JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Induction of Resistance to Gray Mold with Benzothiadiazole Modifies Amino Acid Profile and Increases Proanthocyanidins in Grape: Primary versus Secondary Metabolism

MARCELLO IRITI,[†] MARA ROSSONI,[‡] MICHELE BORGO,[§] LUIGIA FERRARA,[||] AND FRANCO FAORO*,[⊥]

Istituto di Patologia Vegetale, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy, Dipartimento di Produzione Vegetale, Università degli Studi di Milano, C.R.A., Istituto Sperimentale per la Viticoltura, Conegliano Veneto, Treviso, Dipartimento di Scienze della Vita, II Università di Napoli, Caserta, and CNR, Istituto di Virologia Vegetale, Sezione di Milano

Field treatments of grapevine (cv. Merlot) with the plant activator benzothiadiazole (BTH, 0.3 mM) induced resistance against gray mold caused by *Botrytis cinerea.* Both incidence and severity of the disease were reduced. The resistance was associated with an increase of total polyphenols in berry skins, in particular, the proanthocyanidin fraction, that increased up to 36%. The amino acid profile of leaves was also modified by treatments, particularly lysine, that augmented 4-fold. Other amino acids involved in resistance mechanisms to either biotic or abiotic stress increased as well. These results indicate that BTH treatments can be used to control gray mold, thereby limiting an excessive use of fungicides, and could be exploited to increase the content of micronutrients of high nutritional value, arising from both primary and secondary metabolisms.

KEYWORDS: Polyphenols; proanthocyanidins; amino acids; BTH; HPLC; *Botrytis cinerea*; *Vitis vinifera*; nutraceuticals

INTRODUCTION

Chemical defenses, both constitutive and inducible, represent a major plant strategy against pathogens and phytophagi. Plants have evolved an immune system reminiscent of vertebrate innate immunity, in which chemical diversity, mainly arising from secondary metabolism, provides an array of compounds, phytoanticipins and phytoalexins, involved both in abiotic stress tolerance and in disease resistance (*1*).

Secondary metabolites in the plant kingdom derive from three main biosynthetic routes, namely, phenylpropanoid, isoprenoid, and alkaloid pathways. Because of the content of these metabolites in fruits and vegetables, the intake of plant foods, particularly in Mediterranean populations, has been correlated with a low risk of chronic diseases, such as coronary heart disease, ischemic stroke, and cancer. Recent evidences suggest that a plethora of biological properties resides in these pharmaconutrients, as well as in the corresponding functional foods, namely, antioxidant, antimutagenic, anti-inflammatory, antitumoral, antihypertensive, and cardioprotective activities (*2*−*4*).

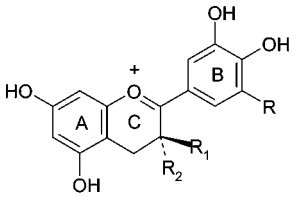
The phenylpropanoid pathway starts from the precursor phenylalanine, a main product of the aromatic amino acid biosynthesis. A first branch in this route directs the substrate, *p*-coumaroyl CoA, to flavonoid and stilbene syntheses via chalcone and stilbene synthase, respectively. Stilbenes include resveratrol, their glucosides, piceides, and polymeric forms, viniferins; flavonoids sensu lato consist of flavanones, flavones, flavonols, flavan-3-ols, and isoflavones, grouped sensu stricto as flavonoids and anthocyanins (*5*).

Proanthocyanidins (PAs), also known as condensed tannins, represent one of the most widely distributed groups of secondary metabolites produced by higher plants (*6*), being the second most abundant phenylpropanoids after lignin. They differ from hydrolyzable tannins, including polyesters of gallic acid, though deriving from the same shikimate pathway. PAs are both oligomeric and polymeric compounds arising from polyhydroxy flavan-3-ol and flavan-3,4-diol units and their epimers, condensed together by C-4→C-8 or C-4→C-6 bonds. Their polymerization degree (PD) ranges mainly between 3 and 11 and up to 17 and more (*7*). The most common monomers include catechins [(+)-catechin and (−)-epicatechin] and gallocatechins [(+)-gallocatechin and (−)-epigallocatechin] (**Figure 1**). Furthermore, a substantial number of polyhydroxyflavan-3-ol esters, known as *O*-gallates, are formed mainly by esterification of the 3-hydroxy group, such as epicatechin and epigallocatechin 3-*O*-gallates that commonly occur in *Vitis* genus (*8*).

The major groups of PAs are procyanidins (catechins and releasing cyanidins) and prodelphinidins (gallocatechins and releasing delphinidin) (*9*). PAs are involved in defense traits,

* Author to whom correspondence should be addressed. E-mail: f.faoro@ivv.cnr.it; phone: +390250316786; fax: +390250316781.
[†] Istituto di Patologia Vegetale, Università degli Studi di Milano.
[‡] Dipartimento di Produzione Vegetale, Università degli Studi di Milano.
[§] Istituto Sperimentale per la Viticoltura.
[||] II Università di Napoli.
[⊥] Istituto di Virologia Vegetale, Sezione di Milano.

10.1021/jf050853g CCC: $30.25
Published on Web 10/12/2005



R=H, R_1=OH, R_2=H	(+)-Catechin
R=OH, R_1=OH, R_2=H	(+)-Gallocatechin
R=H, R_1=H, R_2=OH	(-)-Epicatechin
R=OH, R_1=H, R_2=OH	(-)-Epigallocatechin
R=H, R_1=H, R_2=O-galloyl	(-)-Epicatechin-3-O-gallate

Figure 1. Monomeric proanthocyanidins.

such as protecting leaves from phytophagi, because of their protein-complexing effect, which increases with molecular weight and prodelphinidin/procyanidin ratio (*10*). PAs are stored in the cell vacuole and epidermis of leaves and trichomes. They bind to the collagen of animal hides and to the protein of the mucous membranes; they inactivate enzymes of the herbivore digestive tract and bind to plant proteins, making them less digestible (*11*). PAs inactivate lytic enzymes of some plant pathogens (*12*), such as laccases of *Botrytis cinerea* Pers.: Fr. (*13*, *14*), the etiologic agent of the gray mold, a disease affecting many different crops of agronomic interest.

