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# Isolation and biological activities of lyoniside from rhizomes and stems of

## *Vaccinium myrtillus*

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## Abstract

A lignan glycoside identified as lyoniside (9-*O*- $\beta$ -D-xylopyranosyl(+))lyoniresinol) was obtained from ethanol extracts of rhizomes and stems of bilberry (*Vaccinium myrtillus* L.), on a preparative scale, by droplet counter-current chromatography. The application of this method permitted the isolation of a pure substance in only one chromatographical step. The occurrence of lyoniside in bilberry is reported for the first time. Seasonal fluctuations in the content of this compound in plant organs were demonstrated showing its highest levels in bilberry rhizomes and stems during the winter and their subsequent decrease in the spring. *In vitro*, the purified lyoniside was evaluated for antioxidant, allelopathic and antifungal activities. It showed significant radical scavenging properties in a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with IC<sub>50</sub> of 23  $\mu\text{g}\cdot\text{ml}^{-1}$ . Applied in concentration of 10  $\mu\text{g}\cdot\text{ml}^{-1}$ , it suppressed by 75% the seedling radical growth of *Lactuca sativa* and *Lepidium sativum*, and exerted strong inhibitory effect (55%) on germination of *Larix decidua*. Moreover, the synergistic action of lyoniside and triterpene acids was demonstrated in inhibitory effect exerted on germination and growth of *Pinus sylvestris*. Among 5 tested fungi strains of *Ascomycota*, the highest susceptibility was shown by *Fusarium oxysporum* and *Mucor hiemalis*, with mycelial growth inhibited by lyoniside concentration of 50  $\mu\text{g}\cdot\text{ml}^{-1}$  by 78 and 80%, respectively.

**Keyword index:** *Vaccinium myrtillus*; Ericaceae; bilberry; droplet counter-current chromatography; lignan; lyoniside

## **1. Introduction**

Bilberry (*Vaccinium myrtillus* L., Ericaceae) is a perennial dwarf shrub native to Europe and Northern America, widely known for its tasty fruit of high nutritive value and its use in folk medicine (Anonymous, 2001; Camire 2002). The berries contain high levels of phenolics - mainly anthocyanins (Prior et al., 1998; Valentová et al., 2007) – which, due to their antioxidative properties, are considered to be the pharmacologically active and health-promoting constituents (Martin-Aragon et al., 1999; Yao and Vieira, 2007). The detection of flavonoids and phenolic acids in various bilberry organs including flowers (Riihinen et al., 2008) and even litter accumulated in the soil around bilberry (Gallet and Lebreton, 1995) makes this shrub one of the most phenolic-rich plants known. However, in comparison to berries and leaves, the phenolic content of the rhizomes and stems remains still less characterized. The occurrence of new unusual p-coumarates in bilberry stems has recently been reported (Hybelbauerová et al., 2009), suggesting that the unexplored organs of even well-known plants could represent a source of interesting phytochemicals. This report describes the preparative isolation of a lignan glycoside, lyoniside, from stems and rhizomes of bilberry, the seasonal fluctuations of this compound in plant organs and the surrounding soil, and some of its biological activities, pointing to participation in chemical protection and environmental interactions of this plant.

## **2. Results and discussion**

### **2.1. Isolation and structure elucidation**

Fractionation of a 70% EtOH extract of *V. myrtillus* rhizomes by means of droplet counter-current chromatography, DCCC, with the solvent system CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O (43:37:20,

