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Research Article

## *Vanilla planifolia* Andrews Response against Elicitor *Staphylococcus aureus* and Others Bacterial Compounds

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**Abstract.** Studies done *in vitro* report that plants can trigger a series of responses aimed at limiting the entry of an invader when they are exposed to foreign molecules or microorganisms, for example by increasing the synthesis of nitric oxide. Work aimed at studying the reaction that this plant has to foreign agents is null. The reaction vanilla leaves had against infections with *Staphylococcus aureus*, and components of the bacteria's cell wall was studied. Vanilla plants were grown *in vitro*. The leaves were infected with *S. aureus* and *E. coli*. Researchers searched for bacterial growth and adherence with electron microscopy and for the production of nitric oxide (NO) with fluorescence. NO was also quantified on vanilla leaves treated with bacterial cell components. We detected the expression of defense genes that are induced by NO, was analyzed by RT-PCR. The electronic microscopy showed that *S. aureus* can adhere to the leaves tissues by the formation of biofilm which favors its growth over the tissue. On these leaves it was observed that the NO production was higher when leaves were

infected with *S. aureus* compared with those infected with *E. coli*. The NO levels also increased (time dependent) when leaves were only treated with the bacterial cell wall components such as PGN and LPS. *S. aureus* as well as the PNG of this bacteria induced the mRNA expression of the AOS in leaf, stem and root of vanilla. A Pr1 overexpression was observed when the leaves were treated with both bacteria and with PGN and LPS. *S. aureus* induced an Pr1 overexpression also vanilla's stem and root. These results indicate that *Vanilla planifolia* can be colonized by *S. aureus*, but the plant responded by expressing genes via production of NO that alert the plant to protect itself from possible bacterial internalization.

**Keywords:** vegetable, response defense, elicitor, bacteria.

## INTRODUCTION

Vanilla (*Vanilla planifolia*) is a native plant from the tropical regions in Mexico and Central America. Since pre-hispanic times, vanilla has been used as a spice and to provide flavor to plenty of foods<sup>1</sup>. Currently its laboratory *in vitro* propagation is now feasible, which allows to maintain a stock of vanilla culture in the laboratory.

On other hand, there is a close relationship between plants and microorganisms; therefore, there has been a specific recognition, since the 90s it became clear that the plant's cells were producing molecules to mitigate the damage that could cause the presence of pathogenic bacteria and fungi<sup>2,3</sup>. in addition it was shown that the plant's cells are able to recognize pathogen-associated molecular patterns (PAMPs) activating metabolic pathways and inducing a hypersensitive response, the production of reactive oxygen and nitrogen species, phytoalexins and other defense molecules<sup>4,5</sup>.

In particular, it has been observed that one of the primary recognition events in plant-pathogen interaction is the increased levels of nitric oxide in the plant cells, which is associated with hydrogen peroxide to kill microorganisms and to inactivate the reactive oxygen species protecting the plant is also associated as a messenger in the regulation of genes in defense against oxidative stress, homeostasis of Fe and related to the processes of transcription and translation of genes. In general the following stand out AOS (*Allene oxide synthase*), GST (*glutation S-transferase*), Pr (*patogénesis related 1-5*), Pal (*phe-amonio lyase*), LOX2 (*lipoxigenase*) y OPR3 (*12-oxophytodienoate reductase*)<sup>6</sup>.

Therefore, it has been suggested that the recognition of plants to pathogens is not unique to this group, as there must be a primary recognition of the innate immunity similar in animals that inhibits the recognition mechanisms or activates defense responses for the protection of the plant.

Based on the background, we set ourselves the objective of recognizing the defense response that the species *Vanilla planifolia* leaves had when they receive stimulus of a *Staphylococcus aureus* or cell wall components (PAMPs).