PAs possess an array of biological and pharmacological effects that include antibacterial and antiviral activities, antimutagenic and antitumoral properties, antioxidative and anti-inflammatory effects, and cardiovascular risk-reducing agents (*15*).

Grapevine (*Vitis vinifera* L.) contains a large amount of polyphenols. Among them, PAs are mainly present in skin, seed, and stem tissues of the fruit and, when extracted during winemaking, impart major grape and wine organoleptic properties, such as astringency, bitterness, browning, color stability, and turbidity (*16*, *17*). In particular, PA content in grape berry differs depending on the tissue type. In seeds, PAs represent the major fraction of the total polyphenol extract and are characterized by a lower PD and a higher amount of galloylated derivatives than those present in grape skin (*18*). However, skin PAs are more easily extracted during winemaking, because of their localization in vacuole and cell wall, and are characterized by a high prodelphinidin content (*18*). All the above-mentioned compounds exert a role as defense metabolites against biotic and abiotic stresses (*12*, *19*).

Defense metabolite synthesis, particularly phytoalexins, is also elicited by the application of chemical compounds, namely, plant activators (reviewed in *20*), such as benzothiadiazole (BTH, Bion), recently introduced in agriculture (*21*). BTH is a functional analogue of the plant endogenous hormonelike compound salicylic acid (SA) that, in untreated plants, is required for the induction of defense genes leading to a broad spectrum, long-lasting systemic immunity (SAR, systemic acquired resistance). These genes encode for pathogenesis related (PR) proteins and key enzymes of secondary metabolic pathways, such as phenylalanine ammonia lyase (PAL), thus improving phytoalexin synthesis and, in turn, plant resistance (*22*−*25*). BTH and the related free acid hydrolytic product are completely degraded in plant tissues, not leaving any residues as such (*26*−*27*). An exhaustive history on BTH and its possible applications has been recently published in this journal (*20*).

We have, previously, shown that BTH preharvest treatments prevent postharvest *Botrytis cinerea* infection, correlating the observed protection to enhanced resveratrol and anthocyanin synthesis (*28*). In this work, we show that BTH treatments significantly reduced the incidence of gray mold in natural field infection, modifying the amino acid profile in the treated leaves, and enhancing PA content in berries.

MATERIALS AND METHODS

Plant Materials and Open Field Treatments. Plant (*Vitis vinifera*, cv. Merlot) treatments were carried out in an experimental vineyard at Conegliano Veneto (Treviso, Italy) cultivated following the Sylvoz system, 2 × 3 m one vine for each stake, in an area predisposed to gray mold epidemic. All treatments were applied to three replicates and were arranged in a complete randomized block design, with 10 vines for each replication. To avoid spray drift on neighboring parcels, sprayings were performed with a spray lance, powered by a walking-type motor pump, distributing a volume equivalent to 800−1000 L/ha.

To evaluate the resistance induced by benzothiadiazole against gray mold (*Botrytis cinerea*), plants were sprayed on the 6th, 11th, and 16th of August 2004 and 2nd of September 2004, that is to say, at the beginning and the end of vèraison, with a water suspension of BTH [benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester] (trade name Bion, Syngenta, CH) at the concentration of 0.3 mM, prepared from a wettable formulation containing 50% (w/w) active ingredient (a.i.). This concentration has been selected on the basis of the results obtained in a previous study on bean and grapevine plants (*28*). Control plants were sprayed with a water suspension of wettable powder alone. Both BTH treated and control plants had been protected against downy mildew (*Plasmopara viticola*) and powdery mildew (*Oidium tuckeri*) infections, with programmed treatments from the middle of May to the beginning of July with copper hydroxides and colloidal sulfur.

Observations on gray mold infection were carried out 3 weeks after the last treatment, just before harvesting, assessing 150 bunches per replication and classifying them into seven intensity ranges. The collected data were processed according to the Townsend−Heuberger formula, to calculate the percentage of infection (*I* %).

$$I\,\% = [\Sigma\,(n \cdot v)/z \cdot N] \cdot 100$$

where *I*% = percentage of infection, n = number of leaves or bunches in each class, v = class value, z = highest class value, and N = total amount of assessed leaves or bunches.

For chemical analysis of polyphenolic compounds, five gray mold-free mature clusters per plant were randomly collected 3 weeks after the last spraying from treated and untreated grapevine. Clusters were immediately frozen at −20 °C.

Standards. (+)-Catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, and sephadex 20LH were purchased from Sigma Chemical Co (St. Louis, MO), and gallic acid, methanol, and formic acid were purchased from Fluka (VWR international, Germany). Identification and quantification of monomeric proanthocyanidins were obtained by calibration curve of standards.

Preparative Extraction of Polyphenolic Compounds. Fifty frozen berries selected at random were peeled without thawing and the obtained skins were weighed. Five grams of skin samples was used for each polyphenol extraction with methanol, following the previously reported protocol (*28*).

Proanthocyanidin Isolation. To isolate the crude tannin fraction, the methanol extract (5 mL) was chromatographed on a 20-mL column, packed with 5 g of Lipophilic Sephadex LH-20 (Sigma Aldrich), hydrated for 4 h, and equilibrated with methanol at a 3.5 mL/min flow rate. First elution was performed with methanol/water 20% to remove phenolic acids followed by methanol/water 60% to elute flavonols and anthocyanins. The following two fractions, with methanol 100% and acetone/water 70%, were collected for spectrophotometric and HPLC analysis of monomeric and polymeric proanthocyanidins (*29*−*30*).

Proanthocyanidin HPLC Analysis. Sephadex fractions were monitored with a Shimadzu LC-10ADvp, SIL-10ADvp high-performance liquid chromatograph equipment, with an SPD-10Avp, RF-10Axl detectors.

The HPLC pumps, autosampler, and detectors were controlled via Class vp 3.4 software. A Lichrospher LiChroCART (Merk, VWR international, Germany) Li 100 RP-18 column (4.6 mm × 250 mm,

i.d.; 5 μm), protected with a guard column LiChroCART (4 mm × 4 mm) of the same material, was employed for all analyses at room temperature (24 °C) and 1 mL/min flow rate.

