GENOMICS TO PLANT HEALTH:

EFFECT OF CLIMATE CHANGE ON PLANT SELF-PROTECTION

Beneficial microorganisms could be an effective approach to reduce the use of chemicals in agriculture. In the case-studies here reported, the fungus Trichoderma harzianum T39 *and the bacterium* Bacillus amyloliquefaciens S499 *show promising results in inducing resistance against pathogens.*

1. General mechanism and molecular aspects of plant self-protection

In addition to constitutive barriers (as preformed antimicrobial compounds, pectin, hemicellulose, cellulose, lignin, etc.), plants possess inducible defence mechanisms to protect themselves against pathogens, which also include the enhanced resistance upon treatment with some compounds or microorganisms.

Two phenotypically similar plant-mediated defence responses have been identified: systemic acquired resistance (SAR) and induced systemic resistance (ISR).

Researchers studying these defence response cannot rely on substantial background knowledge: the molecular mechanisms underlying the priming state have been partially studied only on plant model systems and information for crops is particularly scarce.

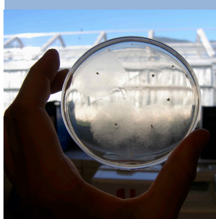
Key-features

Nowadays, the control of plant deseases is mainly based on intensive application of chemicals.

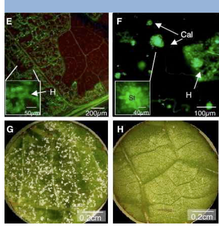
Plants own constitutive barriers to protect themselves against pathogens, but they also possess inducible defence mechanism against pathogens.

Plant self-protection activated by beneficial microorganisms could be an effective approach to reduce chemical applications, for example in the vineyards.

A promising research line in this field is the study of the induced systemic resistance (ISR), a mechanism of the plant immune system activated by beneficial microorganisms. It confers resistance to different microbial pathogens, pests and abiotic stresses.



For example, the fungus *Trichoderma harzianum* T39 and the bacterium *Bacillus amyloliquefaciens* S499 induce resistance against diseases in different plants.



Scarce information is available on the cellular mechanism of ISR in non model plants and on the influence of abiotic stress to plant reaction capacity against biotic stress.

Systemic acquired resistance (SAR)	Induced systemic resistance (ISR)
• It is activated following pathogen recognition or treatment with some chemical inducers (e.g. benzothiadiazole - BTH)	• It is activated by specific strains of beneficial microorganisms or their metabolites
• It is regulated by signalling pathways depending on salicylic acid (SA)	• It is regulated by signalling pathways depending on two substances: jasmonic acid (JA) and ethylene (ET)
• It is activated systemically in parts of the plant that are distant from the site where the stimulus is applied	• It is activated systemically in parts of the plant that are distant from the site where the stimulus is applied
• It usually involves the direct accumulation of plant protection metabolites	• It usually involves the activation of a priming state for enhanced reaction upon pathogen infection
• It entails energy cost for the plant since defence processes are constantly activated.	• It has no energy cost for the plant since defence processes are activated only when the pathogen is present.

The effect of treatments with different chemical inducers and some non-pathogenic microorganisms has been demonstrated on several crops against several plant pathogens. However, ISR activation under field condition seems not stable, affected by environmental conditions and probably by the physiological status of the plants. The inconsistency in the plant protection efficacy prevents a wide use of inducers in agriculture.

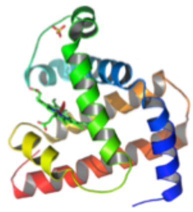
More knowledge on molecular mechanism and on the influence of the abiotic stress is required to improve the efficacy and stability of plant self-protection under field conditions, thus allowing the substitution of chemical pesticides with environmentally friendly resistance inducers.

Here following we show different approaches to the problem:

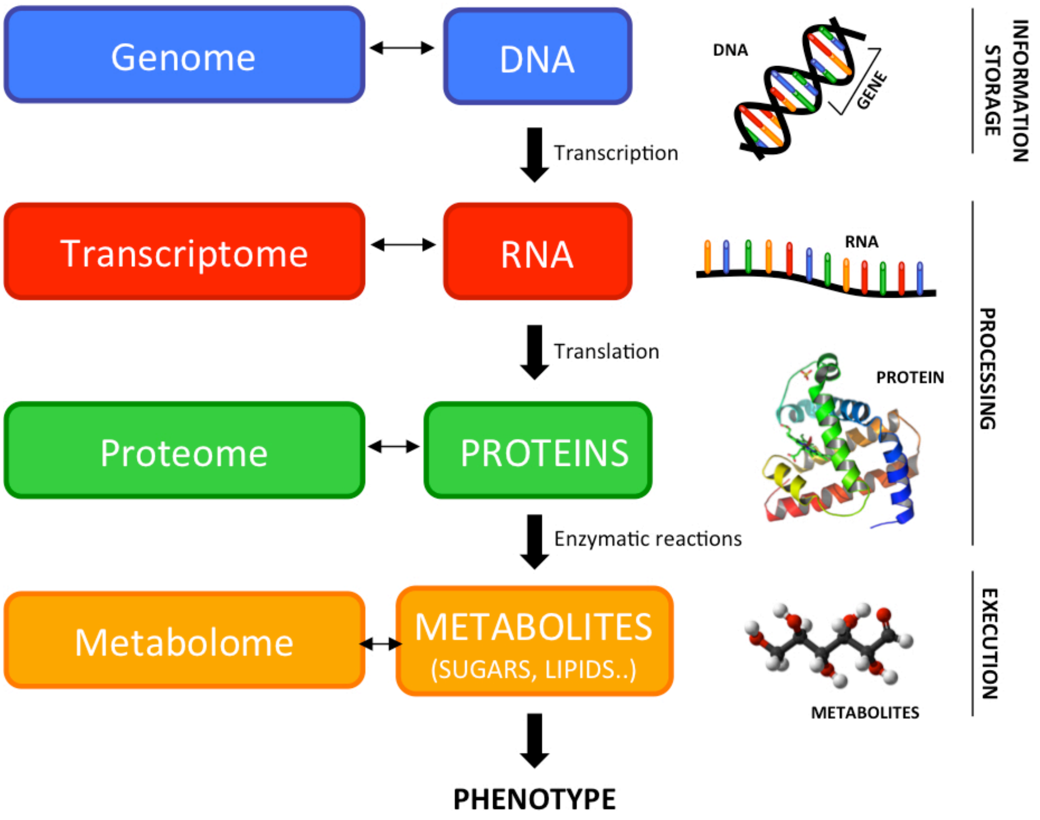
At a transcriptomic level

RNA

At a proteomic and cellular level



PROTEINS



2. Efficacy and molecular mechanisms of grapevine resistance induced by *Trichoderma*

Downy mildew, caused by the biotrophic oomycete *Plasmopara viticola*, is one of the most important grapevine diseases (Fig. 1), and chemical treatments are applied to avoid significant yield losses in warm and wet climates.

