Quantification of stilbene in grapevine leaves by direct fluorometry and high performance liquid chromatography: spatial localisation and time course of synthesis

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QUANTIFICATION OF STILBENE IN GRAPEVINE LEAVES BY DIRECT FLUOROMETRY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: SPATIAL LOCALISATION AND TIME COURSE OF SYNTHESIS

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Abstract

Aim: This work aimed at studying the spatial distribution of stilbenes in grapevine leaves of different genotypes and to determine their time course of synthesis. These highly fluorescent molecules are considered as the main phytoalexin in grapevine.

Methods and results: Two complementary techniques were used to study the synthesis of stilbenes. On the one hand, direct fluorescence of leaves that allowed local evaluation of stilbenes content on 1.6 mm^2, and, on the other hand, a well known precise and accurate method: high performance liquid chromatography. The latter is time consuming and requires extraction of about 1 cm^2 of leaf. This study was performed on different grapevine genotypes cultivated in greenhouse.

Conclusion: Stilbenes content measured by HPLC was highly correlated to the specific fluorescence of stilbene. Fluorescence measured on the abaxial side of the same leaf area increased linearly at least during five days after inoculation. Nevertheless, fluorescence intensity varied depending on the leaf studied and stilbenes are not homogeneously distributed on the abaxial side of the leaf.

Significance and impact of study: The results showed that *in vivo* stilbene fluorometry is an efficient, fast and non destructive tool to evaluate stilbene content of grapevine leaf.

Key words: grapevine, stilbenes, spectrofluorometry, HPLC, *Plasmopara viticola*

Résumé

Objectif : L'objectif de ce travail est d'étudier la distribution spatiale et la cinétique de synthèse des stilbènes dans les feuilles de vigne. Ces molécules fortement fluorescentes sont considérées comme étant les principales phytoalexines de la vigne.

Méthodes et résultats : Deux techniques complémentaires ont été utilisées pour étudier la synthèse des stilbènes. D'une part, par l'analyse directe de la fluorescence sur les feuilles ce qui permet une évaluation globale de la teneur en stilbènes sur 1,6 mm^2 et d'autre part en chromatographie en phase liquide, méthode précise et fiable. Cette dernière technique est longue et nécessite environ 1 cm^2 de feuille. Cette étude a été menée sur différents génotypes de vigne cultivés en serre.

Conclusion : Les teneurs en stilbène mesurées en HPLC sont fortement corrélées à la fluorescence spécifique des stilbènes. La fluorescence mesurée sur la même surface abaxiale d'une feuille augmente linéairement durant au moins cinq jours après inoculation. Cependant, l'intensité de la fluorescence varie selon la feuille étudiée et n'est pas distribuée de façon homogène sur la face abaxiale de la feuille.

Signification et impact de l'étude : Ces résultats montrent que la mesure de la fluorescence des stilbènes *in vivo* est une méthode efficace, rapide et non destructive permettant d'évaluer la teneur en stilbène dans les feuilles de vigne.

Mots clés : vigne, stilbènes, spectrofluorimétrie, HPLC, *Plasmopara viticola*

manuscript received the 18th July 2008 - revised manuscript received the 27th March 2009

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J. Int. Sci. Vigne Vin, special issue Macrowine, june 2010, 27-32