Elution was performed with two solvents, formic acid/water 4% (solvent A) and methanol 100% (solvent B). A linear binary gradient method was applied as follows: 0−5 min, 95% A; 5−45 min, 70% A; 45−60 min, 40% A; 60−75 min, 20% A; 80−90 min washing with 100% B; and, finally, return to the initial elution conditions for 10 min. Twenty microliters of solution for each sample was injected.

Fluorimetric detection was recorded at $\lambda_{ex} = 276$ nm and $\lambda_{em} = 316$ nm; wavelengths of 280 and 520 nm (for anthocyanins) were used for UV detector (*31*).

HPLC Analysis of Stilbenes. HPLC equipment was the same as above. Chromatographic separation was performed with a Purospher LiChroCART RP-18 HPLC column (4.6 mm × 250 mm, particle size = 5 μm), provided with precolumn LiChroCART (4 mm × 4 mm) (Merk, VWR international). Chromatographic analyses were made with a mobile phase linear gradient of absolute methanol, solvent A, and 0.3% perchloric acid in water, solvent B, at a flow rate of 0.45 mL/min (*35*). Gradient elution profile was as follows: 0 min, 27% B, 73% A; 1−32 min, 43% B, 57% A; 32−45min, 68.5% B, 31.5% A; 45−47min, 100% B; 3 min constant 100% B. Samples were centrifuged at 10 000*g* for 30 min and then were filtered using a PTFE 0.45-μm membrane syringae filter (Corning, Inc.). Injection volume of each sample was 10 μL. Fluorimetric detections were recorded for 60 min at $\lambda_{ex} = 330$ nm and $\lambda_{em} = 374$ nm for stilbene quantification. Wavelength of 520 nm was used for absorbance detector in a separated channel.

Polyphenol Spectrophotometric Detection. To evaluate the differences of polyphenolic content (total polyphenols, TP) in berries from untreated and BTH-treated plants, the Folin−Ciocalteau (FC) assay was performed. One milliliter of FC reagent and 4 mL of 10% Na_2CO_3 were added to 1 mL of diluted extract (with acidified ethanol) which was immediately filled up to 20 mL with distilled water. The optical density, after 1 h and 30 min, was evaluated at 700 nm on a Jasco model 7800 UV/VIS spectrophotometer. This colorimetric oxidation/reduction assay determines all polyphenolic compounds without discriminating among them. A gallic acid standard curve was used as reference, and results were expressed as gallic acid equivalents (GAE).

Total flavonoids (TF) and nonanthocyanin flavonoids (NAF) were spectrophotometrically estimated on $^1/_{50}$ diluted extracts with EtOH−HCl−H_2O (70:1:29). A catechin standard curve was prepared, expressing the results as catechin equivalents (CE). Total anthocyanins (TA) were measured at 520 nm, on $^1/_{20}$ diluted extracts with EtOH−HCl−H_2O (70:1:29). A malvidin standard curve was employed, and results were reported as malvidin equivalents (ME) (*32*).

Total proanthocyanidin content was determined with the vanillin assay that is based on the ability of this compound to react in C6 and C8 positions of flavan-3-ols. The absorbance obtained from the vanillin reaction in methanol was, then, converted to catechin equivalents (CE) on the basis of a previous catechin calibration curve (*33*).

Free Amino Acid Analysis. Five apparently healthy and undamaged leaves were collected from each grapevine plant, at the end of August, before the last BTH treatment. Similar leaves were collected from untreated plants as control. They were immediately frozen in liquid nitrogen, powdered in a precooled pestle and mortar, and stored at −80 °C until analysis. All powered leaves from the same treatment were mixed together and samples of 100 mg were used for amino acid extraction. Extraction was performed with 80% ethanol, followed by 6% sulfosalicylic acid. Free amino acids, in the surnatant of the second extraction, were directly analyzed on a Biochrom 20 (Biochrom, U.K.) amino acid analyzer, equipped with a polyvinyl sulfonate cationic-exchange column for physiological fluids, and a postcolumn ninhydrin detection system set at 570 nm (except for proline and hydroxyproline detected at 440 nm), adapting the Moore and Stein procedure (*34*).

Presentation of Results. Values from field trials were analyzed by using MSTAT-C software (Michigan State University). Extractions of metabolites, spectrophotometric, HPLC, and amino acid analysis were repeated at least three times for each replicate, and median values ± SD are given, if not otherwise stated.

Table 1. Gray Mold (*Botrytis cinerea*) Incidence (*I*) and Severity (*S*) in Untreated and Benzothiadiazole (BTH) Treated Plants

treatments	disease incidence (*I*): symptomatic bunches (%)	disease severity (*S*): symptomatic berries (%)
control	33.42 ± 2.75	84.92 ± 8.53
BTH	19.11 ± 2.67	54.34 ± 3.28

RESULTS

Open Field Trials. In midsummer 2004, microclimatic conditions, that is, temperature and humidity, were predisposing for *B. cinerea* epidemic in vineyard (data not shown). The plant activator BTH proved to be effective in inducing resistance to gray mold on grape, decreasing both disease incidence (*I*) and severity (*S*) (**Table 1**). The percentage of symptomatic bunches, namely, the number of bunches with gray mold (*I*), calculated as described in Materials and Methods, revealed a substantial susceptibility of the untreated control plants to the disease (33.42%). BTH treatments significantly reduced disease incidence by 42.81% and disease severity (*S*) by 36.01%. The latter index, being the average percentage of symptomatic berries per bunch, is correlated with pathogen virulence, and its reduction gives an assessment of plant resistance.

No significant differences were observed among the three replicate parcels as assessed by analysis of variance.

Polyphenol Detection. The effect of BTH treatments on the grapevine polyphenol pool, including total polyphenols, stilbenes, flavonoids, anthocyanins, and total proanthocyanidins, as assessed by spectrophotometetric and HPLC analysis, is reported on **Table 2**.

Treatments increased total polyphenols (TP) in berries, as determined by the Folin−Ciocalteau assay, by about 10% compared to the untreated control. Further analyses, carried out with the aim of discriminating the main groups of polyphenolic compounds, showed that BTH enhanced total flavonoids (TF), nonanthocyanin flavonoids (NAF), and total anthocyanins by 2.52, 13.30, and 5.80%, respectively. However, the most outstanding increments, following BTH treatments, involved total proanthocyanidins that increased by 35.89% and stilbenes, particularly *trans*-resveratrol, that doubled in comparison with the untreated control (**Table 2**).

