RESPONSE OF PLANT-BACTERIA INTERACTION MODELS TO NANOPARTICLES

RÉSPONSE DES MODÈLES D'INTERACTION PLANTES-BACTÉRIES AUX NANOPARTICULES

RISPOSTA DEL MODELLO D'INTERAZIONE PIANTA-BATTERI ALLE NANOPARTICELLE

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Abstract

The aim of this study was to evaluate the possibility of using some models developed to study the plant-bacteria interaction mechanisms for the assessment of the impact of chronic exposure to nanoparticles. Rice-associated bacteria showed that some models are sensitive to the presence of NPs and allow a quantification of the effects. Further work needs to be performed in order to set appropriate reference baselines and standards to assess the impact of NPs on the proposed biological systems.
Key-words: *nanoparticles; plant-bacteria interaction; virulence; models; impact; rice*

Résumé

Le but de cette étude était d'évaluer la possibilité d'utiliser certains modèles développés pour étudier les mécanismes d'interaction plantes-bactéries pour l'évaluation de l'impact de l'exposition chronique à des nanoparticules. Rice-bactéries associées a montré que certains modèles sont sensibles à la présence de NP et de permettre une quantification des effets. D'autres travaux doivent être effectués afin de mettre en niveaux de référence et de normes appropriées pour évaluer l'impact des IP sur les systèmes proposés biologiques.
Mots-clés: *nanoparticules; interaction plantes-bactéries; la virulence; des modèles ; l'impact; le riz.*

Riassunto

Lo scopo di questo studio era di valutare la possibilità di utilizzare alcuni modelli sviluppati per lo studio dei meccanismi di interazione piante-batteri per la valutazione degli effetti dell'esposizione cronica alle nanoparticelle. Lo studio di alcuni batteri associati al riso ha dimostrato che alcuni modelli sono sensibili alla presenza di NP e possono consentire la quantificazione degli effetti. Ulteriore

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lavoro deve essere eseguito al fine di impostare le linee di base e gli standard appropriati di riferimento per valutare l'impatto delle NPs sui sistemi biologici proposti.
Parole chiave: *nanoparticelle; interazione pianta-batteri; virulenza; modelli; impatto; riso*

Introduction

Nanoparticles are entities that can be either of natural origin, or generated by chemical engineering processes or unintentionally produced by combustion. They are sized from 1 to 100 nm. Nanoparticle (NP) research is currently an area of intense scientific interest due to a wide variety of potential applications in biomedical, optical and electronic fields. Nanoparticles present possible dangers, both medically and environmentally (Mnyusiwalla *et al.*, 2003). Most of these are due to the high surface to volume ratio, which can make the particles very reactive or catalytic (Ying, 2001). They are also able to pass through cell membranes in organisms, and their interactions with biological systems are relatively unknown (http://ec.europa.eu/health/opinions2/en/nanotechnologies/l-2/6-health-effects-nanoparticles.htm). The importance for the impact they can have on the environment is growing due to an increased unintentional release into the environment or even because they find a practical use in several products already on the market. Therefore, being this increasing importance recognized, it is becoming a need to find some tools for measuring the impact on the environment and on human and animal health of NPs. There are already several reports in which the effect of NPs on biological systems are reported such as on soil microbial biomass (Vittori Antisari *et al.*, 2011), or on sea urchin (Fallugi *et al.*, 2012). The hazard of NPs accumulation and contamination in fetal liver and kidney tissues has also been studied (Gatti *et al.*, 2011), as well as the progressive accumulation and storage within lymphnodes (Iannitti *et al.*, 2010). In this respect some biological models developed to study mechanisms of interaction between different organisms could be evaluated and proposed to study the effects of NPs on biological systems such as those represented by plants and associated microbes.