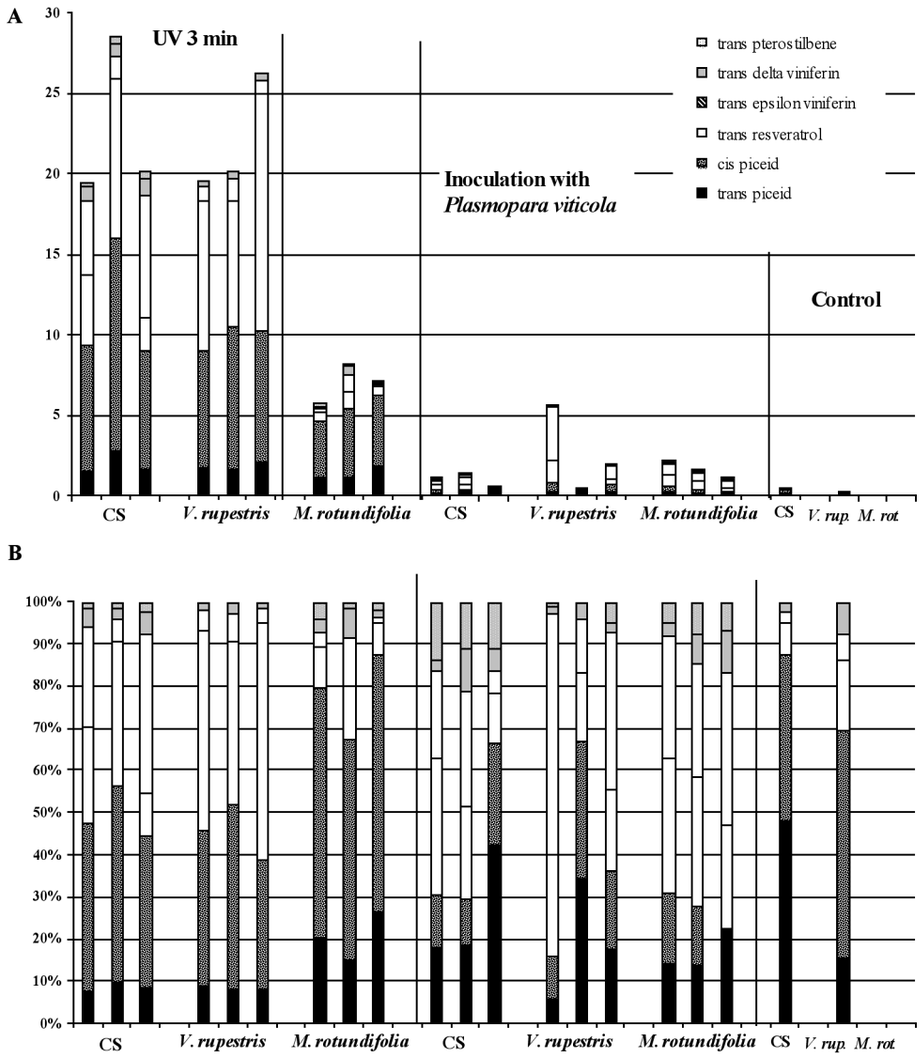

Figure 1 - Stilbenes content (A) and relative content (B) of the six main stilbenes assayed in three leaves of different *Vitis* species treated with UV-C, three days post treatment, or inoculated with *P. viticola*, four days post inoculation. Cabernet-Sauvignon (CS), *V. rupestris*, *Muscadinia rotundifolia.*

INTRODUCTION

Stilbenes, considered as the main phytoalexin in grapevine, belong to the wide phenolic compound family. They are synthesised in grapevine leaves under biotic or abiotic stresses (Adrian *et al.*, 1996) and are intervening in the plant-pathogen interaction (Hain *et al.*, 1993; Langcake, 1981) thus contributing to plant resistance (Morrissey and Osbourn, 1999; Sbaghi *et al.*, 1995). Stilbene synthase (STS) allowed the synthesis of stilbene. The main stilbene accumulated under the action of STS is resveratrol. Numerous derivatives of resveratrol exist like the glucosylated form piceid, oligomeres viniferins or dimethylated resveratrol pterostilbene. Most of these molecules are highly fluorescent (Jeandet *et al.*, 1997; Latouche *et al.*, 2008). Plant transformed by *Vitis vinifera* STS gene generally increases their level of resistance to pathogens (Schwekendiek *et al.*, 2007). In grapevine, level of resistance to *Plasmopara viticola* could be linked to the rate of stilbene synthesis and/or to their local concentration around the infection point (Dercks and Creasy, 1989).