Proanthocyanidin HPLC Analysis. To characterize the proanthocyanidin pool, HPLC analyses were carried out on sephadex-eluted methanol 100% fraction for monomeric and oligomeric PAs (**Figure 2A**) and on acetone/water 70% fraction for polymeric PAs detection (**Figure 2B**).

The increase of both monomeric and oligomeric PAs, following BTH treatments, is shown in the upper profile of the chromatograms in **Figure 2A**. However, monomeric gallocatechin and catechin peaks were higher in PAs fraction from untreated control plants (lower profile in **Figure 2A**).

Polymeric PAs chromatograms have been overlaid in **Figure 2B**. The upper profile, relative to the fraction of berry skin extract from BTH treated grapevines, showed much higher peaks with respect to corresponding fraction from control plants (lower profile). The comparison of chromatograms in **Figure 2A** and **2B** suggests that polymeric PA increase is even greater than that of monomeric and oligomeric PAs.

Free Amino Acid Analysis. BTH treatments modified significantly the pattern of amino acids in grape leaf tissues (**Table 3**). Among them, those of the aspartate pool appeared markedly increased after BTH treatment. In fact, aspartic acid, and its derivative, isoleucine, increased almost 3-fold, while another derivative, lysine, increased 4-fold. The amount of

Table 2. Polyphenol Pool in Berry Skin from Untreated and Benzothiadiazole (BTH) Treated Grapevine

compound group	method	control (mg/L)	BTH (mg/L)	% increment
total polyphenols (TP)	GAE, gallic acid equivalents	1799.4 ± 10.2	1985.5 ± 22.6	10.34
stilbenes: *cis*-resveratrol	HPLC	0.19 ± 0.0	0.29 ± 0.0	52.63
trans-resveratrol		0.68 ± 0.0	1.43 ± 0.1	110.29
total flavonoids (TF)	CE, catechin equivalents	1372.2 ± 5.5	1406.9 ± 8.9	2.52
non-anthocyanic flavonoids (NAF)	CE, catechin equivalents	251.3 ± 4.1	284.3 ± 7.2	13.30
total anthocyanins (TA)	ME, malvidin equivalents	975.9 ± 6.2	1032.5 ± 12.4	5.80
total proanthocyanidins (TPA) (vanillin assay)	CE, catechin equivalents	226.8 ± 3.0	308.2 ± 3.1	35.89

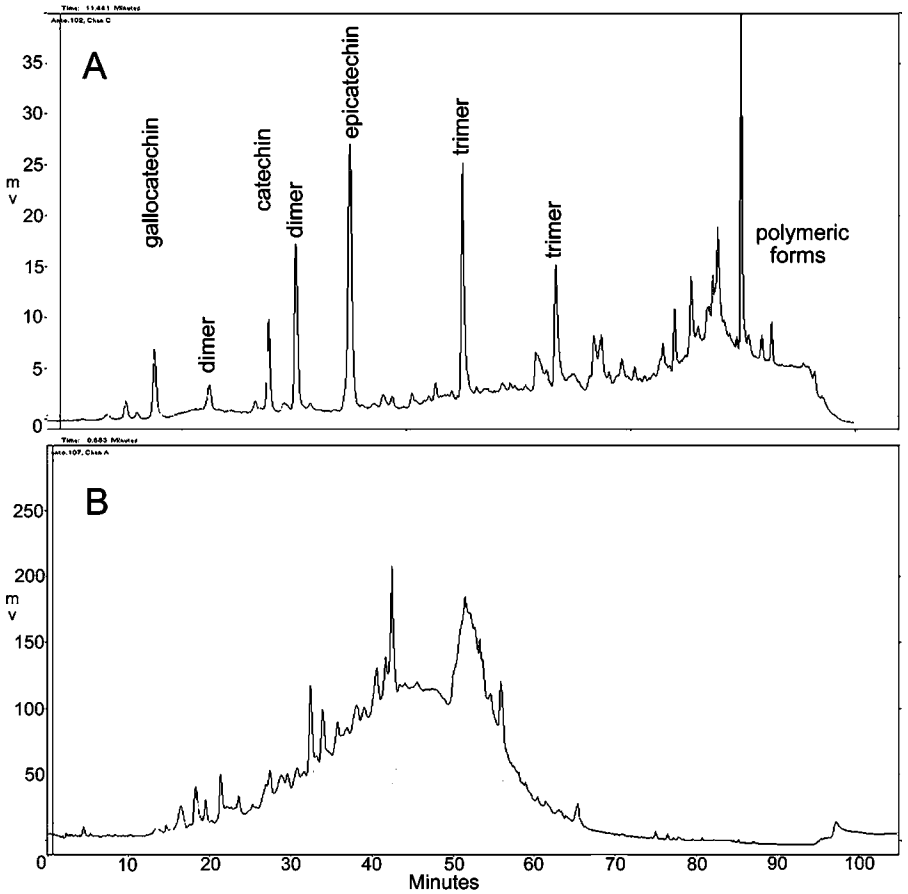


Figure 2. RP-HPLC chromatograms of proanthocyanidins (PAs) present in grape skin extracts fractioned on sephadex LH-20 and detected at 280 nm. (A) Chromatogram overlaid of the monomeric–oligomeric fraction of extract from benzothiadiazole (BTH) treated (upper profile) and untreated control (lower profile) plants. (B) Chromatogram overlaid of polymeric PAs fraction, from BTH (upper profile) and control (lower profile) extracts.

ornithine and proline doubled, whereas their precursor glutamic acid slightly decreased. On the other hand, the increase of glutamine, after treatments, was not significant.

Among unrelated amino acids, BTH treatments increased glycine and histidine almost 2-fold as well as hydroxylysine. Aditionally, aromatic amino acid phenyalanine increased slightly.