In the last ten years a lot of effort has been dedicated to elucidate the interaction between plants and bacteria, both beneficial and pathogenic. One of the most studied crop is rice, because it represents the staple food for more than two billion people every day. There are many bacteria associated to rice, some are pathogenic and cause severe damages to the crop, such as *Xanthomonas oryzae* pv *oryzae* (Shen and Ronald, 2002), *Burkholderia glumae* and *B. plantarii* (Cottyn *et al.*, 1996; Coenye *et al.*, 1999) and *Pseudomonas fuscovaginae* (Mattiuzzo *et al.*, 2011). Other bacteria are less studied but have an important role since they are beneficial and either protect the plant from pathogens, or enhance the growth of the plant by means of produced hormone-like molecules. These are the so called PGRP bacteria (plant growth promoting rhizobacteria), such as *Pseudomonas fluorescens* or *Pseudomonas putida*. Another class of plant associated bacteria has become important and more studied recently, the endophytes, bacteria living in the plant

and having beneficial effects on the plant health. Among them, *Burkholderia kururiensis* is very often isolated from rice and has been studied recently for its potential benefical effects on the plant and the mechanisms of interaction (Suarez-Moreno *et al.*, 2010).

The purpose of this study is to assess the suitability and reliability of some models developed for studying the interaction between rice-associated bacteria and the host, with the aim of using them to investigate the potential effects of NPs on these bacteria, on the host and on the interaction between them. In addition, biological systems such as those reported above could be used as bio-indicators not only to assess the effects of NPs on the environment but also to quantify them. Therefore models previously developed and used for assessing the virulence and pathogenicity of these bacteria, as well as the beneficial effects of some other bacteria, have been used to assess the effects of NPs on the bacteria, the host and their interactions. The work is still in progress and has the aim of assessing the effects of NPs but also to propose models for this assessment.

Materials and methods

Experimental design. The NPs used in this study are the following: iron oxide (Fe_3O_4; 15-20 nm), passivated cobalt (Co, 28 nm), titanium oxide (TiO_2; 20-160 nm), nickel (Ni, 62 nm), silicon oxide (SiO_2, 4-40 nm), cerium oxide (CeO_2, 50-105 nm), tin oxide (SnO_2, 61 nm), nanosilver (Ag, 1-10 nm) (NanoAmor, USA). Stocks of NPs at concentration of 10 mg/ml were used and always sonicated before further dilution or addition to other solutions. The bacteria studied are the following: *Xanthomonas oryzae* pv *oryzae*, *Burkholedria glumae*, *Burkholderia plantarii*, *Burkholderia kururiensis*, *Pseudomonas fluorescens*, *Pseudomonas fuscovaginae*. The plants used for the study of host-bacteria interaction are *Oryza sativa* Italian cultivar Baldo, and *Chenopodium album*. A summary of the experimental plan is reported in figure 1.

