Pythium induced phenolic compounds in the resistance of *Vitis vinifera* to *Botrytis cinerea*

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Grey mold rot in grapevine is caused by a necrotrophic pathogen, *Botrytis cinerea*. There is an enormous loss in the quality and quantity of grapes in the Burgundian vineyards due to *Botrytis* infection. Our studies demonstrate that some species of *Pythium* are capable of inducing disease resistance in grapevine against the grey mold pathogen by accumulating phenolic compounds notably, ellagic, caffeic, gallic, caffeic, o-anisic, 3,4-dihydroxy benzoic and salicyclic acid. This is the first report on *Pythium* induced accumulation of phenolic compounds in the grapevine challenged by *B. cinerea*. Phenolic compounds produced in grapevine at different time periods after exposure to *Pythium* and *B. cinerea* were quantified and their significant increase in quantity was evaluated by statistical analyses.

Key words – *Botrytis cinerea* – disease resistance – HPLC – oomycete – phenolic compounds – *Pythium* – *Vitis vinifera*

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Introduction

Botrytis cinerea is a necrotrophic pathogen that infects a wide variety of plants such as tomato, beans, peppers, strawberries, onion and other crop plants (Elad & Shtienberg 1995). *B. cinerea* has ability to survive harsh environmental conditions in the form of sclerotia. The emergence of fungicide resistant strains of *B. cinerea* (Katan 1982) is challenging for the grape growers. There is an estimated 15-40% annual loss in grapevine due to grey mold disease in France.

Pythium is a diverse genus that consists of approximately 140 recognized saprobic and parasitic species. The plant parasitic species of *Pythium* have devastating impact on economically important crops worldwide (Vanterpool 1938). Some *Pythium* spp. are aggressive mycoparasites of *B. cinerea* (Paul 1999) and non-pathogenic to tomato, beans (Bala et al. 2009) and grapevine.

In the past few decades, phenolic acids have received major attention as antifungal factors in biological control (Lavania et al. 2006). The role of phenolic acids in the defense against bacterial and fungal infections has been demonstrated (Harborne 1993, Ebukanson 1989). Although phenolic acids are known to be present in plants (Hendry 1993), their accumulation is increased under biotic or abiotic stress. This increased production is possibly related to enhanced disease resistance in plants against pathogens (Lattanzio et al. 2006).

Major phenolic compounds like gallic, caffeic, ellagic, ferulic, chlorogenic, protocatechuic acids were induced by *Serratia marces-*

cens NBRI1213 in betelvine roots and shoots challenged with *Phytophthora nicotianae* (Lavania et al. 2006). Phenolic acids have antifungal properties (Sharma & Singh 2003) that confer protection to plants against fungal infections. Phenolic compounds lacking antifungal activity, such as gallic acid, get converted into antifungal compounds that are gallotannins and thereby protect plants against fungal infections (Binutu & Cordell 2000).

Phenolic acids are well known to have key role in the cell wall lignification and phytoalexin synthesis (Wagner 1988). Phenolic acids, particularly coumaric acid, caffeic and ferulic acid are intermediates of lignin biosynthesis (Dixon & Paiva 1995). Lignin accumulation is associated with an increase in cell-wall mechanical rigidity thereby preventing invasion of xylem (Stafford 1988). Lignin and phytoalexin accumulation provides resistance to plants against pathogens. Other phenolic compounds, especially synthetic caffeic acid and rosmarinic acid have a direct inhibitory effect on the germination of zoospores of *Phytophthora capsici*, *P. megakarya* and *P. palmivora* (Wid-mer & Laurent 2006).

The most important signaling molecule, salicyclic acid (SA), is the key compound in local and systemic disease resistance (Mauch-Mani & Slusrenko 1996, Shah & Klessig 1999) and synthesis of pathogenesis related protein (PR) (Dempsey et al. 1999; Shah & Klessig 1999). Systemic acquired resistance (SAR) is induced by the exposure of root or foliar tissues to abiotic or biotic elicitors and is dependent on the phytohormone salicylate that is associated with the accumulation of pathogenesis related proteins. An activated PR expression restricts infection by pathogens and therefore, the transgenic plants that were not able to accumulate salicyclic acid could not activate SAR genes or disease resistance to an array of pathogens that they encountered (Ward et al. 1991, Gaffney et al. 1993).

Our objectives are to assess the synthesis of phenolics in grapevine co-inoculated with *Pythium* and *Botrytis* and to compare their quantity in grapevine inoculated with different species of *Pythium*. We provide the first evidence for *Pythium*-induced increase in the accumulation of phenolic compounds in grapevine leaves during ongoing suppression of *B. cinerea* by *Pythium* spp.