We found that treatments with the beneficial microorganism *Trichoderma harzianum* T39 (T39) significantly reduce downy mildew symptoms on susceptible grapevines (Fig. 2). The biocontrol effect of T39 is mediated by the plant and is systemically activated in untreated leaves of treated plants (ISR-type). Efficacy of T39 leaf treatments shows approximately 70 and 50% of disease reduction locally and systemically, respectively. The systemic resistance is homogeneously activated in the plant, independently of the leaf position on the shoots.

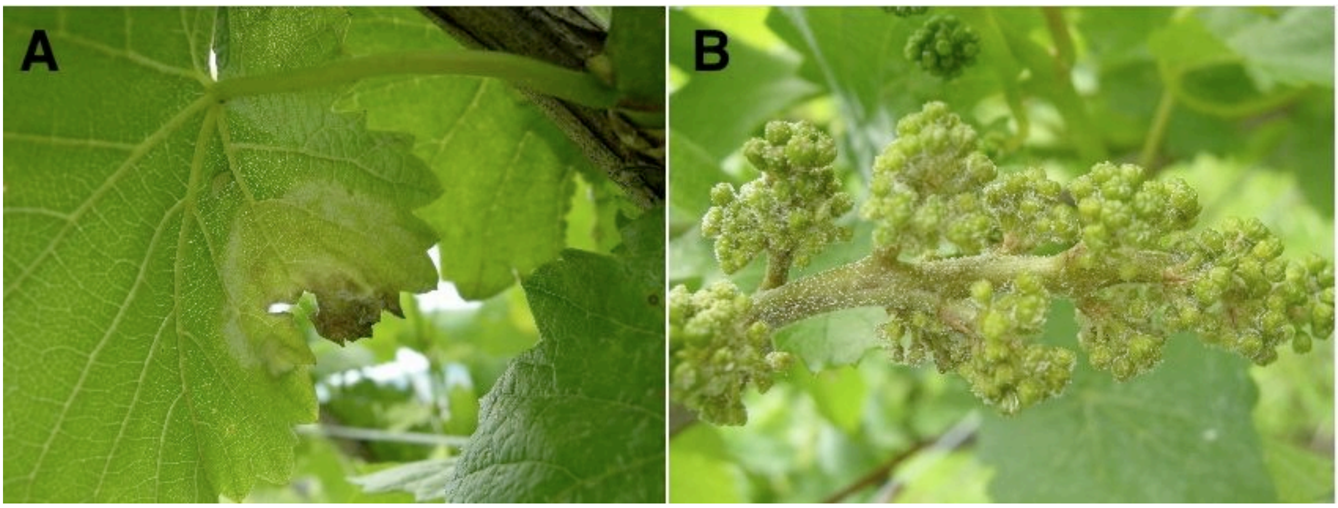


Fig. 1. Disease symptoms of downy mildew infections on grapevine (**A**) leaves and (**B**) flowers (courtesy of Oscar Giovannini).

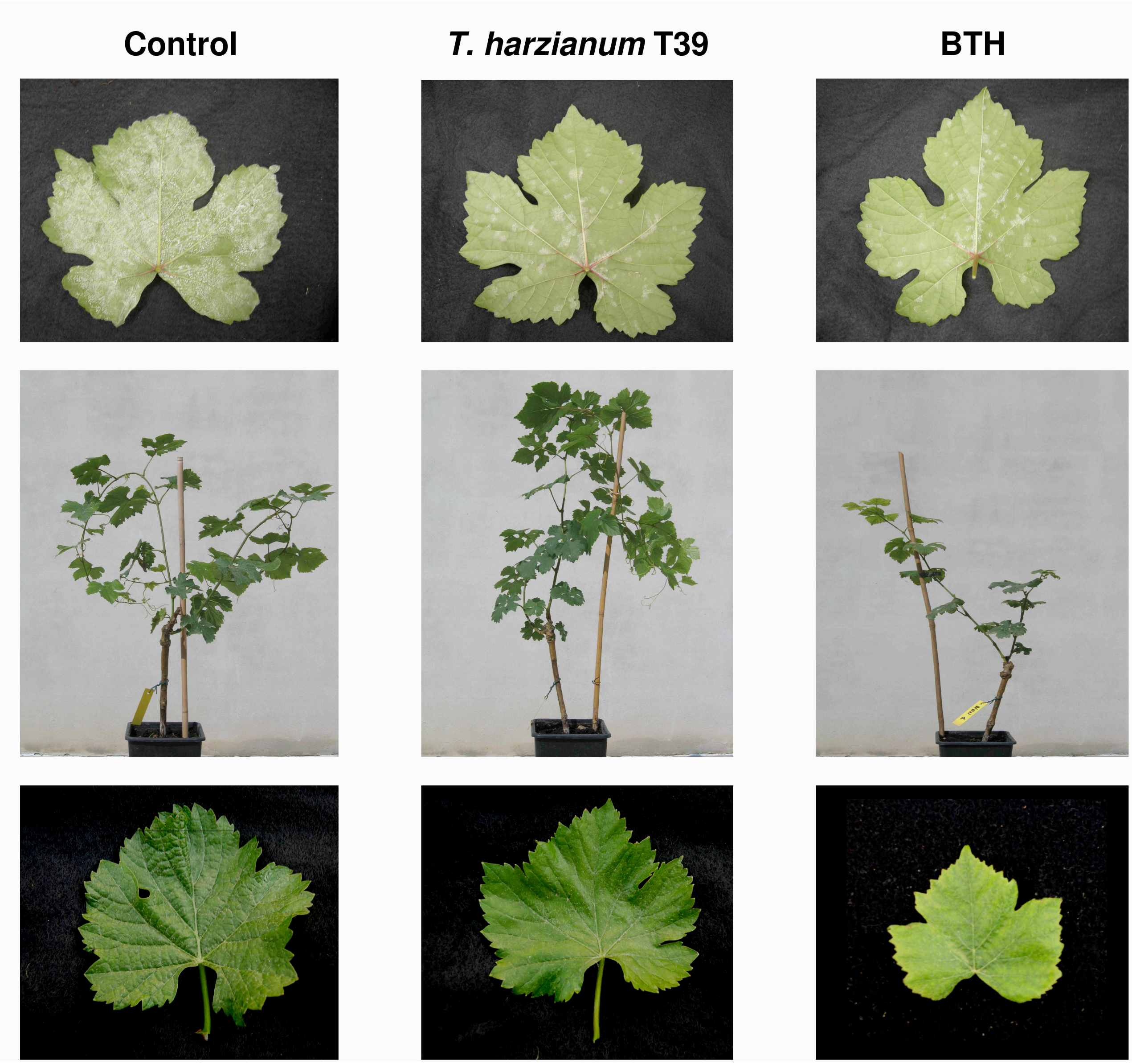


Fig. 2 - Effects of *Trichoderma harzianum* 39-induced and BTH-activated resistance on grapevine plants (adapted from Perazzolli et al., 2011).