## METHODS

For the induction vanilla leaves (obtained from an *in vitro* culture ) were placed into tubes containing 10 ml of liquid MS medium with a pellet of *Staphylococcus aureus* of ( $10^{-8}$  UFC ml<sup>-1</sup>) the interaction between the microorganism and the leaves was maintained at 1 and 3 hours. The same protocol was followed with *Escherichia coli*. For stimulation with bacterial components it was proceeded similarly but using purified commercial bacterial wall in different concentrations (50 µg ml<sup>-1</sup> peptidoglycan of *S. aureus* (PGN) lipoteichoic acid of *S. aureus* (LTA), diaminopimelic acid of *E. coli* (DAP) and 100 µg ml<sup>-1</sup> of lipopolysaccharide (LPS) of *E. coli*). Researchers searched for bacterial growth and adherence (biofilm formation) with electron microscopy (MEB), it was determined biofilm production in function of quantifying the optical density based on the methodology Djordjevic *et al.*<sup>7</sup>

NO was also quantified on vanilla leaves treated with bacterial components of *S. aureus* PGN and LPS of *E. coli*. The production of nitric oxide (NO) was related to the activity of nitric oxide synthase (NOS) which was measured with a NOS kit (Sigma, FCANOS1) using the Zeidler *et al.*<sup>8</sup> technique, the fluorescence intensity was measured in relative fluorescence units (RFU) on a fluorometer with excitation filters at 485 nm and emission at 538 nm.

With total RNA obtained from the treated leaves, the expression of Allene Oxide Synthase (AOS) and pathogenesis related-1 (Pr1), genes that are induced by NO, was analyzed by RT-PCR. The reagent PlantRizol (Invitrogen) was used for RNA extraction. Housekeeping gene (EF1, Elongation factor 1 alfa) and defense response genes primers (AOS and Pr1) were designed from consensus sequences of conserved species in the monocotyledonous class. The BLASTN program was used to find similarities of the sequences obtained in the NCBI and ClustalX2 databases and to compare them and to make sequence consensus of different species.