Methods

Pythium isolation

Pythium species were isolated from soil using hemp-seed baiting technique (Bala et al. 2006). The name of isolates used in this study and their GenBank accession numbers are provided in Table 1.

Growth in culture and inoculations of *in vitro* grapevine plants

Pythium isolates were grown and maintained on potato carrot agar (PCA), corn meal agar (CMA) and potato dextrose agar (PDA). PCA was prepared by boiling 20 g of carrots and 20 g of potato in 1 litre of distilled water followed by adding 15 g of agar (Difco) to the extract and sterilization for 20 min by autoclaving. CMA (Difco) and PDA (Difco) were prepared according to the manufacturer instructions. *Botrytis cinerea* strains were isolated from grapes in the vineyards of Marsannay-la-Côte region that is located 6 km South-West of Dijon. *B. cinerea* strain BC-3 cultures were grown on PDA. Murashige and Skoog media (Murashige & Skoog 1962) was prepared, sterilized and inoculated with the sterile grapevine varieties pinot noir and chardonnay twigs and transferred to growth chamber maintained at 24^{o}C with 16h light and 8h dark photoperiod. Three months old *in vitro* grapevine cultivar chardonnay leaves were inoculated with the *Pythium* spp. only, or *Pythium* spp. and *B. cinerea* simultaneously. The leaves were collected every 24 hours for 3 consecutive days.

Extraction method for phenolics and HPLC analysis

The grapevine leaves were vacuum-dried and the dry weight of leaves was measured. Salicyclic acid and other phenolics were extracted from 10 mg of grapevine leaves using methods of Verberne et al. (2002). Analysis of polyphenol content was done using RP-HPLC-DAD/FD (Reverse Phase High Performance Liquid Chromatography coupled to Diode Array Detector and Fluorescence Detector).

Phenolic compounds were analysed with a Dionex Summit system (Sunnyvale, CA, USA) equipped with a P580 gradient pump, a GINA 50 autosampler, a UVD 340S diode array detector (DAD), a JASCO FP-920 Intelligent Fluorescence Detector and an external BIO-RAD column heater. The separation was performed with a Nucleodur (Macherey-Nagel, Germany) RP C-18 Pyramid (250 x 4.6 mm i.d; 5 µm particle size) operated at a temperature of 40°C. The mobile phase consisted of 0.1% TFA in water (eluent A) and 0.075% TFA in acetonitrile (eluent B). The gradient program was as follows: 0% B to 9% B (15 min), 9% B to 18% B (50 min), 18% B to 100%B (10 min), 100% B (5 min), 100% B to 0% B (5 min) and column equilibration at 100% A during 5 min. Total run time was 90 min. The injection volume for all samples was 10 µL.

Simultaneous monitoring was set at 308 nm (p-coumaric acid, ferulic acid, caffeic acid, o-anisic acid and salicylic acid), 254 nm (ellagic acid, rutin, morin, quercetin-3-beta glucoside), 360 nm (quercetin, myricetin, kaempferol), 280 nm (gallic acid, catechin, epicatechin) for the DAD and excitation and emission wavelengths were set at 300 nm and 374 nm (specific wavelengths for stilbenes family molecules: trans-resveratrol, trans-piceid, trans-pterostilbene and trans-4-hydroxy-stilbene) respectively for the fluorescence detection, at a flow-rate of 1.0 mL/min. Spectra were recorded from 200-595 nm. Phenolic compounds were identified by HPLC by their retention and spectral data as compared by standards and were quantified using six-points calibration curves with custom-made external standard solutions (Sigma-Aldrich Chemical Company) from 1 mg/L to 0.1 mg/L and every 10 injections, a check standard solution was used to confirm the calibration of the system.

Statistical evaluation

All experiments were repeated three times, with three replications for each sample. The presented values are the means of nine determinations ± SD. Statistically significant differences in the mean values were analyzed by two-way ANOVA. Tukey's-test was performed to test the significance in mean differences at $p < 0.05$.