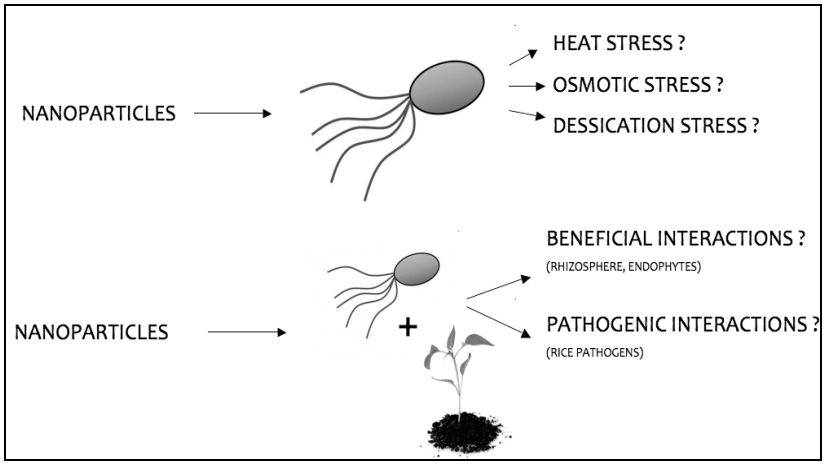

Figure 1

Experimental plan

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To assess the influence of NPs on bacteria, *Burkholderia kururiensis* and *Pseudomonas fluorescens* were grown in the presence of 10 and 100 mg NPs/ml culture, the growth curve determined and compared with the growth in the same media without NPs. The media used were M9 (a minimal defined medium) and King's B (KB, a rich undefined medium). In addition, the bacteria resistance to two stresses was measured when grown with and without NPs: heat and osmotic stress. Heat and osmotic stress were assessed as previously described by Kojic *et al*., (1999), exposing bacterial cells to either 50ºC for 10 min or 2 M NaCl and measuring the survival rate.

The effect of NPs on plant-bacteria interaction was studied using different approaches, depending on the bacteria considered and the host. *B. glumae* and *B. plantarii* were studied at the level of seeds colonization and germination because they are known to reduce seeds germination through seeds colonization. Ten seeds were put on a sterile filter paper into a disposable Petri dish and previously soaked with 5 ml of either KB medium or cultures of the two bacteria. The experiment was repeated for each NP and the controls were: seeds germination without bacteria and without NPs, and seeds germination without bacteria (NPs only). Petri dishes were incubated at 28ºC for 4 days and the length of roots and leaves was measured. The seeds colonization ability of each bacterial pathogen was estimated by measuring the number of colonies obtained from seeds germinated in the presence of NPs. Seeds from the germination experiment were grinded in a tube containing 1 ml of KB. The bacterial suspension was serially diluted and dilutions plated on KB agar for colony forming units (CFU/infected seed) determination.

Pseudomonas fuscovaginae was used to inoculate *Chenopodium album*. Before infection the pathogen was grown in KB with and without the addition of nanosilver at the concentration of 0.1 mg/ml. The inoculated plants were also grown with and without the presence of nanosilver in the soil at the concentration of 0.5 mg/ml.

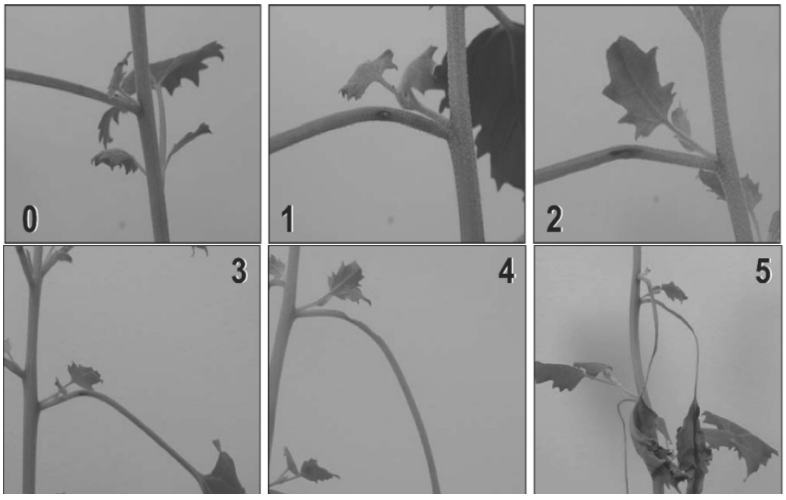

Figure 2

Score scale of the disease symptoms obtained by inoculating Chenopodium with Pseudomonas fuscovaginae

The plant inoculation and infection measurement were performed as previuosly described by Mattiuzzo *et al*., (2011), with a score given to the disease symptoms according to a scale from 1 to 5 (Fig. 2).
Rice leaves were inoculated with *Xanthomonas oryzae* pv *oryzae* (*Xoo*) by the clipping methods previously reported by Ferluga *et al*., (2009). Virulence and symptomes were estimated by measuring the lesion lengths caused by the pathogens along the central vein (Fig. 3).