v/v/v) in the descending mode, separated the compounds into 8 distinct fractions comprised of molecules of increasing polarity. The separate fractions contained 2-3 compounds, except one which contained a single pure substance that could be directly submitted to structural analysis. The fractionation of plant extracts usually requires several steps of separation and purification, which often results in low recoveries of final products due to their irreversible adsorption or decomposition. The application of a liquid-liquid partition technique without solid stationary phase allows non-destructive and high-yield isolation of even labile compounds. DCCC and its modified version, HSCCC, have been successfully applied in the preparative separation of various natural compounds, including two novel anthocyanins from bilberry fruit (Du et al., 2004). In the present study, the application of this method permitted the isolation of a pure substance in only one chromatographical step. The compound was obtained as needle-shaped white crystals;  $[\alpha]_D^{25}$ : +34.2 (*c* 0.75, MeOH); the UV (MeOH) absorption maxima 210 and 278 nm; ESIMS (positive ion mode)  $m/z$  575.3  $[M+Na]^+$ ; HRESIMS (positive ion mode)  $m/z$  575.2114 (calcd. for  $C_{27}H_{36}O_{12}Na$ , 575.2104). Analysis of 1D and 2D NMR spectra (Table 1) identified the purified compound as lyoniside, 9-*O*- $\beta$ -D-xylopyranosyl(+)lyoniresinol (Fig. 1). This is the first report of the isolation of this compound from *V. myrtillus*. Lyoniside-containing fractions obtained from stems were found to be more contaminated and required an additional purification step on preparative TLC plates (silica gel, toluene:acetone 85:15 v/v). The simple isolation of lignans using preparative TLC, with satisfactory results, has been reported previously (Elfahmi et al., 2007). The presence of lyoniside was not detected in extracts obtained from bilberry leaves and fruit.

Lyoniside is a phenyltetralin lignan which was originally isolated and identified as a major component of the wood of *Lyonia ovalifolia* in 1960 (Kashima et al., 2010). It was subsequently detected in woody parts (inner bark, stem bark, roots) of other plants and occasionally also in sempervivent leaves. According to our present findings, the occurrence of lyoniside in *V. myrtillus* is restricted to the winter-persistent part of the plant, including aboveground stems and underground rhizomes.

## **2.2. Free radical scavenging activity**

The exclusive occurrence of lyoniside in winter-persistent organs suggests a possible role in protection against oxidative stress and in chemical defence against pathogens and herbivores. The antioxidant potency of lyoniside was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method to measure free radical scavenging ability (Fig. 2). The scavenging effect on a 0.1 mM DPPH solution exerted by lyoniside concentrations of between 20 and 200  $\mu\text{g}\cdot\text{ml}^{-1}$ , ranged from 48% to almost 90%, respectively. The  $\text{IC}_{50}$  (antioxidant concentration required to quench 50% of the initial DPPH) was 23  $\mu\text{g}\cdot\text{ml}^{-1}$ . At the highest concentration tested, the scavenging activity of lyoniside was 93% of that of  $\alpha$ -tocopherol, thus this compound may be regarded as an effective radical scavenger.

## **2.3. Seasonal fluctuations in plant organs and the surrounding soil**

Quantitative determinations of lyoniside present in bilberry tissues throughout the year showed some seasonal fluctuations (Fig. 3). The highest levels were detected during winter, with concentrations of 1.12  $\text{mg}\cdot\text{g}^{-1}$  dry wt in rhizomes and 184  $\mu\text{g}\cdot\text{g}^{-1}$  in stems in December. In spring, the content of this compound decreased, by 24% (rhizomes) and 12% (stems) in March and even more, by 33% (rhizomes) and 31% (stems) in May. Levels increased again in

September, reaching 91% of the December values in both rhizomes and stems. The content of phenolic compounds in bilberry and other plants is known to change according to the growth season, physiological and developmental stage, and also in response to environmental factors like light, climate and soil fertility. Regulation of the synthesis and accumulation of phenolics in bilberry leaves, by a complex interaction between intrinsic plant factors and external abiotic and biotic stimuli, has been proposed to explain the observed temporal fluctuations in these compounds (Witzell et al., 2003). The parallel changes in the amounts of lyoniside in stems and rhizomes might also be explained by ecological interactions between bilberry and other organisms (neighbouring plants, natural enemies, parasites, pathogenic bacteria and fungi), as well as by variations due to the growth cycle and adaptation to winter conditions.

The presence of high levels of lyoniside in bilberry rhizomes and stems during the winter and their subsequent decrease in the spring, suggest that the accumulated compound may be exudated into the surrounding soil, similarly to many other phenolic allelochemicals. Indeed, lyoniside was detected in samples of soil obtained from the natural bilberry habitat, with the highest level ( $0.11 \text{ mg}\cdot\text{g}^{-1}$  dry wt) found in May and December, and the lowest level ( $0.05 \text{ mg}\cdot\text{g}^{-1}$ ) in July. These amounts are lower than those described for other phenolics originating from falling decomposing leaves (Gallet and Lebreton, 1995), but the possible contribution of lyoniside to known allelopathic activity of bilberry cannot be ruled out. Like other phenolics, lignans are regarded as effective allelochemicals due to their capacity to interfere with the activities of many proteins (enzymes, transporters, ion-channels, receptors) involved in the transport of compounds and biosynthetic pathways in target plants (Wink 2003).