Results

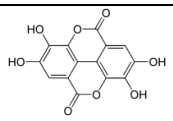
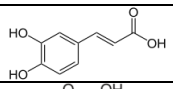
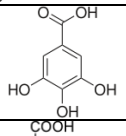
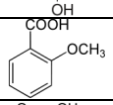
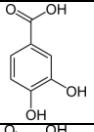
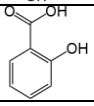
Total phenolic contents Figs 1–6

The total quantity of phenolic compounds accumulated in the grapevine leaves were measured by HPLC analysis. The quantities and other details are provided in Table 1. There was a significant difference ($p < 0.05$) in the total phenolic acid content in grapevine leaves exposed to different *Pythium* spp. as assessed by two-way ANOVA. The highest phenolics content was observed at 72h of post inoculation with *Pythium* and *Botrytis*. The phenolic compounds accumulated were mostly 3,4-dihydroxy benzoic acid, caffeic acid, gallic acid, ellagic acid, o-anisic acid and salicyclic acid. Among these, ellagic acid was produced in the highest concentration. The content was significantly different in other species of *Pythium*. The other phenolic acid contents were significantly lower than ellagic acid. The phenolic content increased with time and varied depending on the species of *Pythium* used for inoculation. The quantity of phenolics was significantly higher ($p < 0.05$) when strains of *P. ultimum* were used for the treatments. The species used in this analysis were non-pathogenic to grapevine and most of these were robust mycoparasites of *Botrytis cinerea*.

The maximally accumulated phenolic compounds in the grapevine leaves were ellagic acid (245 µg/ml dry weight), 3,4-dihydroxy benzoic acid (882 µg/ml dry weight), o-anisic acid (80 µg/ml dry weight), caffeic (6 µg/ml dry weight), gallic (1 µg/ml dry weight) and salicylic acid (0.4 µg/ml dry weight). The caffeic, gallic and salicyclic acid were produced in relatively lower amounts (Table 1). The quantities of all the phenolics amassed in the controls were negligible. The grapevines leaves challenged with *Botrytis* and treated with *Pythium* were many-fold higher in phenolics content than those treated with *Pythium* alone.

The enhanced accumulation of phenolic acids in grapevine challenged with *B. cinerea* and exposed to *Pythium* spp. demonstrates the possible role of phenolic compounds in inducing disease resistance in grapevine. The phenolic acids have an antifungal property that

Table 1 Phenolic acids content in grapevine leaves after co-inoculation with *B. cinerea* and *Pythium* species.

Phenolic compounds	**Formula**	**Biological effects**	**Maximum quantity (µg/ml)**	***Pythium* spp. used in this study**	**GenBank Accessions**
Ellagic acid		antioxidant and antiproliferative	245	*P. ultimum* strain F-1313, *P. ultimum* strain KN-27, *P. paroecandrum* strain B-30, *P. longisporangium* strain B76a, *P. rhizooryzae* strain F-1243	AY737318, AF215686, AY150168.1, AY455804.1, AY207379
Caffeic acid		antioxidant and anticancer	6	*P. ultimum* strain F-1313, *P. ultimum* strain KN-27	AY737318, AF215686
Gallic acid		anti-cancer, anti-inflammatory and anti-neural disorders	1		
o-Anisic acid		Constituent of antiseptic compounds	80		
3,4-Dihydroxy benzoic acid (protocatechuic acid)		Antigenotoxic effect and tumoricidal activity	882		
Salicylic acid		Prevents skin diseases	0.4		

confers resistance to grapevine against the grey mold pathogen.

This study provides first evidence on the role of *Pythium* as a biological elicitor that induces defense system in plants via production of phenolics in grapevine.

The phenolic compounds showed significant differences in their amounts depending on the species and time after application. Salicylic acid was only reported in *Vitis vinifera* cultivar chardonnay and not detected in cultivar pinot noir. Total phenolic acids amount in grapevine shows significant increase at 24 h, 48 h and attains a maximum level at 72 h of post inoculation. The *in-vitro* plants treated with both *Pythium* spp. and *B. cinerea* showed a large increase over those treated with *Pythium* spp. only. The leaves co-inoculated with the *Pythium* spp. and *B. cinerea* did not show any grey mold symptoms supporting the direct mycoparasitic action of *Pythium* on *Botrytis*. The leaves treated only with *B. cinerea* exhibited severe necrotic lesions within a week.

Discussion

The importance of phenolics in health and disease control has been described by Larrosa et al. 2010. Some of these phenolic compounds occur naturally in fruits and vegetables. Ellagic acid is naturally present in berries such as raspberries, cranberries, grapes, pomegranates and has antioxidant and antiproliferative properties (Umesalma & Sudhandiran 2011). Therefore, the consumption of ellagic acid has increased recently considering the health benefits. Our results strongly demonstrate that in the presence of *Pythium*, the grapevine resists the growth of *B. cinerea* and begets phenolics. Ellagic acid is synthesized in the highest quantities among all other phenolics in the grapevine leaves (Fig. 1).