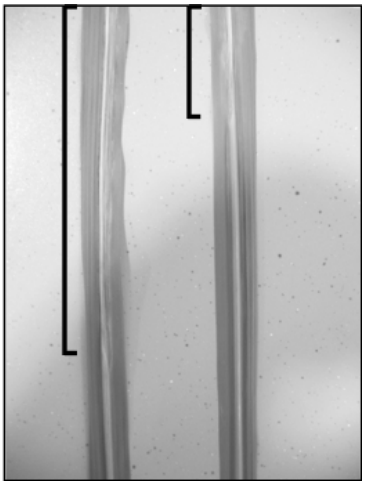

Figure 3

Lesion along the central vein on leaves inculated with Xanthomonas oryzae pv oryzae by the clipping methods.

Xoo was grown in PY medium at 28ºC with and without NPs. Rice plants used for being inoculated were also grown in the presence of NPs at the concentration of 0.5 mg/l of soil.

Rice plants grown in the greenhouse were grown up to the completion of the flowering stage and seeds production. Seeds obtained from rice exposed to NPs at the concentration of 0.5 mg/l of soil were then analyzed for the presence of the NPs to which the rice was exposed by means of the following method. The rice tissues were dried in a forced air oven (T<40°C) and homogenized (in a blender with blades made of pure titanium, carefully checked to not introduce any further metal contamination to samples). Unwashed plant samples were used. Approximately a 0.25 g sub-sample of plant tissues was dissolved in 8 mL of concentrated suprapure nitric acid plus 2 mL of Hydrogen Peroxide (Carlo Erba for electronic use). The mineralization was carried out in Teflon bombs in a microwave oven (Milestone, 2100). After cooling, solutions were made up to 20 mL with milli-Q water and then filtered with Whatmann 42. The accuracy of the instrumental method and analytical procedures used was checked using reference material, which was run after every 10 samples to check for drift in the sensitivity. The analytical quality of the results was checked against the following reference materials, which certify values of the studied elements close to the measured ones: CRM 060 (aquatic plants), CRM 062 (Olive leaves) provided by the European Commission Institute for Reference Materials and Measurements. The solution as analyzed by Inductive

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Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Spectro Ametek, Arcos).Also leaves, stems and roots of rice exposed to NPs were analyzed and the presence and concentration of NPs determined and compared with non-exposed rice. In addition seeds were tested for the germination ability under standard germination condition (in Petri dishes, onto a water-soaked filter paper, at 28ºC for four days).

Results

The experiments were performed with the aim of testing the suitability of some biological models to be used for assessing the influence of NPs on plant-associated and plant-bacteria interactions; following is a summary of the results obtained and some consideration about the reliability of these approaches.

Bacterial growth and stress resistance. When bacteria are grown in the presence of NPs the growth curves of the two bacteria tested are not significantly different from the control for both *Burkholderia kururiensis* and *Pseudomonas fluorescens*.

Pseudomonas fluorescens. No significant differences were observed when *Burkholderia kururiensis* was grown in the presence of NPs. On the other hand, *Pseudomonas fluorescens* showed a slower exponential growth phase when exposed to nanosilver at the concentration of 100 mg/ml; iron oxide seemed to enhance growth at concentrations higher than 10 mg/ml; and Sn seems to anticipate the stationary phase and cell death (Fig. 4).
Influence of NPs on the resistance to osmotic and heat stress was assessed in *B. kururiensis* (Fig. 5A) and *P. fluorescens* (Fig. 5B).
We found that Ce reduces the heat stress resistance in *B. kururiensis* while most of the NPs significantly reduce the heat and osmotic stress resistance in *P. fluorescens.*

Rice seeds colonization and infection by *Burkholderia*. When the rice seeds were inoculated with the two *Burkhoderia* pathogens, the parameter taken into consideration was the colonization ability by means of the CFU/infected seed. Colonization ability of both bacterial pathogens seems to be reduced by most of the NPs and is particularly evident for Ag and *B.glumae* and Si for *B.plantarii*. (Figure 6).
Another parameter considered was the length of the coleoptile in the germinated seeds, as a factor that could be affected by the infection and the NPs interference. Of course the NPs can interfere both in the seeds germination and in the infection of the pathogens, as shown by the results reported in figure 7.