## 2.4. Allelopathic potential

The allelopathic activity of lyoniside was estimated by examining its influence on seed germination and subsequent seedling growth in two commonly used dicotyledon model plants (lettuce and cress), conifer trees (pine, spruce), which occur in forests with bilberry as the dominant understorey, and also larch, which is not a neighbour of bilberry in its natural habitat. The concentration of lyoniside applied was comparable to that detected in the soil obtained from the bilberry habitat. The results are presented in Table 2. Lyoniside inhibited the germination of dicotyledonous plants, with a moderate effect on lettuce (34%), and a weaker effect on cress (18%), but it strongly suppressed the seedling radicle growth of both plants, by 75%. Among the tested conifers, the strongest inhibitory effect was exerted on larch, which showed considerable inhibition of both germination (55%), and radicle and hypocotyl growth (50 and 46%, respectively). The influence on pine and spruce was much smaller, which demonstrates that lyoniside alone cannot be responsible for known allelopathic potential of bilberry against these trees. The relationships between plants in their natural habitats are highly complex due to their long-term coevolution, and cannot rely exclusively on the action of a single compound or even one class of compounds (Jha *et al.*, 2006; Malik and Pellisier, 2000). Therefore, we examined the simultaneous influence of lyoniside and two isomeric triterpenoids, oleanolic and ursolic acids, which also occur in *V. myrtillus* rhizomes and are detected in soil samples, on seed germination and seedling growth of pine. Separate application of these triterpene acids and lyoniside exerted only a slight inhibitory effect on the germination and growth of seedlings. However, a much stronger effect was exerted by their mixture, which inhibited germination by 80% and suppressed growth by approximately 70%



(radicles by 64%, hypocotyl by 75%), thus pointing to synergism between lyoniside and triterpene acids. Phenylpropanoids and triterpenoids occur constitutively in many plant species, acting as antimicrobial phytoprotectants that form a common line of defence in the first chemical barrier to infection. Moreover, they can also be considered as potent allelochemicals supporting the competition with neighboring plants. Our results provide further proof of the synergistic action of compounds within these two large classes of natural products. Free triterpene acids were previously found to act directly on membranes of the target plants (Szakiel and Kabacińska, 2009), and this altered membrane permeability might allow more polar compounds, like lyoniside, to enter the cytoplasm. However, the strong effect of this compound on larch, which is not a natural environmental neighbour of bilberry, shows that lyoniside by itself can be an effective allelochemical against a plant that has not evolved mechanisms of tolerance or detoxification.

## **2.5. Antifungal activity**

The purified lyoniside was tested for its fungicidal activity against 5 strains of *Ascomycota* (Table 3). Mycelial growth of all tested fungi was markedly influenced by lyoniside, although the dynamics of inhibition varied (Table 2). Lignans are known to be potent antimycotics influencing fungal cell synthesis (Hwang *et al.*, 2007), although various strains differ appreciably in their sensitivity to particular compounds. The growth of *A. niger* and *T. viride* was almost completely inhibited at a lyoniside concentration of 50  $\mu\text{l}\cdot\text{ml}^{-1}$  during first 3 days of incubation, but subsequently the mycelia spread rapidly, and after 7 days both strains had developed full colonies covering the entire dish surface (data not shown). In contrast, the inhibition of growth of *A. brassicicola*, *F. oxysporum* and *M. hiemalis* by

lyoniside was less than 100% after 3 days of incubation, but it remained significant until the end of experiment (64, 78 and 80%, respectively) and continued even when the incubation was prolonged for more than 7 days. Thus, lyoniside can be regarded as an antifungal agent that is particularly active against phytopathogens such as *F. oxysporum* and *M. hiemalis*.