Branched amino acid synthesis was increased as well, particularly valine, the second most increased amino acid by BTH treatments, after lysine. Among phosphoamino acid, phosphoethanolamine markedly increased (more then 2-fold), whereas phosphoserine diminished. Sarcosine (*N*-methylglycine) was the most decreased amino acid, while threonine, serine, glutamic acid, tyrosine, γ-aminobutyric acid, and ethanolamine diminished slightly, after treatments.

DISCUSSION

In grape, gray mold is a destructive disease that causes significant yield losses, depending on climatic conditions and varietal sensitivity. The infection is favored by rainy weather, before bloom and from flowering time onward. Immature bunches are naturally resistant, in contrast with the higher susceptibility of ripening berries after véraison (*35*). Agrochemicals traditionally employed in disease control belong to three major groups of fungicides: anilopyrimidimes, carbamates, and the cyclic imides (*36*).

In this study, we showed that field treatments with the plant activator BTH reduced significantly both incidence (*I*) and severity (*S*) of the disease, despite the predisposing climate conditions and the high pathogen diffusion in the field. This resistance was induced by the activation of defense mechanisms, leading to an increased polyphenol synthesis, particularly stilbenes and PAs. Regarding the latter, we demonstrated here for the first time that their synthesis can be modulated by an exogenous elicitor.

In the *Vitis*−*Botrytis* pathosystem, PAs are involved in a fascinating example of host−pathogen coevolution. Vitaceae phytoalexins, particularly stilbenes, are implicated in basic resistance against *B. cinerea* (*37*). Stilbenes consist of a rather

Table 3. Free Amino Acid Profile of Untreated and Benzothiadiazole (BTH) Treated Grapevine Plants (Merlot cv)[a]

amino acid	control	BTH	
Phser (phosphatidylserine)	3.00 ± 0.79	1.96 ± 0.19	*
Pea (*o*-phosphoethanolamine)	0.40 ± 0.09	1.04 ± 0.28	*
Asp (aspartate)	10.41 ± 0.16	28.69 ± 0.69	**
Thr (threonin)	2.21 ± 0.43	2.07 ± 0.14	n.s.
Ser (serine)	2.23 ± 0.07	1.92 ± 0.07	*
Glu (glutamic acid)	33.65 ± 0,17	29.34 ± 0.95	**
Gln (glutamine)	5.41 ± 0.43	5.70 ± 1.24	n.s.
Sarc (sarcolysine)	3.33 ± 0.66	1.93 ± 0.41	*
Gly (glycine)	2.90 ± 0.09	4.30 ± 0.05	**
Val (valine)	0.40 ± 0.24	1.30 ± 0.08	**
Ile (isoleucine)	0.12 ± 0.02	0.30 ± 0.03	**
Leu (leucine)	0.20 ± 0.07	0.38 ± 0.03	*
Tyr (tyrosine)	1.97 ± 0.17	1.76 ± 0.32	n.s.
Phe (phenylalanine)	1.78 ± 0.10	2.16 ± 0.24	*
GABA (γ-aminobutyric acid)	1.95 ± 0.03	1.75 ± 0.06	*
Ethan (ethanolamine)	2.98 ± 0.68	2.54 ± 0.72	n.s.
Hylys (γ-hidroxylysine)	0.90 ± 0.22	2.22 ± 0.72	*
Orn (ornithine)	0.32 ± 0.04	0.59 ± 0.11	*
Lys (lysine)	0.42 ± 0.08	1.61 ± 0.65	*
His (histidine)	0.28 ± 0.01	0.48 ± 0.03	**
Pro (proline)	0.33 ± 0.23	0.66 ± 0.03	**

[a] Data (mean ± SD) are expressed as mg per 100 g of leaf tissue (* = $p \leq 0.05$; ** = $p \leq 0.01$, n.s.= not significant, Student's t).

restricted group of molecules, whose skeleton is based on *trans*-resveratrol (3,5,4′-trihydroxystilbene) structure. Simple stilbenes include resveratrol glucosides, namely, piceids, astringins, and resveratrolosides, in addition to pterostilbenes and dimethylated resveratrol derivatives, whereas viniferins are oligomers more fungitoxic than resveratol (*19*). Thus, the precursor *trans*-resveratrol represents the Gordian node in this plant−fungus interaction, providing a reservoir for an array of defense compounds. In this view, the fitness of the pathogenic stage of *B. cinerea* resides in its capacity to degrade phytoalexins by synthesizing laccases (*13*). The plant, conversely, reacts by enhancing its pool of defense compounds that inactivate the fungal laccases via PAs (*38*).

Laccases are copper-containing glycoproteins having different functions and substrate specifity (*39*). Many fungal laccases catalyze the oxidation of phenolic compounds, and those excreted by *B. cinerea* oxidize stilbenes (*13*, *14*). Thus, resveratrol oxidation, by laccases, plays a pivotal role in *B. cinerea* armamentarium (*14*).

On the other side of the barricade, grape berry PAs competitively inhibit stilbene oxidase activity of laccases, thus attenuating *B. cinerea* attack against stilbenic phytoalexins (*38*). PAs also inhibit the activity of hydrolytic enzymes secreted by the fungus, constraining its development and preventing tissue maceration (*40*, *41*). Even so, phenol oxidase activity of different laccases may be directed against PAs themselves, thus circumventing the plant defense strategy.

In this view, the improved resistance of grape to gray mold because of BTH would result from different, but overlapping, processes in which PA synthesis, greatly enhanced by treatments, may represent the focal step by inhibiting stilbene oxidases and macerating exoenzymes. Nevertheless, BTH effects on phenylpropanoid pathway seem to be rather complex; it upregulates the key enzymes of this biosynthetic route, such as PAL, in bean (*25*) and other crops (reviewed in *42*) besides possibly enhancing catechin polymerization. We previously detected in BTH treated bean leaves a high level of H_2O_2 and enhanced peroxidase activity, both involved in monolignol polymerization and lignin deposition (*25*, *42*). Such conditions are favorable to "head-to-tail" catechin condensation (*43*, *44*),