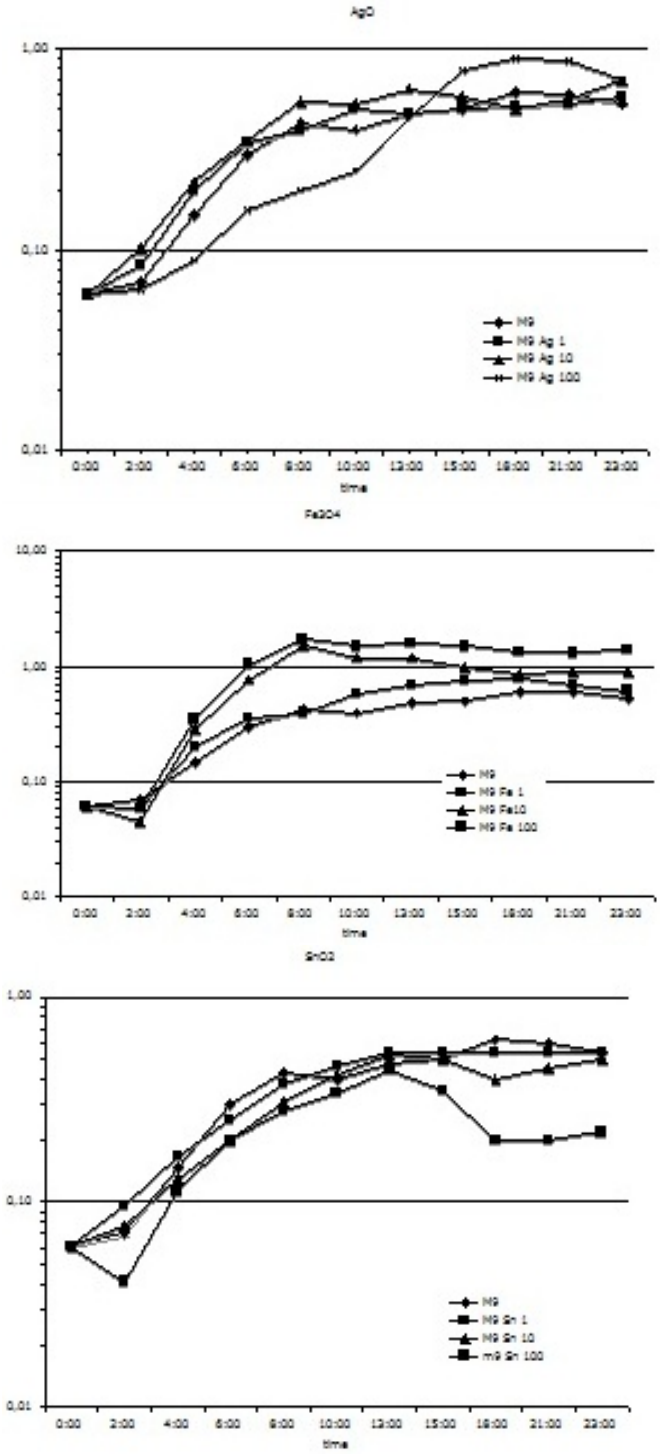

Figure 4

Growth curves of Pseudomonas fluorescens grown in the presence of Ag, Fe and Sn

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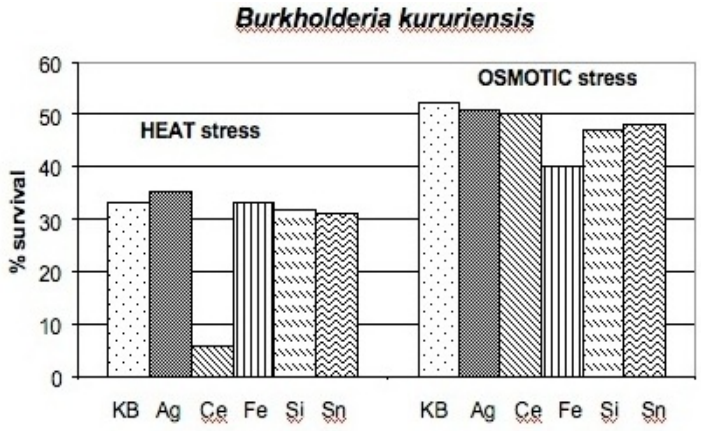
A



B

