Induction of Phytoalexin (Resveratrol) Synthesis in Grapevine Leaves Treated with Aluminum Chloride ($AlCl_3$)

Keywords: *Phytoalexins; resveratrol; aluminum chloride; metallic salts; grapevines*

INTRODUCTION

Phytoalexins are organic metabolites that are produced by plants in response to fungal infection or abiotics such as heavy metal ions or UV light (Bailey, 1982). In grapevines, the response to stresses, *e.g.* fungal infection and UV irradiation, includes the production of a simple stilbene, resveratrol (*trans*-3,5,4′-trihydroxystilbene), and its glucoside (Waterhouse and Lamuela-Raventos, 1994) together with the biosynthetically related compounds viniferins and pterostilbene (*trans*-3,5 dimethoxy-4′-hydroxystilbene) [Langcake and Pryce, 1977; see Jeandet *et al.* (1995b) for a review]. These compounds have been shown to be closely related to the resistance of grapevines to gray mold caused by *Botrytis cinerea* Pers. (Langcake, 1981; Sbaghi *et al.*, 1995).

As part of a current research program, we are investigating a practical means to induce the synthesis of phytoalexins in grapevines to increase their resistance to fungal pathogens. We report here, for the first time, that a metallic salt, aluminum chloride, can act as a potent elicitor of resveratrol synthesis in grapevine leaves.

MATERIALS AND METHODS

Plant Material. Cuttings from *Vitis vinifera* cv. Pinot noir clone 113 (collected at the Viticultural and Enological Experimental Station of the University of Bourgogne) and *Vitis rupestris* cv. Rupestris du Lot clone T110 (supplied by Dr P. Lavie, Institut Jules Guyot) were raised in a climate-controlled room at 25 °C with a 16 h light/8 h dark photoperiod. The upper part of 7-month-old shoots developed from these cuttings was then sectioned between the fourth and the fifth leaf under the apex for resveratrol production assessment.

Elicitation by Aluminum Chloride. $AlCl_3 \cdot 6H_2O$ (Hoechst, Roedel-de-Hain, Germany) was dissolved in deionized water at concentrations of 7, 9, 14, 18, 22, 44, 70, and 90 mM (expressed as millimoles of $AlCl_3$). Grapevine shoots were then dipped in tubes containing water as control or aluminum chloride solutions for 9−26 h and placed in the dark. Each tube was covered with parafilm perforated to position the shoot. Three different experiments were performed with three replicates per experiment.

Since the pH of $AlCl_3 \cdot 6H_2O$ solutions is *ca.* 3, experiments were also carried out by dipping the shoots in deionized water acidified with 1 N H_2SO_4 or 1 N HCl to verify if the phenomena observed were related to the pH of the solution. Similarly, to verify whether the aluminum cation is causing the activity, another control, aluminum sulfate [$(SO_4)_3Al_2 \cdot 14H_2O$; Prolabo, Paris, France], was used at amounts similar to those used as described with aluminum chloride.

Extraction of Resveratrol. Resveratrol was extracted according to a modification of the method of Langcake and Pryce (1976) and Jeandet *et al.* (1991). Material from all sources was ground in a mortar with sand and 30 mL of methanol−water (8:2 v/v). After centrifugation at 10000*g* for 15 min, the supernatant was prepurified on a Sep-Pak C_{18} cartridge (Waters, Milford, MA.). After elution with methanol−water (8:2 v/v), the eluate was evaporated to dryness (<40 °C). Leaf extracts were then redissolved in 10 or 20 mL of methanol/g of fresh weight and analyzed by HPLC. During sample preparation, extracts were constantly protected from light to avoid photochemical isomerization of *trans*-resveratrol to the *cis* form.