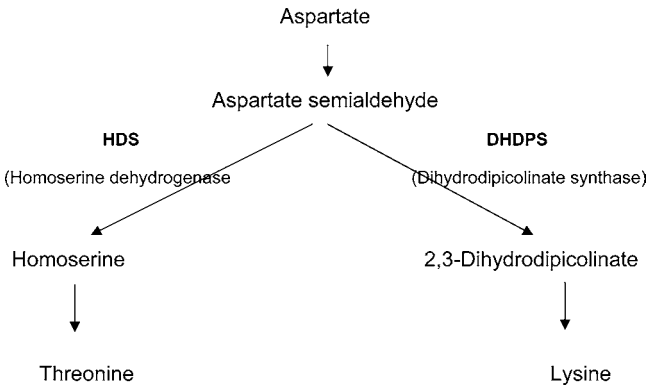


Figure 3. Lysine/threonine pathway from aspartate; intermediate aspartate semialdehyde is a common substrate for dihydrodipicolinate synthase (DHDPS) and homoserine dehydrogenase (HDS); shift toward lysine could be modulated by BTH (see Discussion).

and this would explain the much lower level of catechin and gallocatechin and the enhancement of oligomeric and polymeric PAs in berries from BTH treated plants compared to control berries. Interesting enough, polymeric PAs are reported to be more effective in inhibiting the activity of a stilbene oxidase of *B. cinerea* than the other PA fractions (*45*).

The influence of BTH on primary metabolism has been evaluated on the basis of amino acid composition of treated leaves, with other physiological parameters not significantly altered by BTH, at least in bean plants (*46*).

The aspartate (Asp) synthesis is one of the most enhanced by treatments, as well as the essential amino acids (EAAs) lysine and isoleucine, that arise from aspartate pathway (*47*). The involvement of the aspartate pool, in plant defense, is rather obscure, and, to our knowledge, no study has been carried out about the role of Asp on resistance mechanisms, though the aspartate increase has already been reported in a compatible pathosystem (*48*). Even though lysine and threonine both originate from aspartate (**Figure 3**), only the former increased significantly after treatment. This suggests that BTH directs, in some way, the aspartate-branched pathway toward lysin formation (**Figure 3**), as observed in other physiological conditions (*49*). BTH treatments could be then experimented in the attempt of ameliorating the nutritional values of those plants deficient in lysin, such as cereals (*50*).

Another interesting effect of BTH on primary metabolism was the significant increase of proline and ornithine, both arising from glutamate. In plant, proline is a compatible solute, protecting against water stress caused by drought and salinity (*51*). In addition, proline has been studied in heavy metal stress and as cryoprotectant (*52*−*53*). The latter characteristic could explain the improved chilling resistance, following BTH treatments, observed in vineyards of northeastern Italy in winter 2003 (Borgo, personal communications). Glycine, too, is an osmolyte, especially after methylation of its amino group to form betaines (*51*). It increased significantly following treatments, while sarcosine, a betaine (*N*-methylglycine), decreased. Glycine betaine (*N*,*N*,*N*-trimethylglycine) arises directly from choline whose synthesis starts from ethanolamine via ethanolamine kinase, leading to phosphoethanolamine. Ethanolamine, in turn, arises from serine by serine decarboxylase (*54*). In this view, it is possible that in BTH treated tissues the glycin betain is directly synthesized from sarcosine via glycine methylation pathway (**Figure 4**) as it has been reported for extreme halophilic microorganisms (*55*). Interestingly, sarcosine has never been reported in grape up to now.

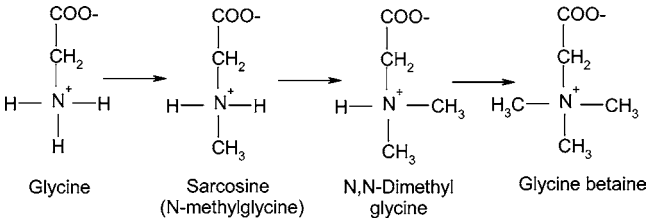


Figure 4. Glycine methylation pathway for glycine betaine synthesis.

Branched amino acids (valine, leucine, and isoleucine) may be a source of an array of phytoanticipins belonging to three main groups and may be involved in plant defense (*56*, *57*); thus, their increase, after BTH treatment, it is not surprising. Among phytoanticipins, cyanogenic glucosides have been recently detected in grape leaves of many cultivars, except Merlot, and cyanogenesis, that is, the release of hydrogen cyanide upon tissue disruption, has been described as a putative mechanism involved in the interaction with pests and pathogens (*58*).

Aditionally, phytoanticipins include several cancer-chemopreventive pharmaconutrients.

One more amino acid involved in defense mechanisms that increased, even if slightly, after BTH treatment was phenylalanine, the precursor of phenylpropanoids. These secondary metabolites include phytoalexins (stilbens, flavonoids, PAs) and monolignols and lignin, antioxidant, antitumoral, cardioprotective, and phytoestrogenic compounds (*5*). During pathogen attack, a major plant defense trait consists of cell wall straightening by lignification and extensin cross-linking, particularly against *B. cinerea ménage*. Interestingly, hydroxylysine, that is involved in cross-linking of extensins, increased as well after treatment, thus contributing with lignin to the cell wall fortification and, in turn, to the observed resistance to gray mold.

In conclusion, plants, for their unique content in micronutrients, such as phenylpropanoids, as well some EAAs, represent a major food and feed resource and many efforts have been carried out to improve their nutritional value by modulating their primary and secondary pathways. Molecular technologies and genetic engineering have been used to this purpose, often with contrasting results. Hence, the use of BTH, a functional analogue of plant endogenous salicylic acid, could represent an innovative approach to protect plants from biotic (*42*) and abiotic stresses (*59*) and to enhance their nutritional value (*28*), eliciting a multistep biochemical response and priming a physiological alert condition. Last, but not the least, because of the very low toxicological risk (*60*) associated with BTH, and considering that it is rapidly degraded in plant tissues and lacks any antibiotic activity (*26*, *27*), its use would greatly reduce the environmental impact of crop treatments.

LITERATURE CITED

(1) VanEtten, H. D.; Mansfield, J. W.; Bailey, J. A.; Farmer E. E. Two classes of plant antibiotics: phytoalexins versus "phytoanticipins". *Plant Cell* **1994**, *6*, 1191−1192.

(2) Oleszek, W. Dietary phytochemicals and human health. *Phytochem. Rev.* **2002**, *1*, 163−166.