Caffeic acid has potential antioxidant and anticancer properties. It occurs naturally in many fruits and vegetables such as cucumber and grapes. It is known to prevent skin irritation, eye swelling, sunburn and dermatitis (Li et al. 2005). Our studies showed that there is an ample increase in the amount of caffeic

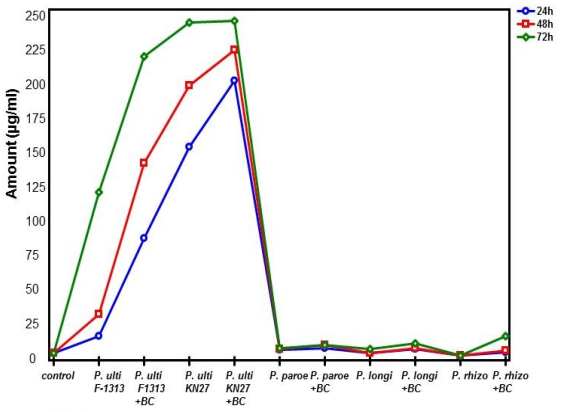


Fig.1 Ellagic Acid

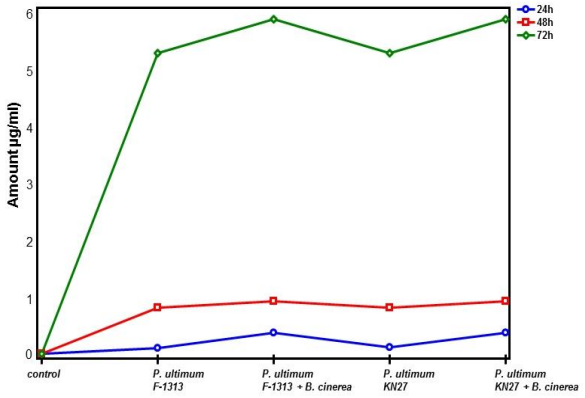


Fig. 2 Caffeic Acid

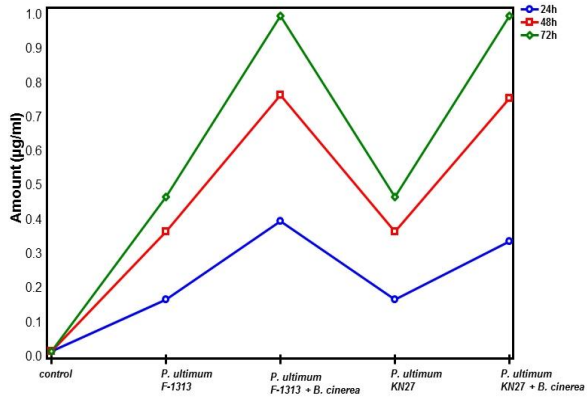


Fig. 3 Gallic Acid

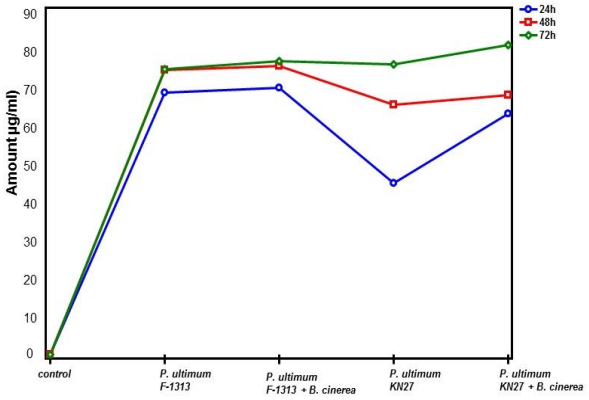


Fig. 4 o-Anisic Acid

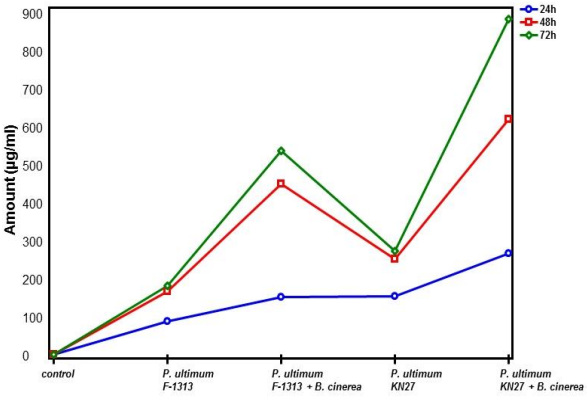


Fig. 5 3,4 Di-hydroxy benzoic acid

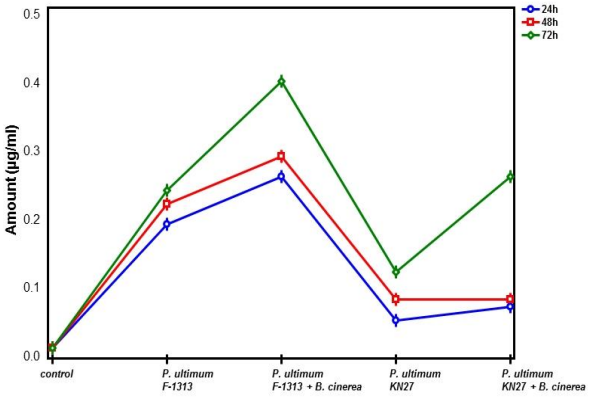


Fig. 6 Salicyclic Acid

Figs 1-6 – Phenolic acid contents in grapevine leaves treated with *Pythium* spp. and co-culture of *Pythium* spp. and *Botrytis cinerea*. Control represents non-treated grapevine leaf**. 1** Ellagic acid content. **2** Caffeic acid. **3** Gallic acid. **4** o-Anisic acid. **5** 3,4-Dihydroxybenzoic acid. **6** Salicyclic acid. Abbreviations: *BC = Botrytis cinerea, P. longi = P. longisporangium, P. paroe = P. paroecandrum, P. rhizo = P. rhizooryzae,. P. ulti = P. ultimum.*

acid when *Pythium* is counteracting *B. cinerea* on grapevine leaves (Fig. 2).

Gallic acid also known as 3, 4, 5 trihydroxy benzoic acid is naturally found in gallnut, sumac, witch hazel, tea leaves and other plants. Previous studies (Kim etal 2011) have shown that it has anti-inflammatory, anti-neural disorders activities and anti-cancer activities against leukemia, certain prostrate, colon and lung cancer cells. Our results show that gallic acid is detected in the grapevine leaves during an ongoing interaction between *Pythium* and *B. cinerea* (Fig. 3). Anisic acids are considered to be parts of antiseptic compounds. The synthesis of o-anisic acid (Figure 2) is observed in grapevine leaves treated with *Pythium and B. cinerea* (Fig. 4).

3,4 Di-hydroxy benzoic acid (DHBA) also known as protocatechuic acid has antigen-toxic and tumoricidal effects on leukemia cells (Tanaka et al. 2011). Our analysis shows an enhanced production of DHBA when *Pythium* mycoparasitises *Botrytis* on grapevine leaves (Fig. 5).