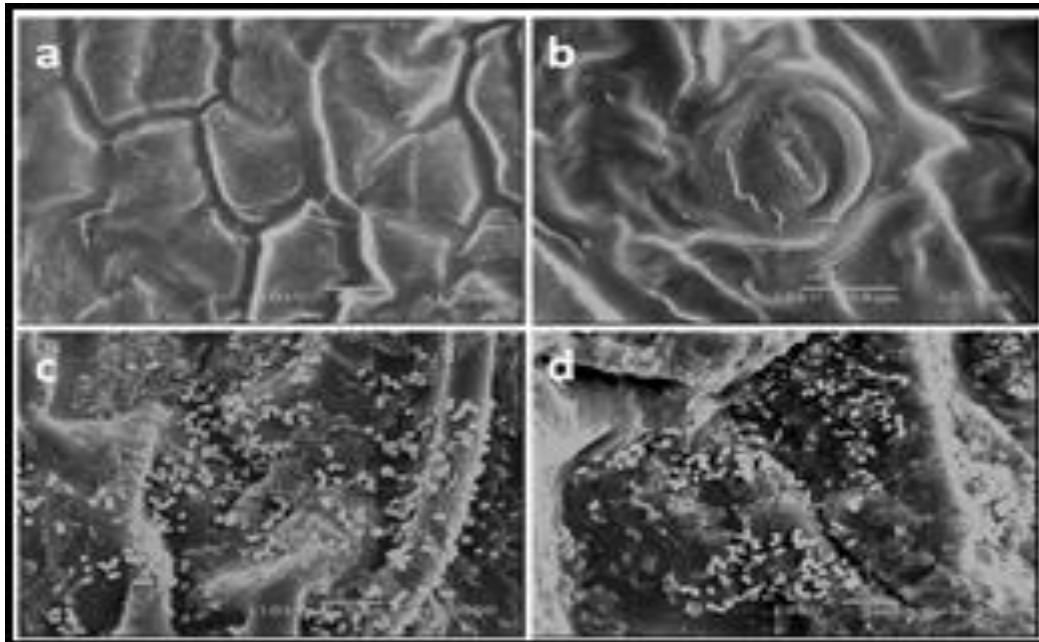
## RESULTS

In our experimental model, *Vanilla planifolia* leaves were placed in liquid MS medium containing *Staphylococcus aureus* and they were allowed to incubate for 1h. To see whether *S. aureus* could colonize the leaf's surface and also reproduced on the leaf, the bacteria and the leaves were allowed to interact for 1 h, afterwards the leaves were vigorously washed and were maintained in liquid MS medium for 24 h. After the incubation time passed, the number of bacteria increased. Bacterial invasion on almost the entire surface of the leaves was observed and it even reached the stomata. Leaves without bacteria showed no bacterial growth or biofilm. The electronic microscopy showed that *S. aureus*, but not *Escherichia coli*, can adhere to the leaves tissues by the formation of biofilm (Figure 1) which favors its growth over the tissue.

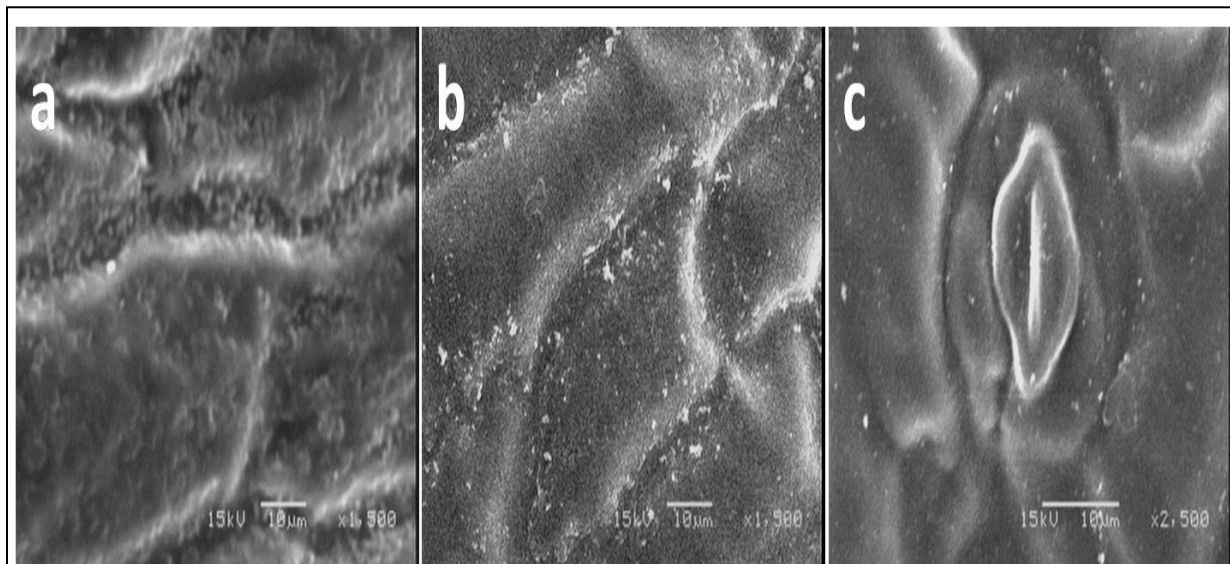
When the vanilla leaves were infected with *E. coli*, which is a Gram negative bacteria and not a biofilm producer, it was observed in the electron microscopy images where the bacteria did not adhere to plant's tissue, as the *S. aureus* did; thus, *E. coli* could not multiply in the vanilla leaves and produce biofilm (Figure 2).

According to the previous references, *S. aureus* is a Gram positive bacteria that infects and adheres mainly to animal hosts; however, like many bacteria, this species produces a carbohydrates matrix called "biofilm" which gives the ability to adhere to any medium in which it is located. Evidence of the work of Prithviraj *et al.*<sup>9</sup> showed that *S. aureus* adhered to *Arabidopsis thaliana* leaves, in addition to show

morphological changes in the leaves such as chlorosis and wilt. Furthermore, it was reported that *S. aureus* was found in the rhizosphere which makes it cosmopolitan and no animal hosts were specified.