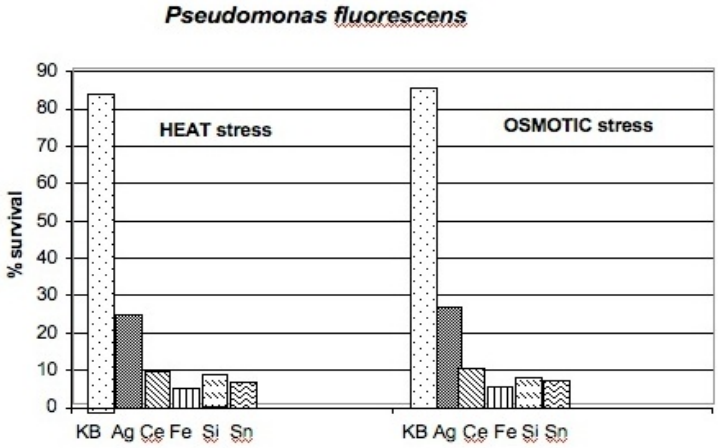
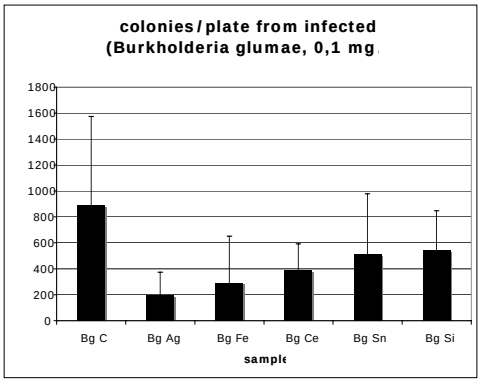
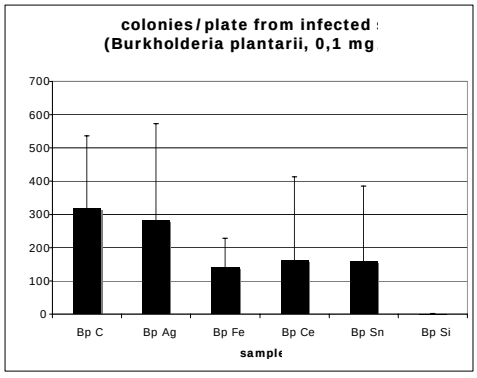


Figure 5

Survival rate of B. kururiensis (A) and P. fluorescens (B) when grown in the presence of NPs under stress conditions.