This work aimed at studying the distribution of stilbene in grapevine leaves of different genotypes and to determine their time course of synthesis. Furthermore, two complementary methods were compared to measure the stilbene content of grapevine leaves following UV-irradiation and *P. viticola* infection:

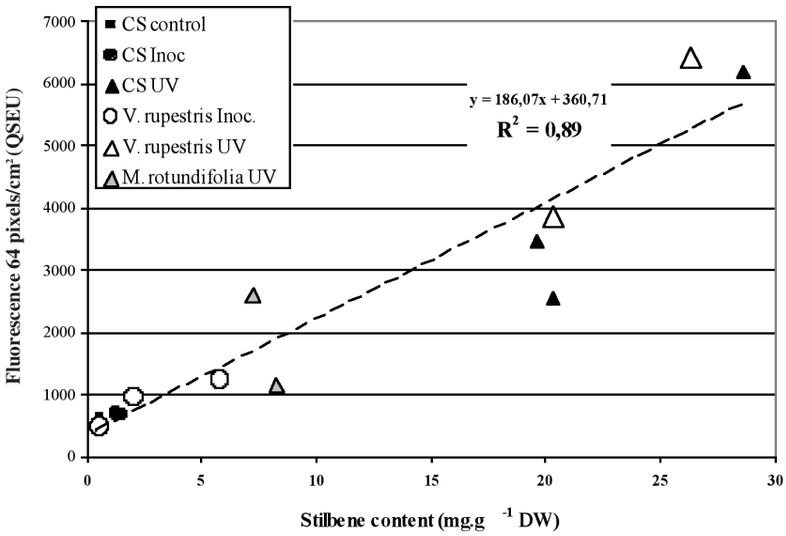

Figure 2 - Relation between fluorescence measurements on the abaxial leaf surface and the stilbene content (for inoculated leaves R^2 = 0.87 and for UV treated leaves R^2 = 0.89).
QSEU: quinine sulfate equivalent unit

- Fluorometry. This simple and fast technique has the advantage to be directly performed on the abaxial side of the leaf *in vivo* (Poutaraud *et al.*, 2007a). The measurement is local and can be repeated on the whole leave giving a mapping of the stilbene distribution.

- High performance liquid chromatography. This widely used method to quantify stilbene is accurate and very informative because of the separation of the molecules, but it is destructive and time consuming (Jean-Denis *et al.*, 2006).

MATERIALS AND METHODS

1. Plant material and leaf sample preparation

Different genotypes were studied that differed on their level of susceptibility to *Plasmopara viticola*: *Vitis vinifera* MO: Muscat ottonel (very susceptible), CS: Cabernet-Sauvignon (susceptible), *V. rupestris* (partially resistant) and *Muscadinia rotundifolia* (totally resistant). Plants were green cuttings grown in greenhouse at 26 °C ± 3 °C, thirteen hours of light (between about 100 and 800 µmol photon $m^{-2}.s^{-1}$), eleven hours of dark, under continuous water and fertilizer applications. The study was performed when plants were at the stage of about fiftheen leaves.

2. Experimentations

To study the localisation of stilbene on the leaf, we used five plants of each of the three following genotypes: CS, *V. rupestris* and *Muscadinia rotundifolia*. The abaxial surface of the fifth leaf (counting from the top) of three plants per genotypes was irradiated by UV-C (3 min) and the sixth leaf of the same plant was immersed in *P. viticola* inoculum at $10^{\wedge}4$ sporangia/ml. Control leaves were dept in water. Five hours later, leaves were placed in transparent boxes on wet paper, the abaxial surface up. The boxes were placed in a culture chamber at 22 °C with eighteen hours of light (about 200 µmol photon m^{-2} s^{-1}) and six hours of darkness. Before treatment and four days post inoculation (dpi) or three days post UV treatment (dpUV), abaxial leaf surface fluorescence was scanned with a microplate fluorometer (Genios Pro Tecan, Lyon, France) equipped with specific stilbene filters: excitation at 330 nm and emission at 389 nm. Four or sixty four individual fluorescence measurements of about 1.6 mm^2 (pixel) were performed per cm^2 of leave (Poutaraud *et al.*, 2007a) then the leave was extracted with MeOH and major stilbenes were measured by HPLC-DAD. Fluorescence is expressed here in quinine sulfate equivalent unit (QSEU) (Poutaraud *et al.*, 2007a).