Lignans are compounds possessing a diverse spectrum of biological properties. The findings of this study suggest that lyoniside is an example of a multifunctional plant secondary metabolite that can be involved solely or synergically in various mechanisms of plant chemical protection and in environmental interactions. Apart from their functions in the host plant, the importance of lignans for humans is due to their potential application in the fields of pharmacy and nutrition. Previous studies have suggested possible therapeutic uses for lyoniside due to its antiinflammatory, anticancer and antioxidant activities (Jin et al., 2006; Sadhu et al., 2007, Song et al., 2007), perhaps it can be useful also in the treatment of some neural disorders (Arai et al. 2009).

### **3. Experimental**

#### *3.1. Plant material*

Whole plants of *V. myrtillus* L. were collected from a natural forest habitat in central Poland. The identity of a voucher specimen was confirmed by the taxonomist Dr. Maja Graniszewska and deposited in the herbarium of the University of Warsaw (accession no. WA 0000017594).

#### *3.2. Extraction and purification*

Air-dried and powdered rhizomes (60 g) were extracted in a Soxhlet apparatus, initially for 10 h with diethyl ether to remove the lipophilic compounds and then for 24 h with 70% aqueous EtOH to obtain the polar compounds. After evaporation at 60°C under reduced pressure, the

latter extract yielded a gummy residue (6.63 g), 1 g of which was submitted to fractionation by DCCC (droplet counter-current chromatography) using the solvent system  $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$  (43:37:20, v/v/v) in the descending mode. DCCC was performed with a Tokyo Rikakikai Eyela model 300 DCCC chromatograph equipped with 95 tubes (400 mm x 2 mm) connected in series. The sample was dissolved in a mixture consisting of 10 ml of lower phase and 10 ml of upper phase of the applied solvent system, and injected using a Merck pump Duramat<sup>R</sup> through the 16 mm injection loop when the mobile phase front had emerged and the hydrostatic equilibrium was established in all the tubes. The flow rate of the mobile phase was set to  $1 \text{ ml} \cdot \text{min}^{-1}$ . Fractions were collected in glass test tubes using a Gilson Microcol<sup>R</sup> TDC 80 automatic fraction collector and analyzed by TLC (thin layer chromatography) using  $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$  (61:32:7, v/v/v). TLC analyses were performed on plastic-backed silica gel 60 (0.20 mm thickness) plates (Merck); chromatograms were visualized by spraying the plates with 10%  $\text{H}_2\text{SO}_4$  in MeOH, followed by heating at  $110^\circ\text{C}$ . A total of 370 fractions of 7 ml each were collected during 40 h and those containing compounds with similar  $R_f$  values were combined into 8 main fractions. After evaporation of  $\text{CHCl}_3$ , fractions 15-31 (main fraction 3) yielded from MeOH/ $\text{H}_2\text{O}$  a 7 mg of a pure crystalized compound which was subjected to structure elucidation.

### 3.3. Spectral analysis

Optical rotations were measured in MeOH using a Perkin-Elmer 341 polarimeter.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded with a Bruker Avance DRX 500 ( $^1\text{H}$  at 500 MHz and  $^{13}\text{C}$  at 125 MHz). 2D experiments were performed using standard Bruker microprograms; the spectra were acquired in  $\text{CD}_3\text{OD}$  at 293K. HRESIMS and ESIMS were recorded using a Finnigan

LCQ deca quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, USA). The samples were introduced by direct infusion in a MeOH solution at a rate of 5  $\mu\text{l min}^{-1}$ .

#### *3.4. Quantitative determination*

Lyoniside was determined spectrophotometrically by absorbance at  $\lambda=278$  nm using a Shimadzu UV-2401PC spectrophotometer. A calibration curve was prepared using MeOH solutions of pure crystallized lyoniside at concentrations ranging from 10 to 250  $\mu\text{g}\cdot\text{ml}^{-1}$ . Extracts of plant organs and soil samples collected in March, May, July, September and December 2008 were fractionated by DCCC as described in 3.2. Fractions containing lyoniside obtained from rhizomes were subjected directly to spectrophotometric quantitative determination, while fractions obtained from the stems and soil were first purified by preparative TLC in the solvent system toluene:acetone 85:15 v/v). Preparative TLC separation was carried out using 20 cm x 20 cm glass plates covered with a 0.25 mm thickness layer of silica gel 60 H (Merck); purified compound was localized by spraying with water and eluted from the gel with MeOH.