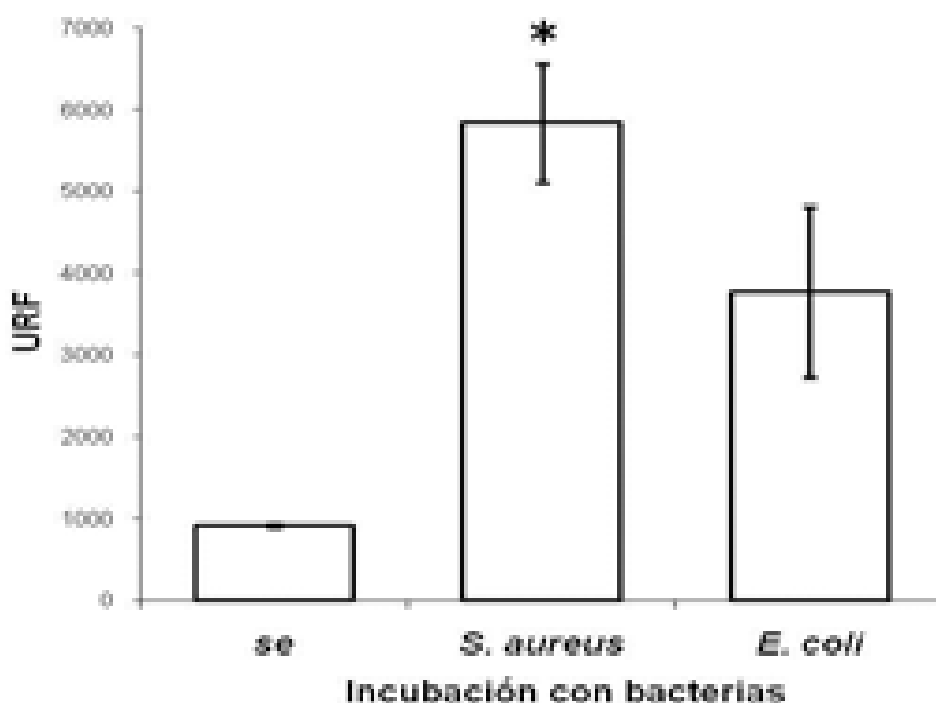


**Fig. 1:** Adherence of *Staphylococcus aureus* on *Vanilla planifolia* leaves (MEB). Negative test, foliar tissue without bacterial treatment. (a) and stoma (b). Leaves that were in contact with bacteria for 1 hr of incubation multiply and showed adherent to the foliar surface (c) and the stoma (d). Scanning electron micrograph 2500x.



**Fig. 2:** *Vanilla planifolia* leaves inoculated by *Escherichia coli* (MEB). (a) Leaves without bacteria, negative test. Leaves that were in contact with *E. coli* for 1 hr of incubation, does not showed bacterial growth in (b) foliar surfaces and (c) estoma.

To demonstrate if the interaction bacteria-leaf induces any response to activate the plant's defense, then the NO production in the infected leaves with whole bacteria and in cells treated with some components of the bacterial cell wall leaves was determined. On these leaves it was observed that the NO production was higher when leaves were infected with *S. aureus* compared with those infected with *E. coli* (**Figure 3**). The NO levels also increased (time dependent) when leaves were only treated with the bacterial cell wall components such as PGN and LPS (**Figure 4**).