To follow the time course of the synthesis of stilbene, the fluorescence was measured each day on exactly the same cm^2 area of leaf of the fifth leaf of MO from day one to day five on three inoculated and three control leaves. The last day, the zones studied were extracted with MeOH at 60 °C for 45 min and the presence of *trans*- and *cis*-isomers of resveratrol, piceid, ε and δ viniferin and pterostilbene was measured by HPLC-DAD (Poutaraud *et al.*, 2007b).

3. Statistical analyses

Statistical analyses were performed using the software StatBox Pro (version 2.5, Grimmer softwares, Paris, France).

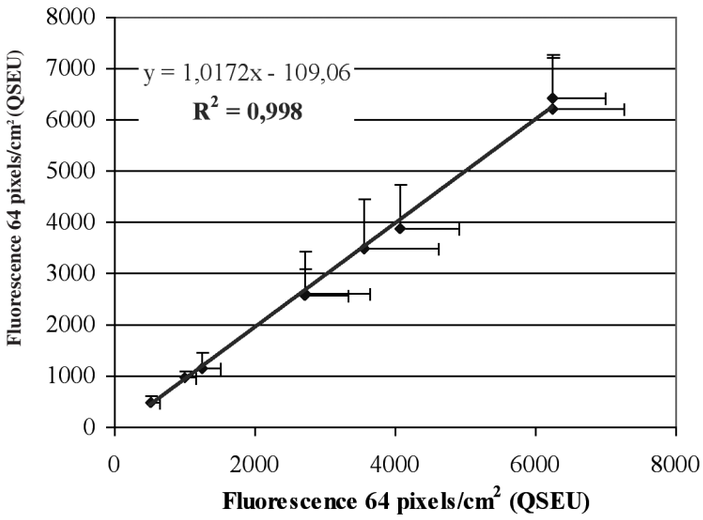

Figure 3 - Relation between fluorescence of abaxial surface of leaves studied at 64 and at 4 pixels per cm².
Bars represent standard deviation of the mean value measured on 1 cm².

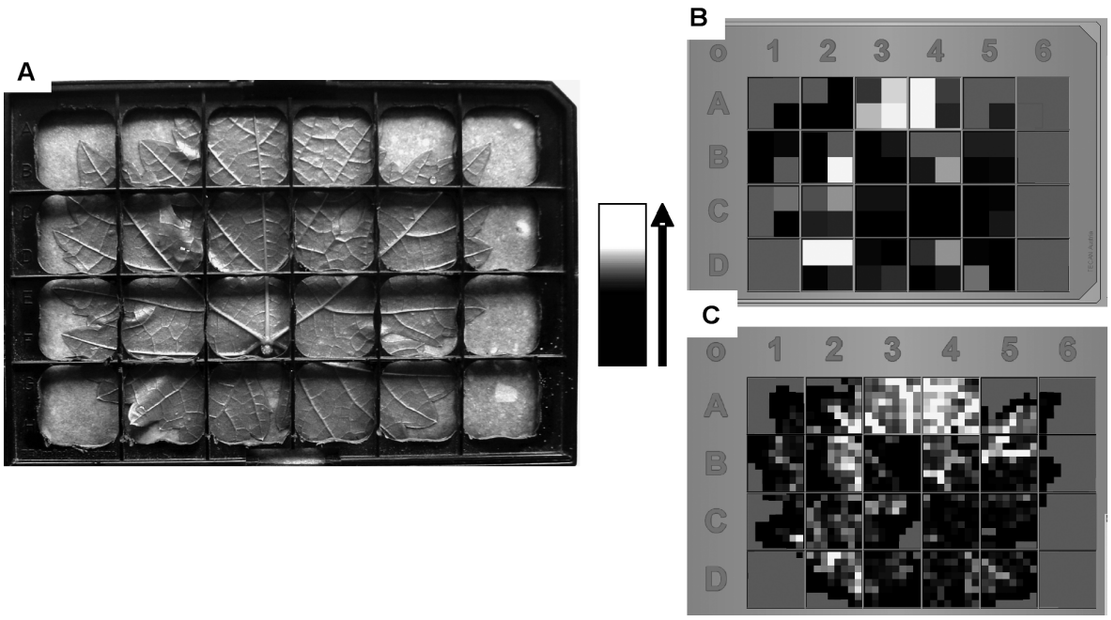

Figure 4 - A - Cabernet-Sauvignon leaf three days post UV-C treatment.
B - Fluorescence distribution on abaxial side at 4 pixels per cm².
C - at 64 pixels per cm².
In black : low fluorescence, in white : high fluorescence.
For B and C, the background of the leaf was colored in gray which doesn't correspond to fluorescence.