Salicyclic acid has dual benefits, both cosmetic and medicinal, and is used to treat skin diseases, acne, psoriasis and remove warts (Paterson & Lawrence 2001). It is used in the form of aspirin to treat fevers and to prevent pain, clots and cardiac disease. Salicylic acid is also known to mediate systemic acquired resistance (SAR) and induce host defenses during pathogen infection on plants (Métraux et al. 1990). Our analysis sheds light on its induction in grapevine (Fig. 6) by *Pythium* in the process to suppress *B. cinerea* and its possible involvement in the activation of disease resistance.

Phenolic compounds with antioxidant and anti-proliferation activities help in prevention and treatment of cancer (Grassmann et al. 2002). Hence, consumption of fruits with increased phenolics content could have potential health benefits. The grapes treated with biological agents should be investigated for phenolics composition and should be explored for medicinal purposes. Grapevine leaves that are thrown away instead should be studied for usefulness in nutritional, health benefits and disease cure. Consumption of biologically treated grapevine and improved quality wine might have beneficial human health effects. Content of antioxidant compounds in chemically and biologically treated wine could be measured for further validation. Further research on medicinal aspects will elaborate more applications in human health benefits.

Thus, we conclude that with the application of *Pythium* spp., it is possible to induce the synthesis of phenolic compounds in grapevine that are believed to have a role in disease resistance and control of the pathogen, *B. cinerea*. The synthesis of phenolic compounds is possibly correlated with the activation of pathogenicity resistance (PR) genes in grapevine that further inhibits infection by *B. cinerea*. Molecular study on pathogenesis at gene level will uncover the genes involved in the resistance mechanisms.

Moreover, investigating underlying molecular mechanisms in induced resistance will open new windows for disease control and crop biosecurity.

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