Optimal disease control is obtained with at least three consecutive applications of T39 before pathogen infection. The persistence of T39-induced resistance is shorter than one week under greenhouse controlled conditions.

Gene expression analysis shows direct induction and enhanced expression of genes encoding pathogenesis-related (PR) proteins after pathogen inoculation (Fig. 3), indicating a dual effect of T39-induced resistance.

The stronger local than systemic modulation of defence-related genes corresponds to higher local than systemic disease control in T39-treated plants.

Comparable expression profiles of defence genes are observed by application of a commercial formulation and purified conidia of T39, indicating possible activation of resistance with low cost resistance inducers. The modulation of marker genes indicates the involvement of ET/JA signals in the defence processes induced by T39, in contrast to the SA pathway activated by the chemical inducer benzothiadiazole (BTH). Indeed, BTH directly induces the expression of *PR-1*, which is a marker of SA signals.

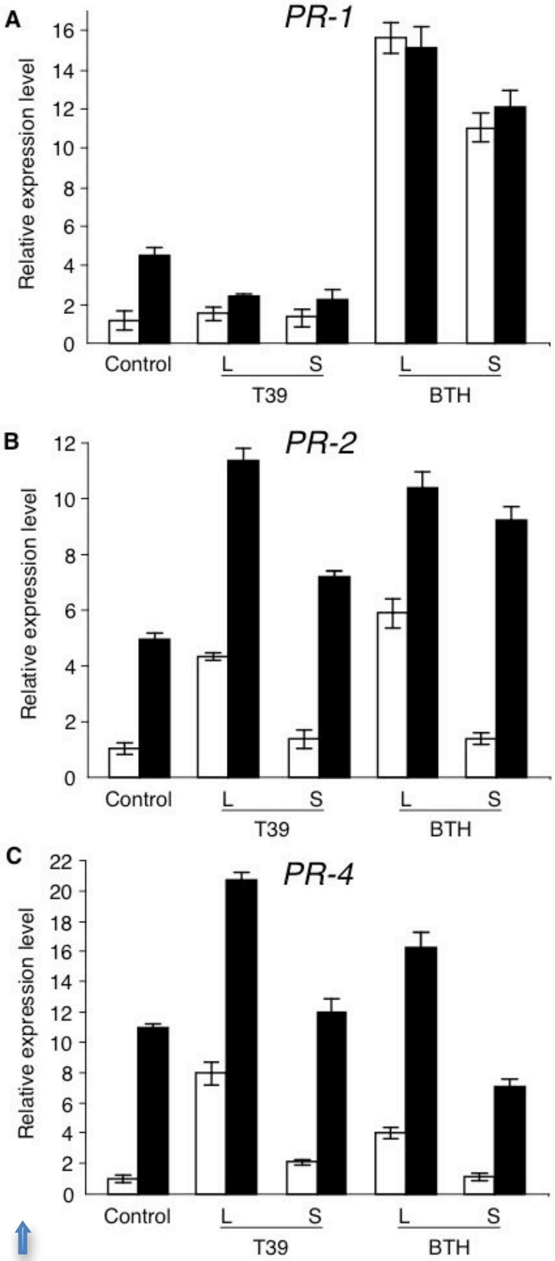


Fig. 3 - Expression analysis of defence-related genes in grapevine plants treated with *Trichoderma harzianum* T39 (T39) or benzothiadiazole (BTH) and in control plants (Control). Expression of genes encoding *PR-1* (**A**), *PR-2* (**B**) and *PR-4* (**C**) was analyzed before (white) and 24 h after (black) *Plasmopara viticola* inoculation in treated (L, local effect) or untreated (S, systemic effect) leaves (adapted from Perazzoli et al., 2011)

In order to identify key genes and processes responsible for the activation of grapevine self-defence, the study of the global transcriptional dynamics of T39-induced resistance is in progress.

Leaf samples collected before and 24 h after *P. viticola* inoculation on T39-treated and control leaves were analyzed.

More than 7000 genes are significantly modulated in at least one pair-wise comparison and their functional annotation revealed complex transcriptional reprogramming and involvement of different functional categories during T39-induced resistance (Fig. 4).

Genes related to microbial recognition are directly induced by T39 or primed for enhanced expression after *P. viticola* inoculation, confirming the dual effect of T39-induced resistance.

P. viticola-responsive genes specifically modulated in control plants are manly down-regulated and involved in process of primary metabolism and energy.

On the other hand, T39-treated plants react to *P. viticola* inoculation by inducing genes related to defence and secondary metabolism, indicating that T39 treatment alters the grapevine response to *P. viticola* inoculation.