RESULTS AND DISCUSSION

Different stilbene content and composition were obtained four days after leaf inoculation by *P. viticola* or three days post UV-treatment of different grapevine genotypes (figure 1). Stilbene content was measured three days post UV treatment. It is late compare to previous studies but it was done on purpose to better visualize the difference of synthesis between genotypes. Inoculated leaves were studied even latter because this way of induction of stilbenes synthesis is softer than UV. Almost no constitutive stilbene was found in non-induced leaf (control) showing that plants were not stressed or infected before the experimentation. Stilbene synthesis was always higher after a three minutes UV treatment than after inoculation by *P. viticola* whatever the genotype. Analysis

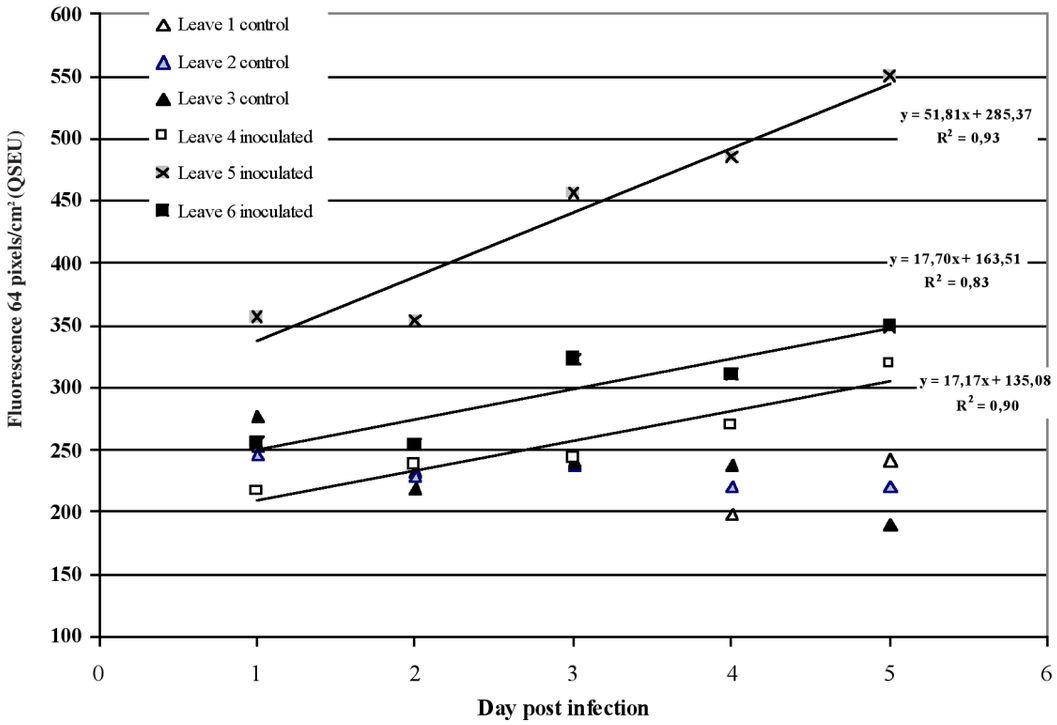

Figure 5 - Evolution of the fluorescence on the abaxial surface of Muscat ottonel leaves infected by *P. viticola* and non infected controls.