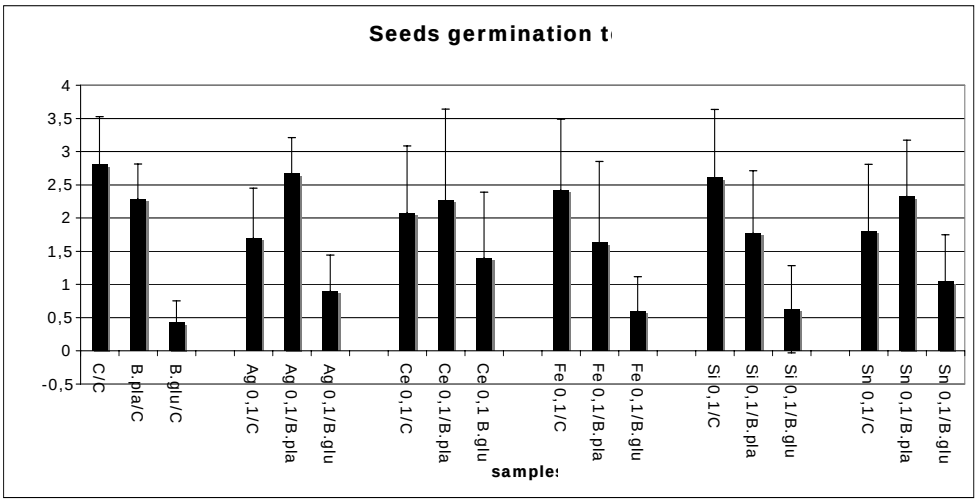
Figure 6 - *Colony forming units (CFU) per infected rice seed in the presence of NPs.*





The results suggest that some of the NPs tested seem to be able to reduce the germination of rice (i.e. Ag) while others seem to reduce the bacterial virulence (i.e. Ce with *B.glumae*). In addition, we found that Co reduces seeds germination and *B. glumae* virulence, while Ni and Ti seem not to have significant effects (data not shown).

Figure 7 - *Seeds germination and length of the coleoptile in the presence and absence of the two bacterial pathogens and of different NPs.*



Chenopodium infection by *Pseudomonas fuscovaginae*. The rice pathogen *Pseudomonas fuscovaginae* was used to inoculate the petioles of Chenopodium and the infection level determined using a disease score from 0 to 5. The results were not satisfactory due to the low sensitivity of the methods and the score used. However there were no significant differences when the experiment was performed in the presence and absence of nano silver (Fig. 8).

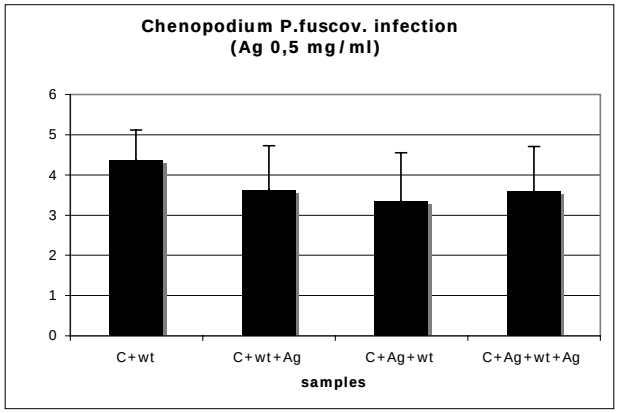


Figure 8

Chenopodium infected by P. fuscovaginae and related disease score. C+wt, no nano silver; C+wt+Ag, P. fuscovaginae grown in the presence of nano silver; C+Ag+wt, rice grown in the presence of nano silver; C+Ag+wt+Ag, rice and P. fuscovaginae grown in the presence of nanosilver

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Nanosilver and seeds germination. The rice grown in the green house in the presence and absence of nano silver was brought to seeds production. Seeds from plants exposed and non-exposed to nano silver were then germinated and the percentage and efficiency of germination estimated by means of the root length The results reported in figure 9 and table 1 show a sligthly reduced germination of seeds from rice exposed to nano silver with no significant difference in term of root development.

	experiment			
Germination rate (%)	**1**	**2**	**3**	**average**
- control	**72**	**79**	**46**	**65.6**
- nanosilver	**60**	**71**	**54**	**61.6**

Table 1

Germination rate of seeds from rice non-exposed and exposed to nano silver.