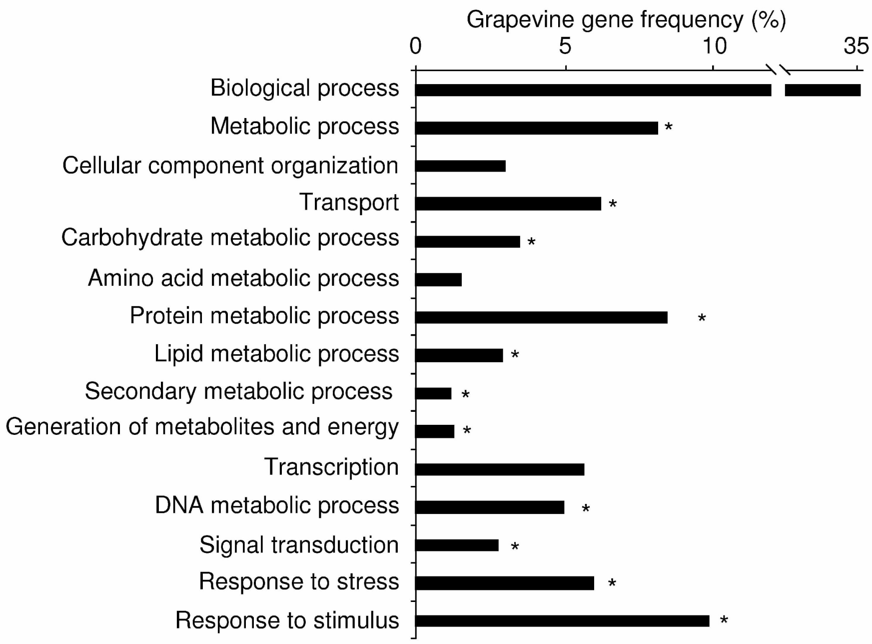


Fig. 4 - Distribution of ISR-modulated genes among the 15 selected gene ontology (GO) functional categories. Asterisks indicate categories differently represented in the ISR-modulated genes compared to the entire grapevine transcriptome.

3. Dissecting grapevine resistance induced by *Trichoderma harzianum* T39: from protein to cells phenotype

In the future, the effects of climate change could overcome the natural capability of plant to respond to pathogens and activate disease resistance. Thus, a deep understanding of resistance mechanism is an essential step to improve and control plant protection.

Combining microscopic observation of cells phenotype and plant proteome analysis we investigated the resistance mechanism induced by the biocontrol agent T39 in grapevine and its ability to reduce downy mildew symptoms (Fig. 2).

Observations of plant tissues revealed that T39-induced resistance could be related to activation of early signalling events, which begin as soon as germ tubes of *P. viticola* zoospores have penetrated the stomata and involve mostly plugging and closure of stomata. Indeed stomata are the natural aperture for fungus and oomycetes to penetrate plant tissues (Fig. 5).

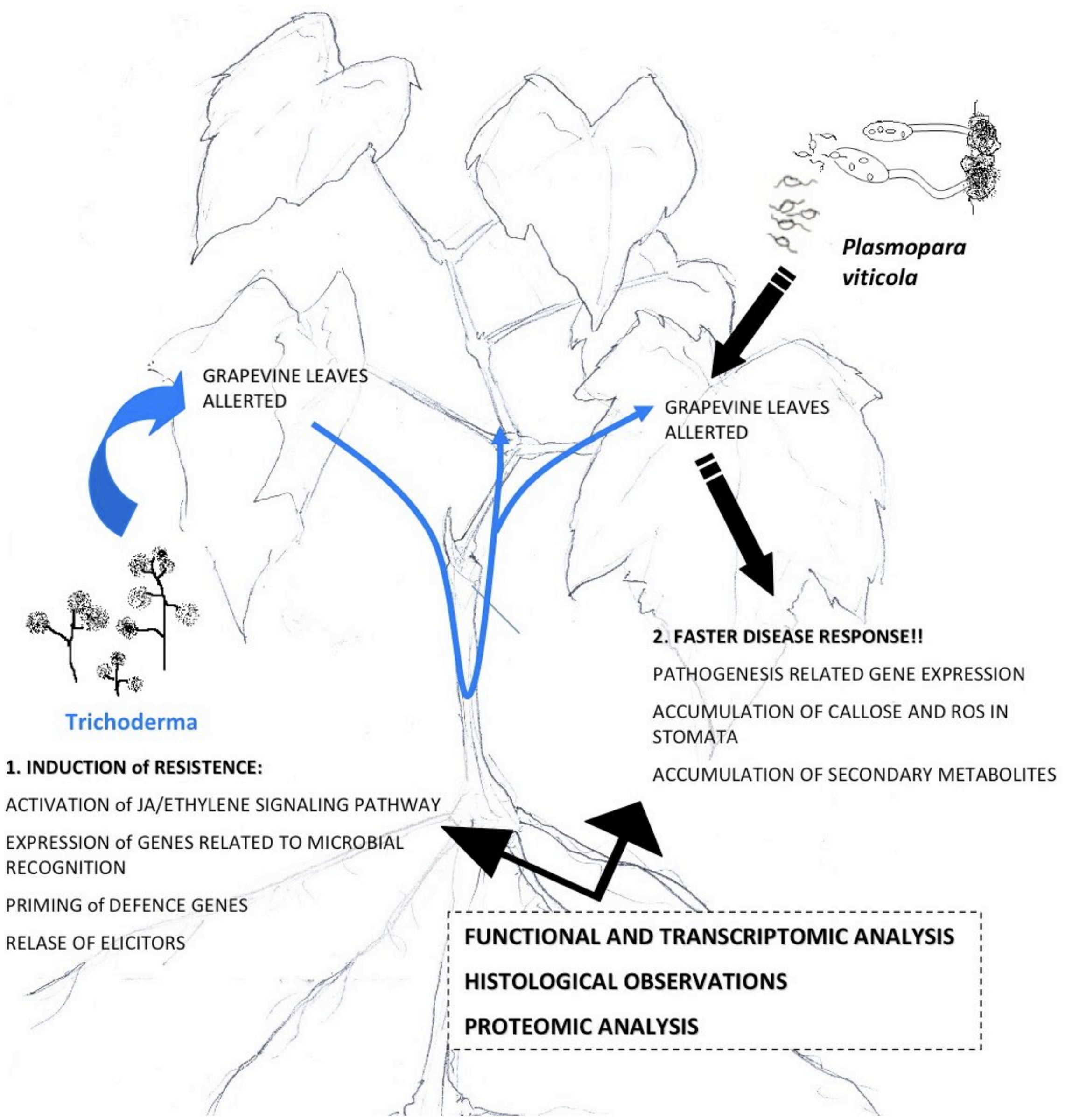


Fig. 5 *Trichoderma harzianum* T39 enhanced grapevine resistance against downy mildew by directly inducing a complete microbial recognition machinery. Moreover, upon pathogen arrivals T39 induce the expression of genes related to defence and secondary metabolism and cause the accumulation of cell wall-related defence compounds (ROS and callose) at the site of the attempt of *P. viticola* penetration.

An accumulation of callose (a plant polysaccharide) in the stomatal guard cell was observed at 24h post *P. viticola* in T39-treated plants and diffuse in the area around the attempt of penetration in the following days (Fig. 6). Moreover, an accumulation of reactive oxygen species (ROS) was also observed by fluorescence microscopy (Fig. 7).