(3) Andlauer, W.; Fürst, P. Nutraceuticals: a piece of history, present status and outlook. *Food Res. Int.* **2002**, *35*, 171−176.

(4) Simonetti, P.; Ciappellano, S.; Gardana, C.; Bramati, L.; Pietta, P. Procyanidins from *Vitis vinifera* seeds: in vivo effects on oxidative stress. *J. Agric. Food Chem.* **2002**, *50*, 6217−6221.

(5) Iriti, M.; Faoro, F. Plant defense & human nutrition: the phenylpropanoids on the menu. *Curr. Top. Nutraceut. Res*. **2004**, *2*, 47−65.

(6) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds - nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric*. **2000**, *80*, 226−230.

(7) Guyot, S.; Doco, T.; Souquet, J. M.; Moutounet, M.; Drilleau, J. F. Characterization of highly polymerised procyanidins in cider apple (*Malus sylvestris* var kermerrien) skin and pulp. *Phytochemistry* **1997**, *44*, 351−357.

(8) Porter, L. J.; Hrstich, L. N.; Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223−230.

(9) Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. J. N. Plant proanthocyanidins - Part 1- Introduction: the isolation, structure and distribution in nature of plant procyanidins. *J. Chem. Soc., Perkin Trans. 1* **1972**, 1387−1399.

(10) Porter, L. J.; Woodruffe, J. Haemanalysis: the relative astringency of proanthocyanidin polymers. *Phytochemistry* **1984**, *23*, 1255−1256.

(11) Ayres, M. P.; Clausen, T. P.; MacLean, S.; Redman, A. M.; Reichardt, P. B. Diversity of structure and antiherbivore activity in condensed tannins. *Ecology* **1997**, *78*, 1696−1712.

(12) Swain, T. Secondary compounds as protective agents. *Annu. Rev. Plant Physiol.* **1977**, *28*, 479−501.

(13) Pezet, R.; Pont, V.; Hoang-Van, K. Evidence for oxidative detoxification of pterostilbene by a laccase-like stilbene oxidase produced by *Botrytis cinerea*. *Physiol. Mol. Plant Pathol*. **1991**, *39*, 441−450.

(14) Pezet, R. Purification and characterization of a 32-kDa laccase-like stilbene oxidase produced by *Botrytis cinerea* Pers.: Fr. *FEMS Microbiol. Lett*. **1998**, *167*, 203−208.

(15) De Bruyne, T.; Pieters, L.; Deelstra, H.; Vlietinck, A. Condensed vegetable tannins: biodiversity in structure and biological activities. *Biochem. Syst. Ecol*. **1999**, *27*, 445−459.

(16) Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seed. *Phytochemistry* **1994**, *36*, 781−784.

(17) Souquet, J. M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509−512.

(18) Gabetta, B.; Fuzzati, N.; Griffini, A.; Lolla, E.; Pace, R.; Ruffilli, T.; Peterlongo, F. Characterization of proanthocyanidins from grape seeds. *Fitoterapia* **2000**, *71*, 162−175.

(19) Jeandet, P.; Douillet-Breuil, A.-C.; Debord, S.; Sbaghi, M.; Adrian, M. Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity and metabolism. *J. Agric. Food Chem*. **2002**, *50*, 2731−2741.

(20) Gozzo, F. Systemic acquired resistance in crop protection: from nature to a chemical approach. *J. Agric. Food Chem*. **2003**, *51*, 4487−4503.

(21) Kunz, W.; Schurter, R.; Maetzke, T. The chemistry of benzothiadiazole plant activators. *Pestic. Sci*. **1997**, *50*, 275−282.

(22) Friedrich, L.; Lawton, K.; Ruess, W.; Mesner, P.; Speker, N.; Gut Rella, M.; Meier, B.; Dincher, S.; Staub, T.; Uknes, S.; Métraux, J. P.; Kessmann, H.; Ryals, J. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant J*. **1996**, *10*, 61−70.

(23) Görlach, J.; Volrath, S.; Knauf-Beiter, G.; Hengy, G.; Beckhove, U.; Kogel, K. H.; Oostendorp, M.; Staub, T.; Ward, E.; Kessmann, H.; Ryals, J. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* **1996**, *8*, 629−643.

(24) Lawton, K.; Friedrich, L.; Hunt, M.; Weymann, K.; Delaney, T.; Kesmann, H.; Staub, T.; Ryals, J. Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant J*. **1996**, *10*, 71−82.

(25) Iriti, M.; Maffi, D.; de Ascensao, A. R.; Rabotti, G.; Farina, G.; Mignani, I.; Faoro, F. Mechanisms of BTH-induced resistance against bean rust. *Proc. XIth Int. Congress on Molecular Plant-Microbe Interactions*, St.-Petersburg, Russia, July 18−26, 2003.