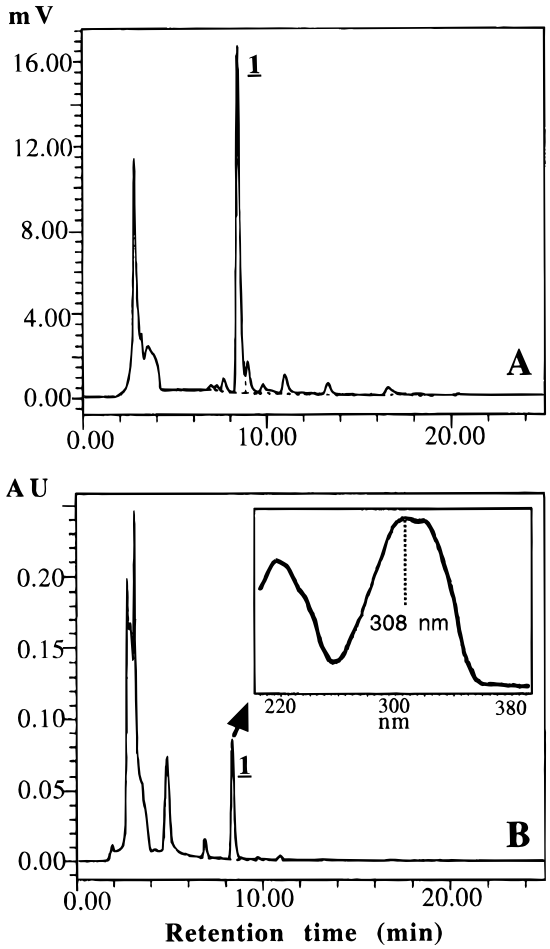

Figure 1. HPLC analysis of a *V. rupestris* leaf extract treated with 14 mM $AlCl_3$, using fluorometric detection (A) (ex = 330 nm, em = 374 nm) or UV detection (B). Peak 1 is *trans*-resveratrol (retention time = 8.40 min). (Inset) UV spectrum of compound **1** (maximum absorbance = 308.9 nm). For chromatographic conditions see Materials and Methods.

HPLC Analysis. Samples were injected on a lichrocart Merck C_{18} (Merck-Clevenot Corp., Darmstadt, Germany) reversed phase column (250 × 4 mm; 5 μm) and analyzed isocratically with 40% acetonitrile/60% water eluant at a flow rate of 0.6 mL/min using a Waters system comprising a Model W600 system controller, a Model W717 sample injector, a Model W996 photodiode array detector, and a Model W474 fluorometer. Resveratrol was detected at 308 nm (Jeandet *et al.*, 1995a). For fluorometric detection, maximum excitation wavelength was measured at 340 nm and emission at 374 nm. The specificity of the fluorometric parameter detection, linked to the chemical structure of stilbene compounds, decreases the risk of peak confusion (Pezet *et al.*, 1994).

Identification and Quantification of Resveratrol. Identification of *trans*-resveratrol in extracts was carried out by comparison of the retention time of pure resveratrol, synthesized as described in Jeandet *et al.* (1991), and that within the extracts. Resveratrol was also characterized by its UV spectrum (Figure 1). A standard calibration curve was established using peak area *vs* different concentrations of resveratrol (*i.e.* 10, 20, 50, 100, and 200 ng). Linear correlation for fluorometric detection was excellent (correlation coefficient $r^2 = 0.9990$).

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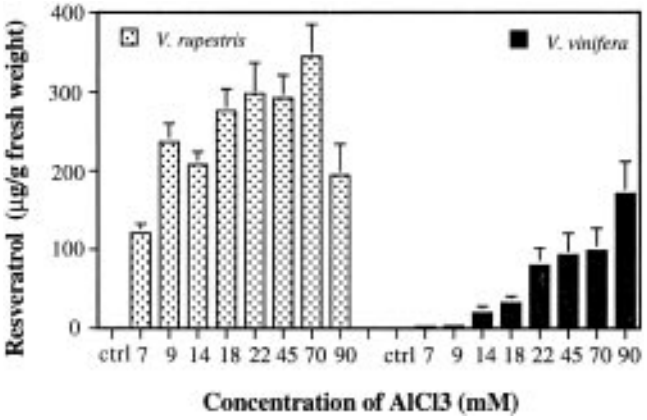

Figure 2. Effect of various concentrations of aluminum chloride (expressed as millimoles of $AlCl_3$) on resveratrol accumulation in grapevine leaves of two *Vitis* spp. (*V. vinifera* and *V. rupestris*). Grapevine shoots were dipped in tubes containing $AlCl_3 \cdot 6H_2O$ solutions (from 7 to 90 mM $AlCl_3$) for 22 h at room temperature in the dark. Leaves were then extracted and analyzed by HPLC (see Materials and Methods). Ctrl, controls (water and H_2SO_4-acidified water). Experiments were made in triplicate with similar results obtained. We present here the results of one experiment (three replicates/experiment). Vertical bars indicate standard errors for the three replicates.

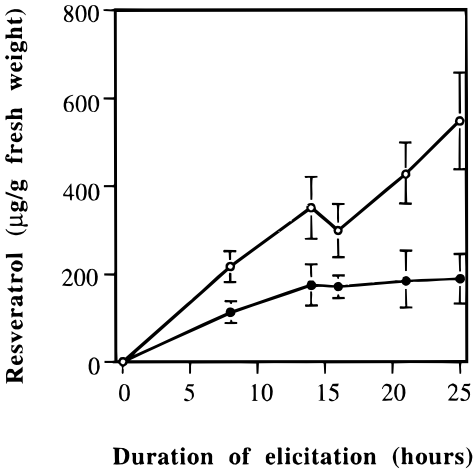

Figure 3. Time course changes in resveratrol synthesis by grapevine leaves: *V. vinifera* (●) and *V. rupestris* (○) in response to induction by aluminum chloride at a concentration of 70 mM $AlCl_3$. Grapevine shoots were dipped in tubes containing $AlCl_3$ and then incubated for various times (9, 15, 30, 17, 22, and 26 h) at room temperature in the dark. Experiments were made in triplicate with similar results obtained. We present here the results of one experiment (three replicates/experiment). Vertical bars indicate standard errors for the three replicates.