ROS are reactive molecules derived form oxygen and they have different roles in plant defence mechanism. Firstly they function as signal molecules responsible of the induction of defence genes and programmed cell death. Secondly they induce stomata closure and contributie to cell-wall strengthening by increasing lignifications and cross-liking of proteins and. Callose deposition and ROS production induced by T39 seemed to be required but not sufficient to explain the level of protection conferred, confirming that stomatal perturbation is just part of a more complex defence mechanism.

To study the hypothesized wide diversity of cellular pathways that T39 induces in grapevine, we investigated changes in grapevine proteome prior and after downy mildew infection (Fig. 5).

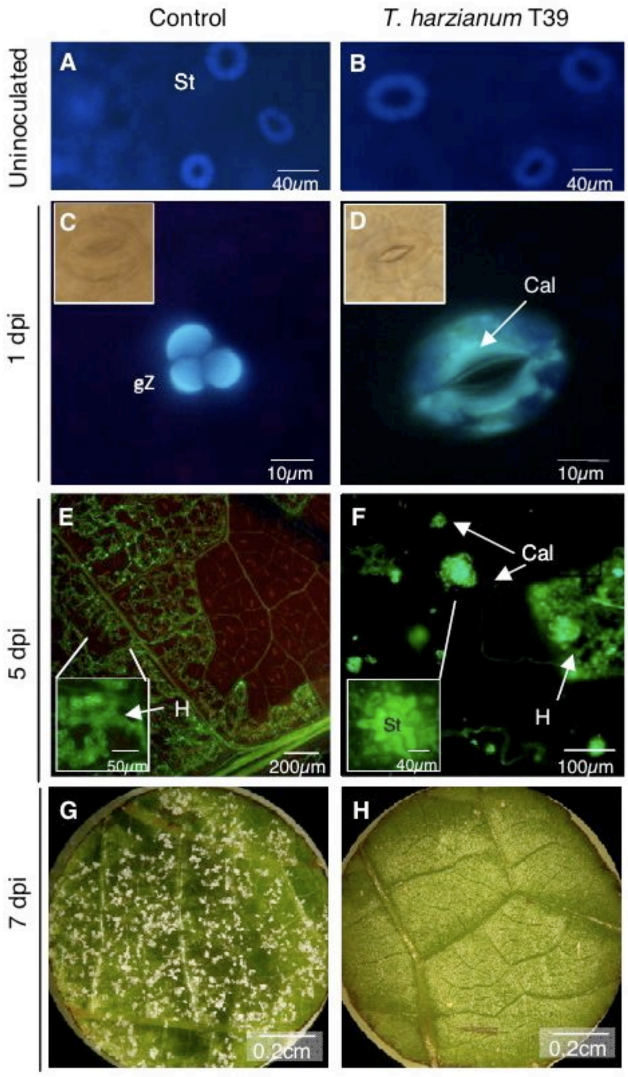


Fig. 6 - Time course of intercellular colonization of *Plasmopara viticola* during T39-induced resistance. Susceptible *V. vinifera* cv. Pinot Noir leaf disks were treated with water (Control) or *Trichoderma harzianum* T39.
(A-F) Pathogen development and callose deposition were monitored before (Uninoculated), one and five days post inoculation (1, 5 dpi) of *P. viticola* by epifluorescence microscopy after aniline blue straining. gZ: germinated zoospores; Cal: callose; H: *P. viticola* hyphae; St: stomata. (G-H) Disease symptoms on grapevine leaf disk at 7 dpi.

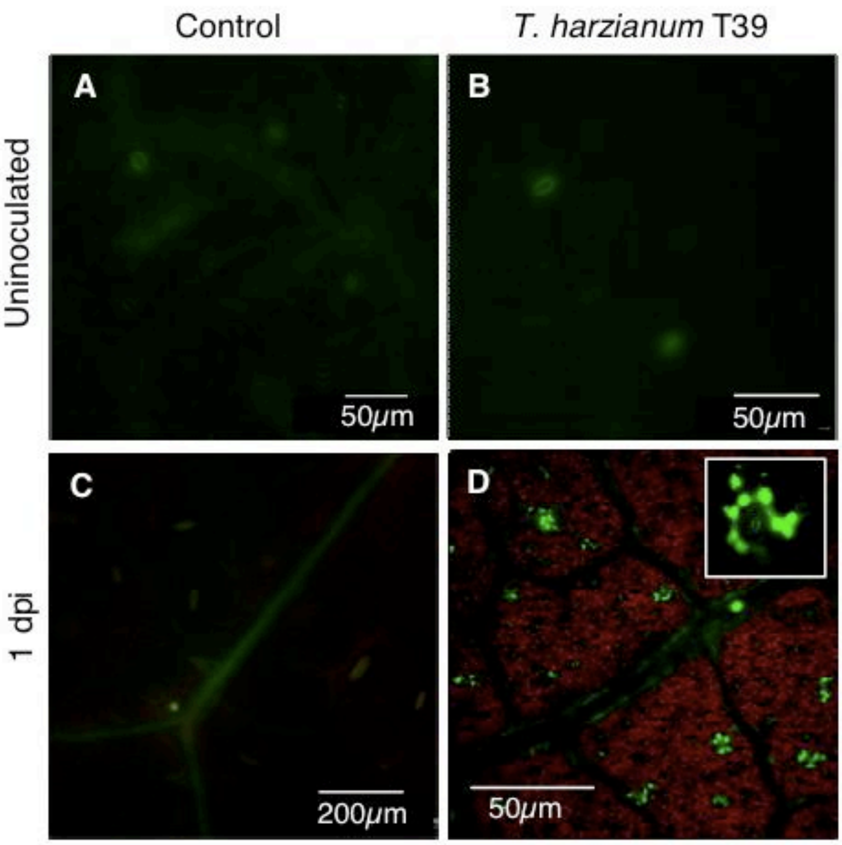


Fig. 7 - Accumulation of reactive oxygen species (ROS) after *Plasmopara viticola* inoculation. Leaf disks of susceptible *V. vinifera* cv. Pinot Noir were treated with water (Control) or *Trichoderma harzianum* T39. ROS accumulation was monitored before (Uninoculated, A-B), and seven days post inoculation (7 dpi, C-D) of *P. viticola* by epifluorescence microscopy using the cH_2DCF-DA fluorescent dye.