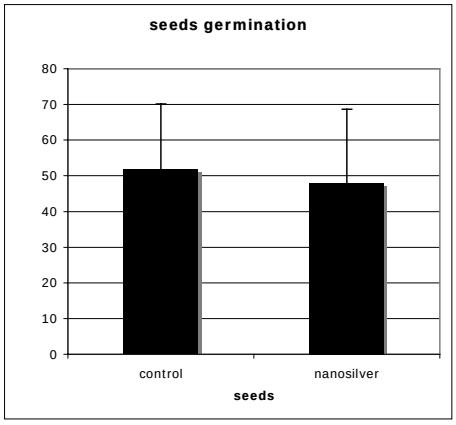

Figure 9

Root development in germinating seeds from rice exposed and non-exposed to nano silver.

Nanosilver tissue accumulation. The tissues from rice exposed and non-exposed to nano silver were analyzed after mineralization and the concentration of silver measured. The results are shown in Table 2. Silver concentrations are statistically different ($p<0.05$) between control and the two samples exposed to nano silver. Only in stems and leaves from experiment where nano silver was dissolved into the soil there is no accumulation. When nano silver was sprayed an higher concentration was found in the panicles.

	Test	**Ag added to**	**Ag spray**
Panicles and seeds	**0.23 a**	**0.61 b**	**1.45b**
Stems and leaves	**0.38a**	**0.29 a**	**0.92 b**
Roots	**0.34 a**	**1.4 b**	**0.97 b**

Table 2

Concentration of Ag in different rice tissues from plants exposed and non exposed to nanosilver.

NPs interference in Xoo rice infection. Interference of NPs in the rice infection by *Xanthomonas oryzae* pv *orzyae* was assessed by measuring the lesion length in infected leaves of rice plants exposed to NPs. Also Xoo used for infection was grown in the presence and absence of NPs. When *Xoo* was grown in the presence of Co, a reduced lesion length was found when compared to non-exposed rice, suggesting a inhibitory role of Co on *Xoo* growth and rice infection (Fig. 10).

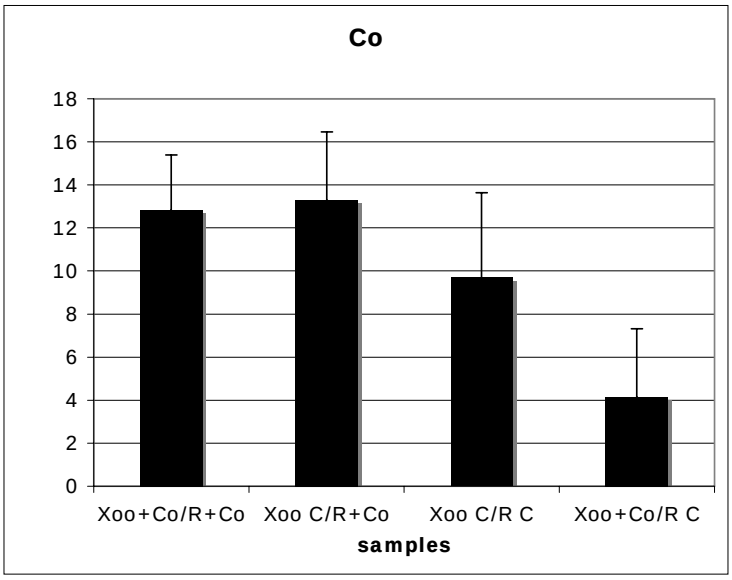

Figure 10

Lesion lengths in infected leaves of rice exposed (R+Co) and non-exposed (R C) to Co, and infected with Xoo grown in the presence (Xoo+Co) and absence (Xoo C) of Co.

Conclusion

The aim of this work was to developed and evaluate to be used for the assessment of NPs influence on bacteria, plant and plant-bacteria interaction. We reported several experiments; the results obtained are encouraging and suggest that additional work needs to be performed in order to optimize the sensitivity of the models and the reproducibility of results. However initial results shows that some NPs have an influence on bacterial growth and stress resistance, on plant sensitivity to bacterial infection, and on the mechanisms of interaction between plant and associated bacteria.

Acknowledgments

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