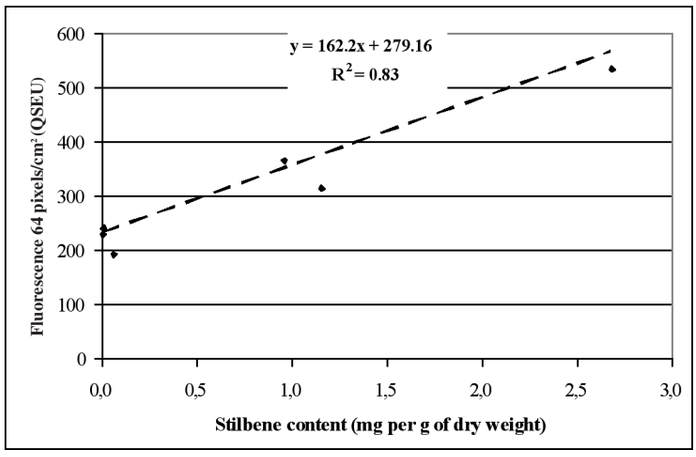

Figure 6 - Relation between fluorescence acquired with the microplate fluorometer (Genios Pro) on Muscat ottonel abaxial leaf side and the HPLC-DAD analysis of the corresponding samples (leaves infected by *P. viticola*, and non infected controls).

of variance (data not shown) showed no significant difference between the three inoculated genotypes although their level of resistance to *P. viticola* is very different. In UV treated leaves, *Muscadinia rotundifolia* presented a lower significant stilbene content.

Highly significant linear relation was found between stilbene content measured by HPLC-DAD and fluorescence measured on the abaxial side of the leaves, at 64 pixels per cm² (figure 2). This result validated the non destructive fluorescence measurements for the local evaluation of stilbene content. The small difference observed between fluorescence and HPLC measurements could be attributed to two major phenomenon. Firstly, stilbenes produced in the inner leaf tissues are likely not detectable by fluorometry. Secondly, as the proportion of

the six main stilbenes assayed in the leaves is different depending on the samples (figure 1B) it could be attributed to the difference in the stilbene fluorescence spectra (Poutaraud *et al.*, 2007a).

The relation between stilbene content and the fluorescence measured with 4 pixels/cm² was also highly significant. We obtained a coefficient of determination R^2 of 0.86 (data not shown) for this last relation and of 0.99 for the relation between the measurements of the fluorescence at 4 pixels/cm² and at 64 pixels/cm² (figure 3) showing that a rapid fluorescence scan of the leaf (4 pixels/cm²) is efficient enough to determine the global content of stilbenes on the leaf.

Measurement of stilbene fluorescence in microplate with a spectrofluorometer allows the study of the distribution of these molecules on an entire leaf. As shown in figure 4 for the abaxial side of a Cabernet-Sauvignon leaf treated 3 min with UV-C light, the distribution of fluorescence is not homogenous. In that case, the ratio between the 1 cm² area presenting the highest (orange) and the lowest fluorescence (green) is 1.7. For the other samples, this ratio can reach 2.5. Therefore, particular attention must be paid during sampling for stilbene quantification.

To follow the time course of the synthesis of stilbene, the fluorescence was measured each day on exactly the same cm² area of the fifth leaf of Muscat ottonel (64 fluorescent pixels per cm²) from day one to day five on three inoculated and three control leaves (figure 5). The last day, the zones studied were extracted and stilbene content measured by HPLC-DAD. The relation between these two methods was, as in the previous experimentation (figure 2), very significant (figure 6, $R^2 = 0.83$).

Fluorescence increased linearly after infection at least during five days. As already shown by HPLC-DAD (Poutaraud *et al.*, 2007b), the time course of production of stilbene is linear. It looks that after induction of the synthesis the mechanism of synthesis is engaged and run linearly. The fluorescence intensity depended on the particular leaf even though all leaves were of the same node number on the plant and as the inoculation was identical.

CONCLUSION

These experiments validate *in vivo* stilbene fluorometry as an efficient, fast and non destructive tool to evaluate stilbene content of grapevine leaf at different scales (from the total leaf area to only 1.6 mm²). For the same induction treatment the synthesis of stilbene is not homogeneously distributed on leaf surface and leaves of the same node on the plant could react differently. Stilbene leaf fluorescence appears to be a good marker of the early presence of pathogen attack. It is a good indicator for several biotic or abiotic stresses but completed with the climatic conditions to determine the kind of stress, it could be use as a decision making tool to optimise the positioning and the rate of phytochemical applications on grapevine or in breeding programs to rapidly screen population of grapevine for their capacity to produce stilbene.

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