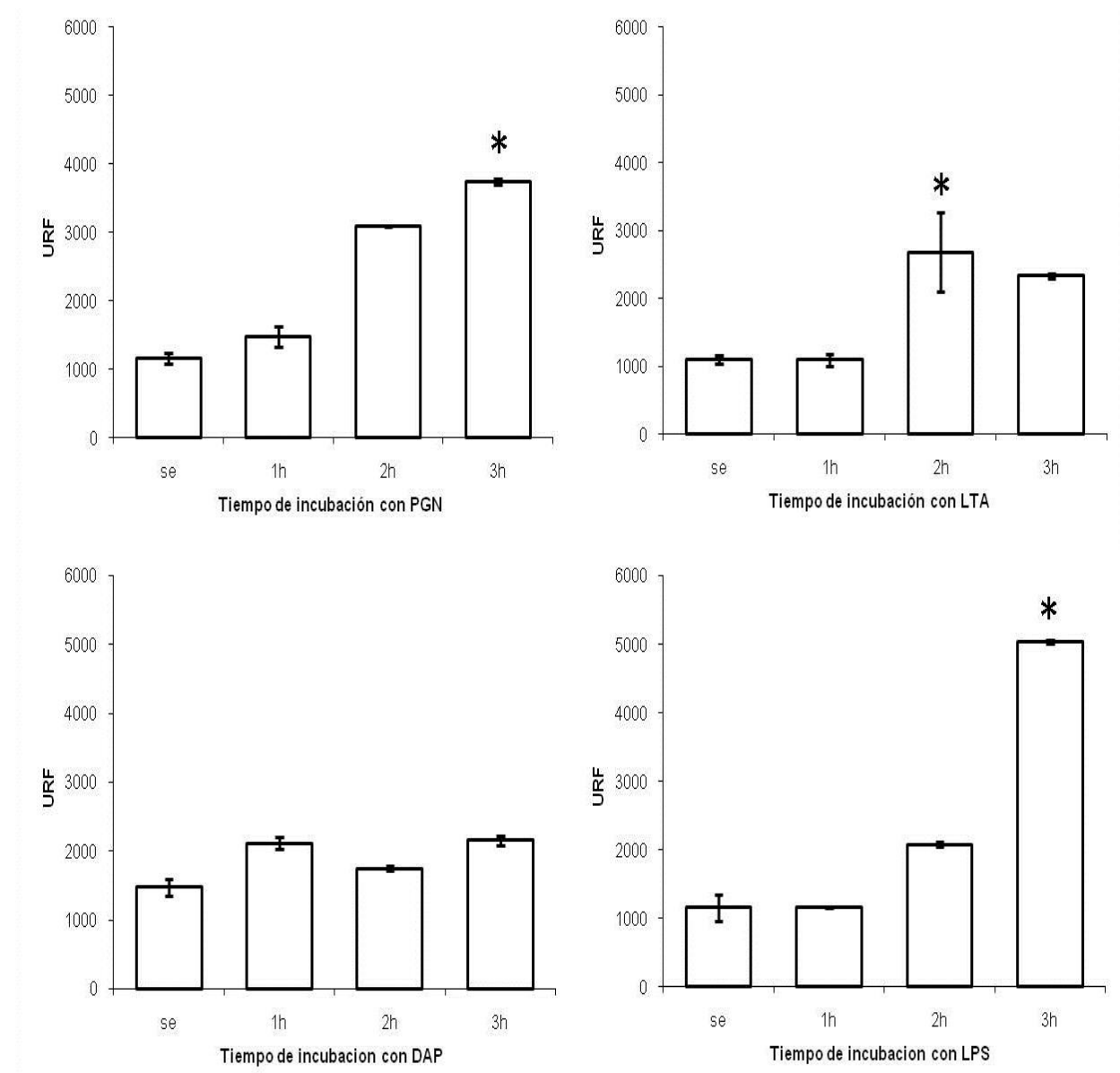
**Fig. 3:** Nitric oxide production in vanilla leaves were stimulated with *Staphylococcus aureus* and *Escherichia coli*. \* significant statistical difference  $p < 0.05$ .

Because the bacteria and bacterial components induced NO production in the vanilla leaves, it was decided to examine whether this NO production led to the activation of responsive genes regulated by this metabolite, as it does in *A. thaliana* and other plants. *S. aureus* as well as the PNG of this bacteria induced the mRNA expression of the AOS in leaf, stem and root of vanilla. A Pr1 overexpression was observed when the leaves were treated with both bacteria and with PGN and LPS. *S. aureus* induced a Pr1 overexpression also vanilla's stem and root (**Figure 5**).