T39 enhanced grapevine resistance against downy mildew by modulation of biotic and abiotic stress-related proteins. Particularly, a microbial recognition machinery was directly induced by T39 and processes related to signal transduction and oxidative stress were induced after *P. viticola* inoculation in T39-treated plants. Moreover, in agreement with the phenotypic observations, proteins related to oxidative stress (ROS production) and cell wall-related defence were significantly modulated during T39-induced resistance (Figure 5). Compatible interaction can be achieved by pathogenic oomycetes through the secretion of molecules, which can suppress host defences and favour pathogen colonization. In our study, a global protein down- regulation (mainly proteins related to the plant primary metabolism) was observed in inoculated control plants, possibly reflecting the establishment of a compatible interaction. Moreover, susceptible grapevine plants appear to activate a week resistance reaction, which is neither fast nor robust enough to prevent the pathogen from spreading into plant tissue.

4. Effect of temperature and water stress on grapevine induced resistance

Under field conditions, the expression of induced resistance is often inconsistent and likely to be influenced by the environment, plant genotype and its physiology. The perception of all extracellular stimuli by plants and the activation of defence responses require a complex interplay of signalling cascades that might be influenced by its physiological state. Since abiotic stresses (such as drought, extreme temperatures and oxidative stress) leads to physiological and molecular changes that may interfere with the ability of the plant to assemble the resistance against pathogens, the aim of this project is to investigate the effect of a short heat and/or drought exposure of grapevine plants on the systemic resistance activated by T39 and BTH against downy mildew (*Plasmopara viticola*).

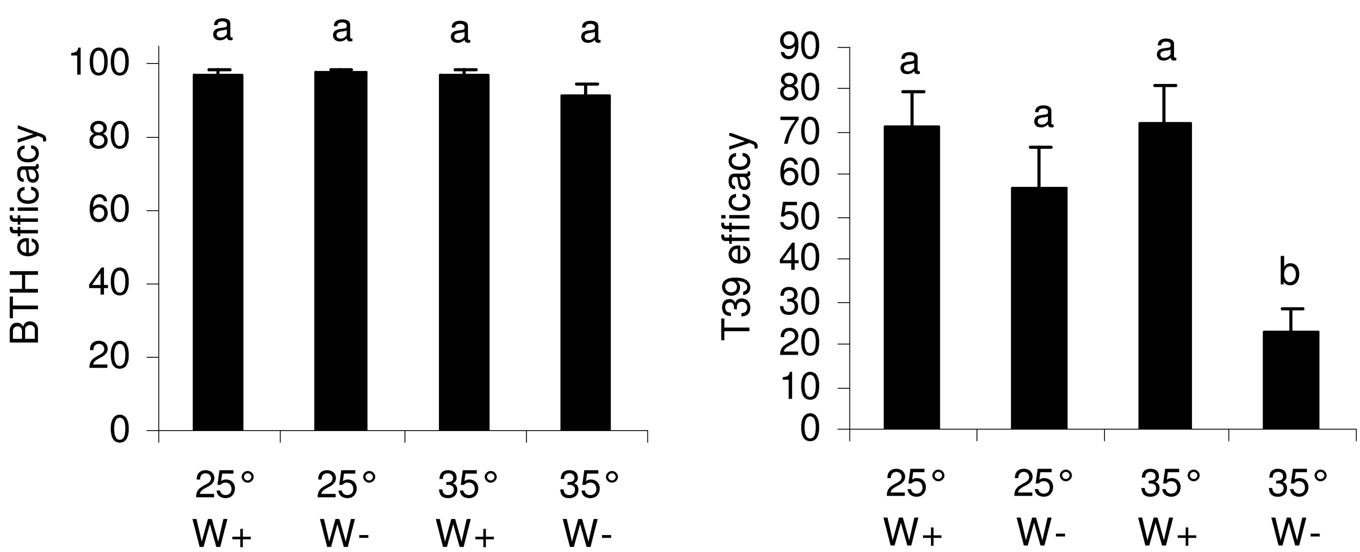


Fig. 8 - Efficacy of foliar treatments with benzothiaziadole (BTH) and *Trichoderma harzianum* T39 against *Plasmopara viticola* on grapevine plants incubated for a period of 15 days at different temperatures (25° and 35° C) and irrigation regimes (irrigated W+ and non irrigated W-). Letters according to Tuckey test.

Conditions of drought (irrigation suspended for 15 days at 25° C) and/or high temperatures (plant incubation at 35° C for 15 days) did not affect the efficacy of BTH-activated resistance.

The efficacy of T39-activated resistance was not affected by abiotic stress, when the stressing factors were given singularly, but it was reduced when plants were submitted to the combination of both stressing factors (Fig. 8).

In our system, the reaction to the combination of both abiotic stresses had any effect on the BTH-mediated resistance, but negatively affected the resistance induced by T39 against downy mildew. Therefore, T39-induced resistance might be a useful tool in the control of downy mildew, but it can be modulated by the physiological state of the plant.

Genes expression analysis on six resistance genes known to be modulated in plants by the application of T39 were studied using Real Time PCR. These genes are upregulated by the application of T39 in control conditions, but only three of them are upregulated when plants are submitted to heat and drought stress. Drought stress together with heat shock may interfere with the T39-induced resistance leading to a reduction of the level of protection against downy mildew. Further characterization of the molecular pathways in the T39-induced resistance under abiotic stress is currently object of study in order to predict the possible effect and stability of T39-induced resistance under field conditions, especially in a view of climate change.

In molecular biology, Real Time Polymerase Chain Reaction is a laboratory technique based on the principles of PCR that enables both detection and quantification of a targeted gene, in presence of specific primers and a fluorescent probe. Quantification can be absolute (data interpolated on a calibration curve) or relative (as in the present study): based on values normalized to a standard constitutive gene (Actin) and a reference sample (Control).

5. Effect of temperature and water stress on *Bacillus*-induced resistance

The genus *Bacillus* is a reservoir of bacterial strains able to effectively control plant diseases caused by pathogenic microorganisms. Until now, the antagonist aptitudes of *Bacillus* spp. strains have been commonly evaluated under conditions that are favourable to plants and microbes and the role played by the temperature have not been taken into account so far.

The ability to protect plants against phytopathogenic fungi exhibited by *Bacillus* spp. strains mainly relies on the production of surfactins, lipopeptides (involved in motility, biofilm formation and plant root colonization) and ISR activation.

We evaluated how temperature may modulate the biocontrol activity of *B. amyloliquefaciens* strain S499, by affecting the synthesis of surfactins and mechanisms associated with the production of these lipopeptides.

The results obtained during the project showed that low temperatures determine an increase in the quantity of surfactin produced by S499 single cells, although they do not sustain a faster growth of the bacterium. On the contrary, the bacterial growth is positively influenced by the higher temperatures while surfactin synthesis results to be impaired.