#### *3.5. Free radical scavenging activity*

The DPPH (2,2-diphenyl-1-picrylhydrazyl) method was used to measure free radical-scavenging activity. 2 ml of 0.1 mM DPPH in MeOH was added to 2 ml of MeOH containing different amounts of lyoniside to produce final concentrations of 0 (control), 20, 40, 60, 120 and 200  $\mu\text{g}\cdot\text{ml}^{-1}$ . The absorbance at 517 nm was measured after 10, 20 and 30 min.  $\alpha$ -Tocopherol in the same concentrations was used as the reference compound. Triplicates of each sample were run and the mean values calculated. The scavenging of DPPH radical [%]

was calculated according to the formula  $[(A_0 - A_1)/A_0 \times 100]$ , where  $A_0$  is the absorbance of the control reaction and  $A_1$  is the absorbance of reactions containing lyoniside or  $\alpha$ -tocopherol.

### 3.6. Allelopathic bioassays

Sheets of Whatman No.1 filter paper were placed in Petri dishes (100 mm diameter) and impregnated with 10 ml of  $10 \mu\text{g}\cdot\text{ml}^{-1}$  lyoniside in EtOH. The solvent was evaporated and 30 seeds of tested plants (lettuce *Lactuca sativa*, cress *Lepidium sativum*, pine *Pinus sylvestris*, spruce *Picea abies* or larch *Larix decidua*) were distributed evenly on the prepared sheets moisturized with 10 ml of pure sterile water. Control dishes without lyoniside were prepared in parallel. In the bioassays examining possible synergism between lyoniside and a mixture of oleanolic and ursolic acids, the tested compounds were applied to 30 seeds of *P. sylvestris*, either separately or mixed, at the final concentration of  $10 \mu\text{g}\cdot\text{ml}^{-1}$ . All dishes were then closed and placed in the dark in a thermostat (22°C). Germinating seeds of lettuce and cress were counted after 3 days and those of pine, spruce and larch after 7 days; the radicle and hypocotyl lengths were measured after 7 and 15 days.

### 3.7. Antifungal bioassays

Fungi strains, obtained from the Institute of Fermentation Technology and Microbiology, Technical University of Łódź, were grown on Sabouraud agar (*Aspergillus niger*, *Alternaria brassicicola* and *Mucor hiemalis*) or potato dextrose agar (*Fusarium oxysporum* and *Trichoderma viridae*). Petri dishes (55 mm), filled with 6 ml of sterile medium containing lyoniside at concentrations of 20 or  $50 \mu\text{g}\cdot\text{ml}^{-1}$ , were inoculated with 5 mm agar plugs containing mycelia and incubated at 25°C in darkness for 5 days. Three replicates were prepared for each lyoniside concentration. Commercial fungicide, captan, was used as the

reference compound. The radial growth of mycelium (colony diameter) was measured after 1.5, 3 and 5 days for each culture, and compared with controls without lyoniside.

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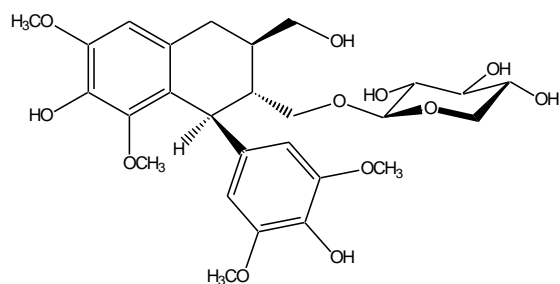


Fig. 1. Chemical structure of lyoniside (9-*O*-β-D-xylopyranosyl(+))lyoniresinol).