The expression of genes that are induced by the production of NO, such as AOS and Pr1, in infected leaves with *S. aureus* is a proof that this bacteria provokes an activation reaction possibly of warning

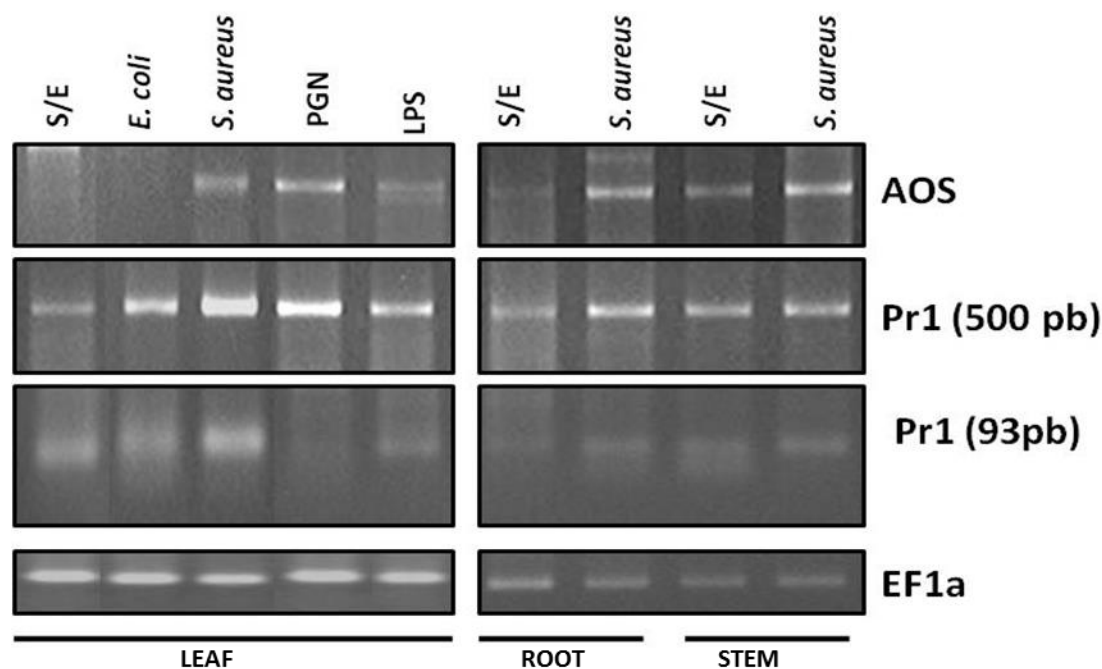
since this bacteria did not cause damage as when confronted with *A. thaliana*<sup>9</sup>. The mechanism by which *S. aureus* induces this cell activation through NO is possibly via PGN, since this molecule as well the LPS of the *E. coli* induced the expression of these genes when leaves were treated with these compounds.

These results are similar to those obtained by Zeidler *et al.*<sup>8</sup> which reported that the treatment with LPS is an enhancer in the *A. thaliana* leaves with PR gene expression, assuming that this induction can be a pretreatment to increase the defense response, the work of Gust<sup>5</sup> also confirmed that there is a stimulus of *A. thaliana* innate immunity due to the recognition of PGN, moreover it observes an increase in the expression of transcripts hence deriving the most relevant genes corresponding to the group At1g and the PR1 gene.





**Fig. 4:** Nitric oxide production in vanilla leaves were stimulated with bacterial cell wall compounds (PGN, LTA, LPS y DAP) \* significant statistical difference  $p < 0.05$ .



**Fig. 5:** mRNA expression of AOS and Pr1 on vanilla's leaf, stem and root stimulated with bacteria and bacterial components.

## CONCLUSIONS

These results indicate that *Vanilla planifolia* can be colonized by *S. aureus* which has been considered pathogenic only for humans, but the plant responded by expressing genes via production of NO that alert the plant to protect itself from possible bacterial internalization.

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