Moreover, it has been shown that higher is the temperature, faster is the bacterium movements across solid surfaces. At 35°C, S499 (with a cell diameter of ≈2μ) covers a distance of 45 mm in less than 16 hours while it moves slowly at 15°C (only 5 mm) (Fig. 9).

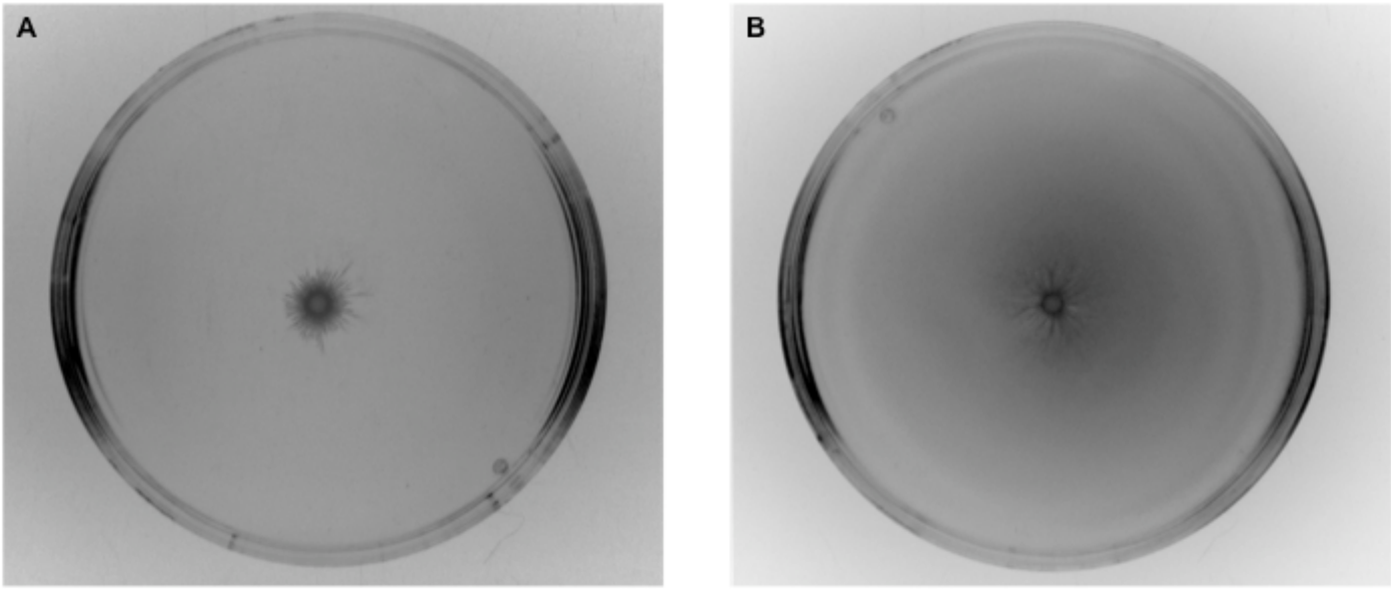


Fig. 9 - Effect of temperature on the motility of *Bacillus amyloliquefaciens* strain S499. The bacterium has been incubated at 15°C (A) and 35°C (B). The high temperature prompts a faster movement of S499 cells.

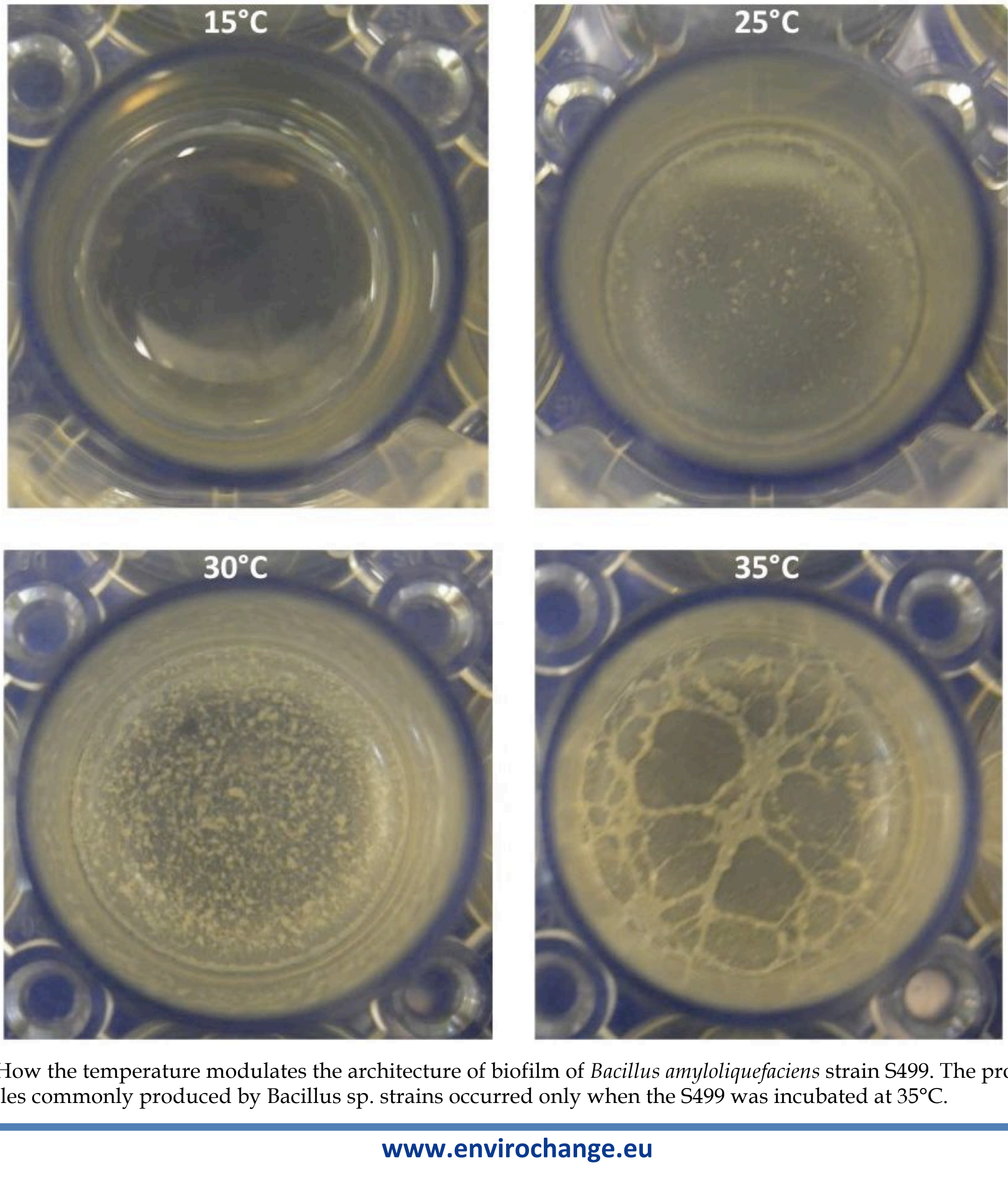


Fig. 10 - How the temperature modulates the architecture of biofilm of *Bacillus amyloliquefaciens* strain S499. The production of the pellicles commonly produced by Bacillus sp. strains occurred only when the S499 was incubated at 35°C.

