful colonization. Our study aimed to understand the contribution of *Bursaphelenchus xylophilus*-associated bacteria in interaction with the nematode, and as well independently, under oxidative stress conditions in an attempt to mimick their behaviour in the oxidative burst conditions of the host tree in the early stages of pine wilt disease (PWD). Thus, we begin by examining the oxidative stress resistance of three *B. xylophilus*-associated bacteria (*Serratia* spp. LCN-4, LCN-16 and PWN-146) (Vicente et al., 2011 and 2012), and as well *Escherichia coli* OP50 (control strain), in increasing concentrations of hydrogen peroxide (H_2O_2) ranging from 15 to 40 mM in a 24h-exposure period. We could see that all *Serratias* were able to tolerate the strong and prolonged H_2O_2 conditions, in contrast with control *E. coli*. Following, we checked the mortality of two isolates of *B. xylophilus* (virulent Ka4 and avirulent C14-5) in absence and presence of associated-*Serratia* and control strain in the same stressful conditions. Without bacteria (surface sterilized nematode), Ka4 and C14-5 presented significant differences in their ability to tolerate H_2O_2, being Ka4 clearly more resistant than C14-5. With *Serratia* spp., both Ka4 and C14-5 were able to survive at all H_2O_2 concentrations tested, with mortality rates lower than 10 %. In the presence of the *E. coli* OP50, mortality percentage of avirulent C14-5 was higher and closer to the values obtained in nematode alone conditions, with no statistical differences between treatments. These results indicate a beneficial and potential helper effect towards *B. xylophilus*, suggesting that these associated *Serratia* spp. are able to express several antioxidant enzymes and detoxifying systems, which explain their high tolerance to H_2O_2-mediated stress. Next, we focused on *B. xylophilus* catalase transcript levels to target H_2O_2. Two catalases were predicted in the *B. xylophilus* genome, BxyCTL-1 (BUX.s00579.159) and BxyCTL-2 (BUX.s01109.377), with a high protein similarity with other nematode catalases. Relative gene expression of catalase genes of *B. xylophilus* Ka4 and C14-5 in both absence and presence of *Serratia* spp. PWN-146 were studied under stress conditions (24h-exposure to 15mM H_2O_2) and

compared with non-stress condition. Bacterial effect was transversal to virulent and avirulent *B. xylophilus.* Relative gene expression of catalase genes of *B. xylophilus* show that, without bacteria, the virulent isolate Ka4, for both non-secreted *Bxyctl-1* and secreted *Bxyctl-2* genes, presented a 1.5-fold difference to avirulent C14-5. When in interaction with bacteria (*Serratia* spp. PWN-146), both virulent and avirulent *B. xylophilus* catalase levels decreased to levels comparable to normal conditions without oxidative stress, which is also in agreement with mortality test results.

Futher, we explored the bacterial interaction with *B. xylophilus*, namely the attachment to the nematode cuticle, an important characteristic in bacteria dissemination and that, to our knowledge, has not been studied before. We performed co-culturing of *B. xylophilus* and GFP-labelled bacteria in *Botrytis cinerea* plates. We observed that after 24-hour contact with *Serratia* spp. LCN-16, the density of nematode-attached bacteria was sparse, and no GFP fluorescence signal was detected from inside the nematode. From these results, adhesion of these bacteria to the nematode surface and organs seems to be weak and non-specific. Previously, Shinya *et al.* (2010) have shown, through scanning electron microscopy (SEM), the presence of few bacteria on the nematode cuticle even after the nematode was vigorously washed. *B. xylophilus*-associated bacteria are reported to be carried on the nematode's surface, and in average 290 were counted on the cuticle of PWN isolated from diseased trees (Zhao et al., 2003). If bacteria are not attached to the nematode surface, how can they be transported by *B. xylophilus* from and into a pine tree?

New insights into the nematode-bacteria interaction are given in this study. We report, for the first time, that *B. xylophilus* associated bacteria may assist the nematode opportunistically in the disease, and that a virulent *B. xylophilus* isolate was able to better tolerate OS conditions than an avirulent isolate.

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