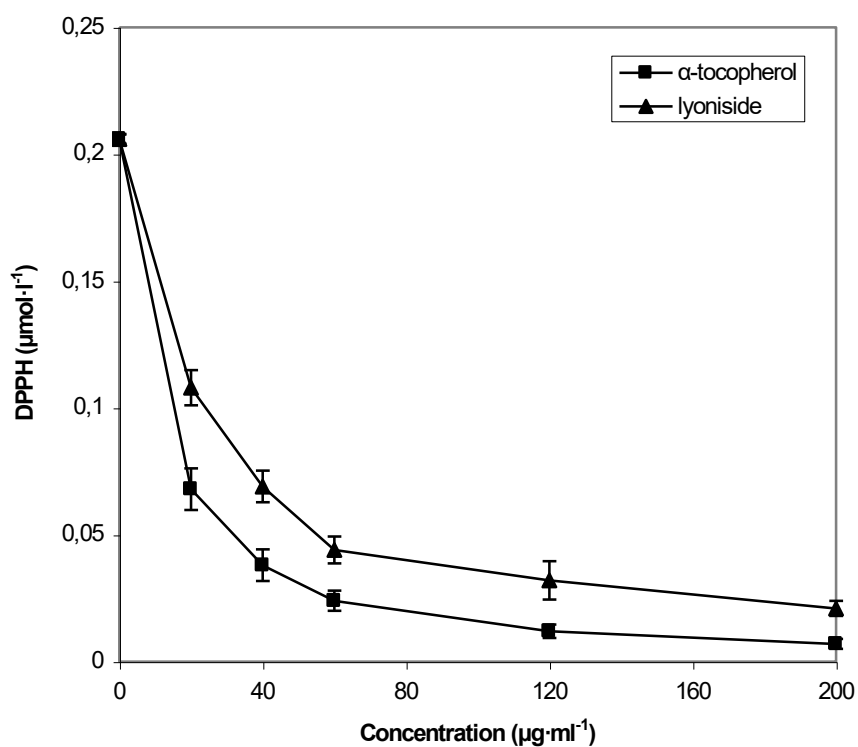


Fig. 2. DPPH free radical scavenging activity of lyoniside. α-Tocopherol was assayed as the positive control. Results are means of triplicates ±S.D.

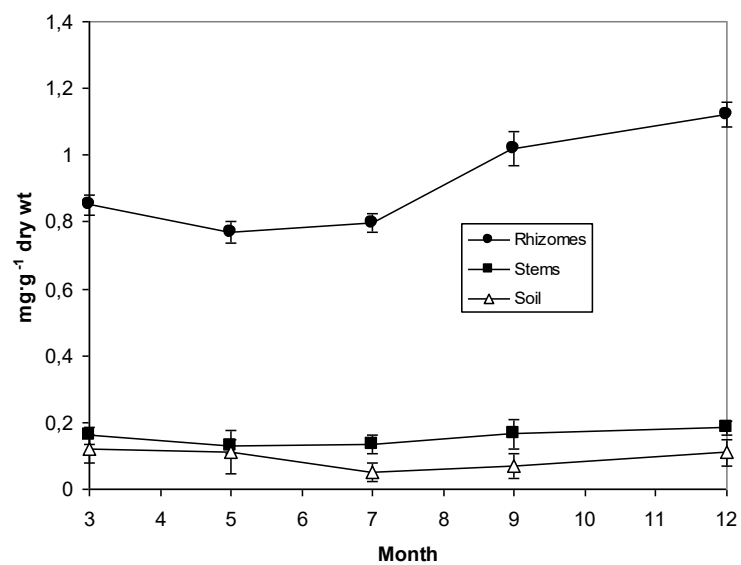


Fig. 3. Seasonal fluctuations of lyoniside in *V. myrtillus* plant and the surrounding soil. Results are means of triplicates  $\pm$ S.D.

Table 1.  $^1\text{H}$  (500 MHz) and  $^{13}\text{C}$  NMR (125 MHz) spectral data of lyoniside in  $\text{CD}_3\text{OD}$ .