Furthermore, temperature influences also the ability to form microcolonies onto inert surfaces such as polystyrene and the production of pellicles at air-liquid interface occurs only when the S499 grows at 35°C (Fig. 10).

During this work, S499 has been applied to the roots of bean, tomato and zucchini plants previously exposed to temperature and water stress and then inoculated with the pathogens. Low and high temperatures combined with water stress reduced the level of induced resistance on plants, even with some differences among the three types of crops.

This behavior indicates that the physiological status of the plant is crucial for the level of expression of plant induced resistance. On the contrary when plants are treated with S499, subsequently exposed for seven days to 35, 25 and 15°C and then inoculated with the pathogens, no differences in plant protection among the different environmental conditions have been observed.

The application of S499 gave an appreciable reduction of disease severity in all the conditions assayed (Fig. 11). For example the higher cell density registered onto tomato roots grown at 35°C determined a reduction of 46% of late blight severity. Interestingly, we have observed an overall ability of S499 to better protect the plants through root colonization and ISR induction when these plants have been grown at non favourable temperatures such as 15 and 35°C. Probably the bacterium is helping plants adapt to these temperatures and relieve the abiotic stress caused by high, low temperature and water stress.

These results are important if we consider the ongoing climate changes, indeed the exploitation of a biocontrol agent could at the same time counteract problems coming both from the temperature changes and from phytopathogenic microorganisms.

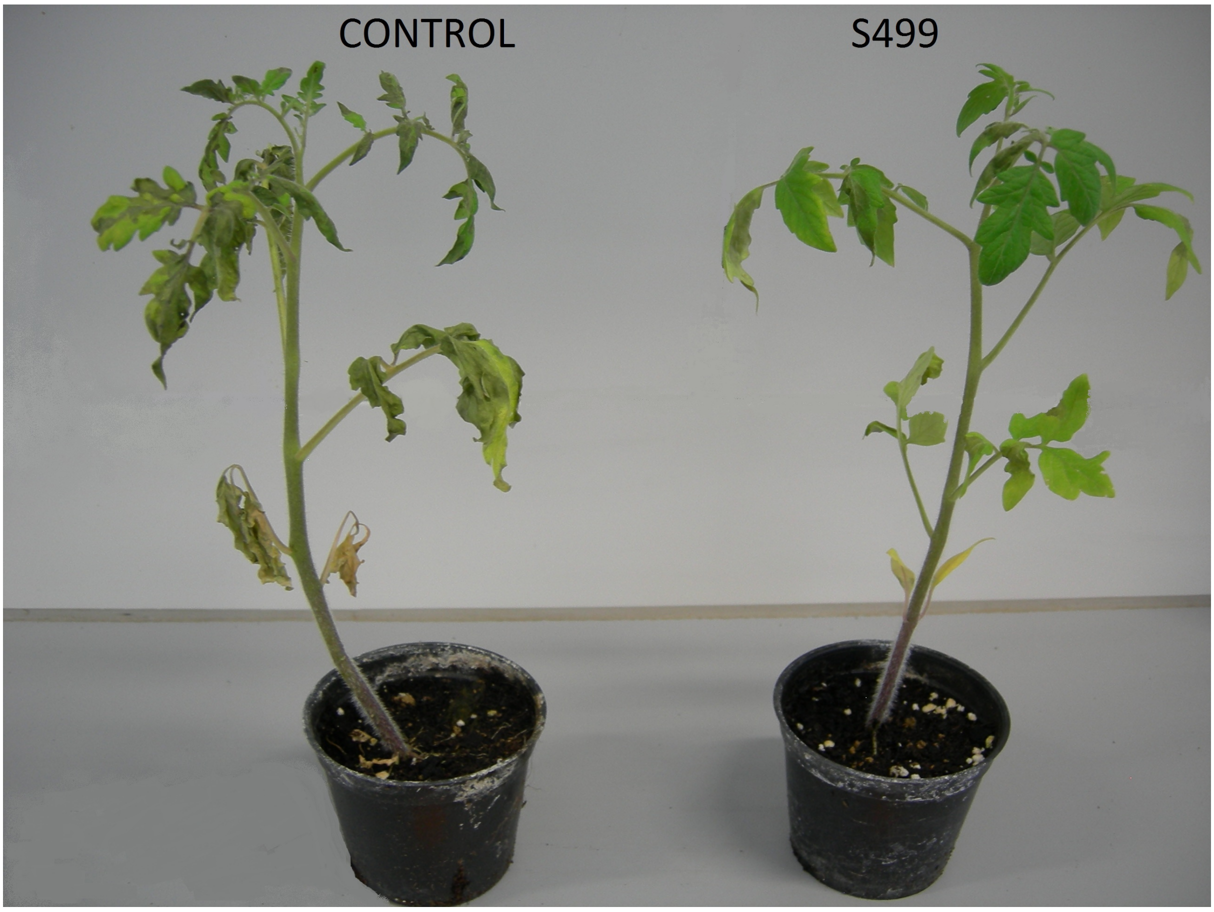


Fig. 11 - Effect of *Bacillus amyloliquefaciens* strain S499-induced resistance on tomato plants in the control of *Phytophthora infestans.*

To know more

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THE ENVIROCHANGE PROJECT

The EnviroChange project focuses on global change and sustainable management of agriculture in highly developed mountain environment.
It aims at assessing the short-term biological, environmental and economic impact of climatic change on agriculture at the level of the Trentino region particularly on quality and pest management that are more likely to be influenced by climate change in the short term. The final aim is to preserve and improve the quality of life of habitants, protecting environment and biodiversity for the future generations, as well as to represent a model for sustainable development of mountain areas.

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