RESULTS AND DISCUSSION

After 15 h of incubation, $AlCl_3$-treated leaves (with the exception of the one or two youngest ones per cutting, which produce only very low amounts of resveratrol; Sbaghi *et al.*, 1995) exposed to long-wave UV light exhibited a bright blue fluorescence that is characteristic of *trans*-resveratrol (Langcake and Pryce, 1976). This fluorescence appeared first in principal veins and then in fine veination and generally covered the entire adaxial and abaxial leaf surfaces, thus indicating a systemic action of aluminum chloride. Both the adaxial and the abaxial surfaces of the leaves showed a bright fluorescence. This fact has never been previously reported. Past studies have shown (Langcake and Pryce, 1976; Pool *et al.*, 1981) that only the abaxial surface of leaves was fluorescent after treatment with UV light, suggesting that the sites of stilbene synthesis were located abaxially within the leaf. In comparison, no fluorescence could be seen on leaves of shoots dipped in acidified water (pH 3) or in the control.

$AlCl_3$-treated leaf extracts analyzed by HPLC using UV and fluorometric detection revealed a compound with a retention time identical to that of authentic *trans*-resveratrol (Figure 1). The intensity of blue fluorescence was directly related to the quantity of resveratrol accumulated by leaves. As suggested by long-wave UV light observations, no resveratrol was found in leaf extracts obtained from shoots dipped in water or in acidified water.

The two species evaluated exhibited a differential response in their ability to synthesize *trans*-resveratrol in response to $AlCl_3$ treatment. All concentrations of $AlCl_3$ (from 7 to 90 mM) were capable of inducing a high resveratrol production in the leaves of *V. rupestris*, while greater concentrations of $AlCl_3$ (from 22 to 90 mM) were required to obtain a similar response in the leaves of cv. Pinot noir (Figure 2). It appears that *V. rupestris* generally produces greater amounts of resveratrol than does Pinot noir (Figures 2 and 3). These results are in agreement with those of Sbaghi *et al.* (1995), who reported that *V. rupestris* has a higher potential to produce resveratrol in response to UV irradiation (up to 500 μg/g of fresh weight) than *V. vinifera* cv. Pinot noir (up to 200 μg/g of fresh weight): *V. rupestris* also exhibits higher disease resistance, particularly against *B. cinerea*. Thus, stimulation of phytoalexin synthesis in susceptible or tolerant species seems to be strictly determined by the inherent capacity for phytoalexin production, whatever the elicitor (Dercks and Creasy, 1989).

The content of resveratrol in leaves of both species was enhanced, at all concentrations of $AlCl_3$, with increasing durations of elicitation (Figure 3). However, at high concentrations of $AlCl_3$ (>90 mM) symptoms of toxicity (*i.e.* a withering of the leaf and a bright yellow fluorescence of parts of the leaf when examined under long-wave UV light) were noted with a corresponding decrease in resveratrol induction in *V. rupestris*. On the other hand, the minimal concentration required to obtain a phytoalexin response in leaves was 7 mM, with a minimal elicitation duration of 9 h.

Aluminum sulfate used at the same concentration as $AlCl_3$ was found to have effects similar to those of $AlCl_3$ on phytoalexin induction (as shown by the blue fluorescence on leaves and HPLC analysis), suggesting that the aluminum cation is responsible for the activity observed.

The data thus clearly show that $AlCl_3$ can stimulate phytoalexin accumulation in grapevine leaves. To our knowledge, no metallic salt has yet been reported to induce resveratrol synthesis in grapevines. Previously published results have shown that the salts of mercury or copper can induce the production of isoflavonoids by *Pisum sativum* (Perrin and Cruickshank, 1965; Sweigard *et al.*, 1986), wyerone and other furanoacetylenes by *Vicia faba* (Mansfield *et al.*, 1980), sesquiterpenes by *Solanum tuberosum* (Tomiyama and Fukaya, 1975), and stilbene compounds by *Veratrum grandiflorum* (Hanawa *et al.*, 1992). In grapevines, however, much interest has been focused upon the effect of fosetyl-aluminum, a fungicide used for the control of downy mildew in the vineyard, on phytoalexin accumulation. Nevertheless, application of this compound alone does not appear to result in *de novo* phytoalexin synthesis

(Raynal *et al.*, 1980; Fenn and Coffey, 1985; Dercks and Creasy, 1989).

CONCLUSION

Strong evidence is provided to suggest that $AlCl_3$ is a potent elicitor of resveratrol production in grapevine leaves. This is the first time that a metallic salt has been shown to act as a direct inducer of phytoalexin response in grapevines, resulting in systemic production of resveratrol. Thus, it would be of great interest to determine the potential for $AlCl_3$ treatment for control of *B. cinerea* in the vineyard.

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