

Quantitative detection methods for evaluating induction of disease resistance against *Verticillium dahliae* correlated with increased defense responses in tobacco plants treated with coactyl.



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Introduction

Verticillium wilt remains one of the most serious soil borne diseases worldwide. An array of different strategies to reduce the consequences of pathogen pressure is available. Among these methods, application of natural compounds, which stimulate locally and systemically plant defense reactions, is becoming an industrial alternative to the application of chemicals with deleterious side effects.

Our objective was to investigate, through the quantitative and very sensitive real-time RT-PCR technology, the potential of Coactyl™, composed of humic acid associated to a phenolic acid, to act as a plant defense inducer conferring resistance to *Verticillium dahliae* wilt in tobacco used as a model.

Material and Methods

We investigated by real time PCR how the organic amendment Coactyl delayed the appearance of disease caused by *Verticillium dahliae*. On one hand the effect of coactyl on defense mechanisms was determined by quantifying defense-related gene transcripts in a time course experiment (Fig 1), and on the other hand, the effectiveness of coactyl was quantified in presence of *V. dahliae* by molecular detection of pathogen before any symptom could be observed (Fig 2).

For RT-PCR experiments, RNA analysis, total RNA were extracted from 60 mg of crushed apical root using Nucleospin RNA plant kit (Macherey-Nagel, Germany) following manufacturer's instructions. DNase-treated RNA were quantified by measuring the absorbance at A_{260nm} and RNA quality was assessed by OD₂₆₀/OD₂₈₀ ratios and by electrophoresis on 1% formaldehyde agarose gels stained with Sybr Gold. First strand cDNA synthesis was carried out in 20 µl reactions containing 1 µg RNA with iscript (MMLV-derived) reverse transcriptase and mix of oligo(dT) and random primers from iscript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real time RT-PCR was performed on 25 ng cDNA using iQ Sybr green supermix in an iCycler iQ (Bio-Rad Laboratories, Hercules, CA). The reaction efficiency of PCR (1.99 - 2.02) was determined on the slope of serial dilution curves of pooled cDNAs with each specific primer pairs at harvested times.

For PCR diagnostic assay, plant/fungus genomic DNA were extracted using genomic DNA from plant kit (Macherey-Nagel, Germany) following manufacturer's instructions. To evaluate the resistance of a plant to a pathogenic fungus it was necessary to quantify the degree (ng fungus DNA/ng plant DNA) to which it is colonized by that fungus. Specific primers, even more in greenhouse condition, were required. Sequencing amplicon identified only the internal transcribed spacer ITS of *V. dahliae*, target of *V. dahliae* sequence and no tobacco sequence demonstrated the specificity of the reaction. Calibration curve (CT/ng ADNg VD: 25ng-2.5 pg) were performed with 10ng/ul gDNA of *V. dahliae* extracted from conidia cultivated in messtiae medium and 100ng/ul gDNA *N. tabacum* from healthy plants. After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 60°C to 95°C.

Primer pairs used were designed with Beacon Premier Biosoft Software as shown in Table 1. Target gene expression was normalised to COX 1, EF2 and TUA expression (NF 0.9-1.1 statistically no significant). Relative expression (n-fold) of the normalised target gene in the treatments was calculated by comparison to control plants considering efficiency values according to the mathematical model proposed by Pfaffl, 2001 [1].

Fig. 1. Experimental designs: experiments on gene expression of plant defense molecular markers.

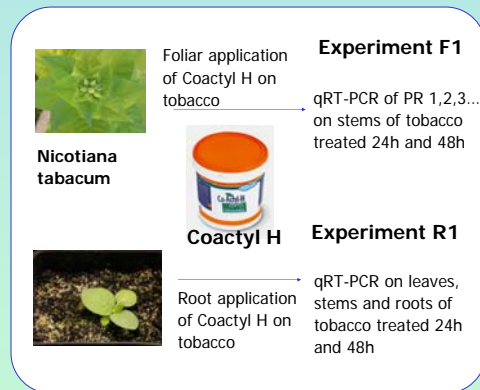
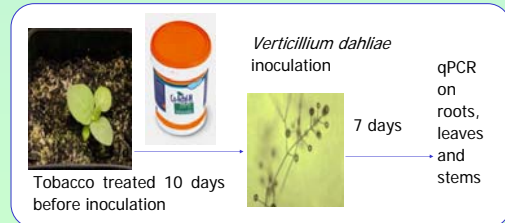
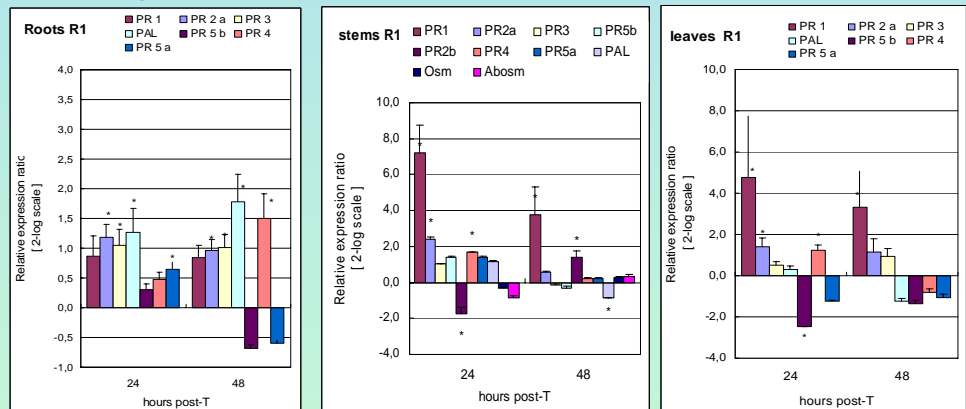


Fig. 2. Experimental design of disease resistance quantification by PCR of fungus colonization.



Results of gene expression of plant molecular markers: Experiments F1 and R1

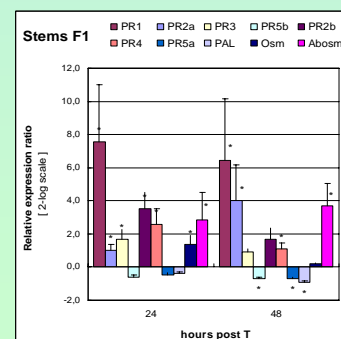


Results of PCR diagnostic assay

Detection and quantification of *Verticillium dahliae* in *Nicotiana tabacum* plant 7 days post inoculation (pg fungus DNA/ng DNA)

Treatment	Mock- treated plants (positive control)	Coactyl H- treated plants	No-infected plants (Negative Control)
Roots	5.5±0.75	0.4±0.17	Nd
Stems	Nd	Nd	Nd
Leaves	Nd	Nd	Nd

Nd : no amplicon detected after 40 cycles of PCR.



Discussion

First, the use of the real-time RT-PCR technology allowed to quantify the level of plant defense genes, thus assessing the level of plant defense mechanisms. Second, PCR quantifying the level of fungus highlight the potential of Coactyl as a novel compound able to provide commercially significant resistance (93%) of tobacco against *V. dahliae* through the activation of molecular defense responses. In conclusion, these molecular techniques provide useful tools to evaluate both the action of "inducing agent" in plant defense mechanism", and the effectiveness of the commercial product against pathogen infection.

[1] Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real time PCR. *Nucleic Acids Research* vol 30, n°9e36.