(26) Scarponi, L.; Buonaurio, R.; Martinetti, L. Persistence and translocation of a benzothiadiazole derivative in tomato plants in relation to systemic acquired resistance against *Pseudomonas syringe* pv *tomato*. *Pest Manage. Sci*. **2001**, *57*, 262−268.
(27) Buonaurio, R.; Scarponi, L.; Ferrara, M.; Sidoti, P.; Bertona, A. Induction of systemic acquired resistance in pepper plants by acibenzolar-S-methyl against bacterial spot disease. *Eur. J. Plant Pathol*. **2002**, *108*, 41−49.
(28) Iriti, M.; Rossoni, M.; Borgo, M.; Faoro, F. Benzothiadiazole enhances resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to *Botrytis* cinerea. *J. Agric. Food Chem.* **2004**, *52*, 4406−4413.
(29) Perret, C.; Pezet, R.; Tabacchi, R. Fraction of grape tannins and analysis by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Phytochem. Anal*. **2003**, *14*, 202−208.
(30) Sànchez-Moreno, C.; Cao, G.; Ou, B.; Prior, R. L. Anthocyanin and proanthocyanidin content in selected white and red wines. Oxygen radical absorbance capacity comparison with non traditional wines obtained from highbush blueberry. *J. Agric. Food Chem.* **2003**, *51*, 4889−4896.
(31) Merghem, R.; Jay, M.; Brun, N.; Voirin, B. Qualitative analysis and HPLC isolation and identification of procyanidins from *Vicia foba*. *Phytochem. Anal*. **2004**, *15*, 95−99.
(32) Di Stefano, R.; Cravero, M. C. I composti fenolici e la natura del colore dei vini rossi. *L'Enotecnico* **1989**, *Ottobre*, 81−87.
(33) Di Stefano, R.; Cravero, M. C.; Gentilizi, N. Metodo per lo studio dei polifenoli dei vini. *L'Enotecnico* **1989**, *Maggio*, 83−89.
(34) Stein, W. H.; Moore, S. *Methods Enzymol*. **1963**, *6*, 819−831.
(35) *EPPO Bull.* **2002**, *32*, 371−392.
(36) Couderchet, M. Benefits and problems of fungicide control of *Botrytis cinerea* in vineyards of Champagne. *Vitis* **2003**, *42*, 165−171.
(37) Langcake, P. Disease resistance of *Vitis* spp. and the production of stress metabolites resveratrol, ϵ-viniferin, α-viniferin and pterostilbene. *Physiol. Plant Path*. **1981**, *18*, 213−226.
(38) Goetz, G.; Fkyerat, A.; Métais, N.; Kunz, M.; Tabacchi, R.; Pezet, R.; Pont, V. Resistance factors to grey mould in grape berries: identification of some phenolics inhibitors of *Botrytis cinerea* stilbene oxidase. *Phytochemistry* **1999**, *52*, 759−767.
(39) Thurston, C. F. The structure and function of fungal laccases. *Microbiology* **1994**, *140*, 19−26.
(40) Hill, G.; Stellwaag-Kittler, F.; Huth, G.; Schlösser, E. Resistance of grapes in different developmental stages to *Botrytis cinerea*. *Phytopathol. Z.* **1981**, *102*, 328−338.
(41) Jersch, S.; Scherer, C.; Huth, G.; Schlösser, E. Proanthocyanidins as basis for quiescence of *Botrytis cinerea* in immature strawberry fruits. *J. Plant Dis. Prot*. **1989**, *96*, 365−378.
(42) Iriti, M.; Faoro, F. Benzothiadiazole (BTH) induces cell-death independent resistance in *Phaseolus vulgaris* against *Uromyces appendiculatus*. *J. Phytopathol*. **2003**, *151*, 171−180.
(43) Lòpez-Serrano, M.; Ros Barcelò, A. Reversed-phase and size-exclusion chromatography as useful tools in the resolution of peroxidase-mediated (+)-catechin oxidation products. *J. Chromatogr., A* **2001**, *919*, 267−273.
(44) Hosny, M.; Rosazza, J. P. N. Novel oxidation of (+) catechin by horseradish peroxidase and laccase. *J. Agric. Food Chem*. **2002**, *50*, 5539−5545.
(45) Pezet, R.; Viret, O.; Perret, C.; Tabacchi, R. Latency of *Botrytis cinerea* Pers.: Fr. and biochemical studies during growth and ripening of two grape berry cultivars, respectively susceptible and resistant to grey mould. *J. Phytopathol.* **2003**, *151*, 208−214.
(46) Iriti, M.; Faoro, F. Does benzothiadiazole-induced resistance increase fitness cost in bean? *J. Plant Pathol.* **2003**, *85*, 265−270.
(47) Azevedo, R. A.; Arruda, P.; Turner, W. L.; Lea, P. J. The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. *Phytochemistry* **1997**, *46*, 395−419.
(48) Shaw, M.; Colotelo, N. The physiology of host parasite relations: the effect of stem rust on the nitrogen and amino acids in wheat leaves. *Can. J. Bot*. **1961**, *39*, 1351−1358.
(49) Frankard, V.; Ghislain, M.; Jacobs, M. Two feedback-insensitive enzymes of the aspartate pathway in *Nicotiana sylvestris*. *Plant Physiol*. **1992**, *99*, 1285−1293.
(50) Galili, G.; Tang, G.; Zhu, X.; Gakiere, B. Lysine catabolism: a stress and development super-regulated metabolic pathway. *Curr. Opin. Plant Biol*. **2001**, *4*, 261−266.
(51) Rai, V. K. Role of amino acids in plant responses to stresses. *Biol. Plant.* **2002**, *45*, 481−487.
(52) Whiters, L. A.; King, P. J. Proline: a novel cryoprotectant for the freeze preservation of cultured cells in *Zea mays*. *Plant Physiol*. **1979**, *64*, 675−678.
(53) Alia, M.; Saradhi, P. Proline accumulation under heavy metal stress. *J. Plant Physiol.* **1991**, *138*, 504−508.
(54) Rontein, D.; Nishida, I.; Tashiro, G.; Yoshioka, K.; Wu, W.-I., Voelker, D. R.; Basset, G.; Hanson, A. D. Plant synthesize ethanolamine by direct decarboxylation of serine using a pyridoxal phosphate enzyme. *J. Biol. Chem*. **2001**, *276*, 35523−35529.
(55) Chen, T. H. H.; Murata, N. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol*. **2002**, *5*, 250−257.
(56) Fahey, J. W.; Zalcmann, A. T.; Talay, P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* **2001**, *56*, 5−51.
(57) Zagrobelny, M.; Bak, S.; Vinther Rasmussen, A.; Jørgensen, B.; Naumann, C. M.; Lindberg Møller, B. Cyanogenic glucosides and plant-insect interactions. *Phytochemistry* **2004**, *65*, 293−306.
(58) Franks, T. K.; Hayasaka, Y.; Choimes, S.; van Heeswijck, R. Cyanogenic glucosides in grapevine: polymorphism, identification and developmental patterns. *Phytochemistry* **2005**, *66*, 165−173.
(59) Iriti, M.; Rabotti, G.; De Ascensao, A.; Faoro, F. BTH-induced resistance modulates ozone tolerance. *J. Agric. Food Chem.* **2003**, *51*, 4308−4314.
(60) Tomlin, C. D. S. *The Pesticide Manual*, 12th ed.; British Crop Protection Council: London, 2001.

Received for review April 14, 2005. Revised manuscript received July 26, 2005. Accepted July 28, 2005. This study has been funded by the Italian National Research Council, Agrifood Department.

JF050853G