Position	$\delta_{\text{H}}$ (m, $J$ in Hz)	$\delta_{\text{C}}$
Lyoniresinol		
1	-	139.1
2	6.45 s	106.6
3	-	148.6
4	-	133.6
5	-	148.6
6	6.45 s	106.6
7	4.35 (d, 6.3)	42.4
8	2.11 (tt, 6.3-5.4)	46.2
9	3.47 (dd, 9.9-4.1)	71.4
	3.84 (dd, 9.9-5.4)	
1'	-	125.8
2'	-	147.1
3'	-	138.1
4'	-	148.4
5'	3.71 s	108.3
6'	-	130.4
7'ax	2.61 (dd, 15.2-11.6)	33.2
eq	2.78 (dd, 15.4-4.6)	
8'	2.11 (dt, 11.6-6.4-4.4)	39.8
9'	3.53 (dd, 11.0-6.8)	65.7
	3.66 (dd, 11.0-4.3)	
$\text{OCH}_3$	3.36 s	60.5
2 $\text{OCH}_3$	3.77 s	57.0
$\text{OCH}_3$	3.89 s	56.8
Xylose		
1''	4.32 (d, 7.7)	105.0
2''	3.30 (dd, 9.1-7.7)	74.4
3''	3.42 (t, 9.1)	77.3
4''	3.57 (ddd, 10.6-9.1-5.4)	70.6
5''	3.25 (dd, 11.5-5.4)	66.4
	3.90 (dd, 11.5-10.6)	

Table 2. The influence of lyoniside on seed germination and growth of model plants. Values are means of triplicates  $\pm$ S.D.

Plant/ compound	Germination		Seedling growth			
	Number of germinating seeds	Inhibition (%)	Radicle length [mm]	Inhibition (%)	Hypocotyl length [mm]	Inhibition (%)
Lettuce						
control	29 $\pm$ 1		22.83 $\pm$ 2.01		10.67 $\pm$ 2.15	
lyoniside	19 $\pm$ 2	34	5.71 $\pm$ 2.24	75	8.14 $\pm$ 2.87	24
Cress						
control	28 $\pm$ 2		38.13 $\pm$ 2.05		24.5 $\pm$ 1.55	
lyoniside	23 $\pm$ 1	18	9.6 $\pm$ 0.74	75	10.4 $\pm$ 3.70	58
Pine						
control	26 $\pm$ 3		5 $\pm$ 2.8		2 $\pm$ 0.36	
lyoniside	23 $\pm$ 2	15	3.9 $\pm$ 0.63	22	1.5 $\pm$ 0.1	25
OL/UR	19 $\pm$ 2	27	4.1 $\pm$ 0.57	18	1 $\pm$ 0.02	50
Lyoniside + OL/UR	5 $\pm$ 1	80	1.8 $\pm$ 0.46	64	0.5 $\pm$ 0.02	75
Spruce						
control	23 $\pm$ 2		6.28 $\pm$ 1.6		2.84 $\pm$ 0.62	
lyoniside	14 $\pm$ 1	39	4.24 $\pm$ 2.35	32	1.85 $\pm$ 0.2	35
Larch						
control	22 $\pm$ 2		7.12 $\pm$ 1.26		3.08 $\pm$ 0.89	
lyoniside	10 $\pm$ 1	55	3.54 $\pm$ 1.47	50	1.65 $\pm$ 0.32	46

Table 3. The influence of lyoniside on mycelial growth of tested fungi. The growth of mycelium is measured as a diameter of colony without inoculation plug. Values are means of triplicates  $\pm$ S.D.

Fungi	Lyoniside concentration [ $\mu\text{g}\cdot\text{ml}^{-1}$ ]	Diameter of colony [mm]			Inhibition [%]		
		Days of incubation					
		1.5	3	5	1.5	3	5
<i>Alternaria brassicicola</i>	control	35 $\pm$ 1.98	50	50			
	20	2 $\pm$ 0.82	16 $\pm$ 1.28	21 $\pm$ 1.45	94	68	58
	50	0	12 $\pm$ 1.42	18 $\pm$ 1.98	100	76	64
<i>Aspergillus niger</i>	control	15 $\pm$ 1.6	40 $\pm$ 1.10	50			
	20	4 $\pm$ 0.52	10 $\pm$ 0.95	33 $\pm$ 1.68	73	75	44
	50	0	0	19 $\pm$ 1.23	100	100	62
<i>Fusarium oxysporum</i>	control	14 $\pm$ 1.86	32 $\pm$ 3.78	50			
	20	10 $\pm$ 1.04	18 $\pm$ 1.75	28 $\pm$ 2.06	29	44	44
	50	6 $\pm$ 1.25	7 $\pm$ 1.03	11 $\pm$ 1.63	57	78	78
<i>Mucor hiemalis</i>	control	40 $\pm$ 2.36	50	50			
	20	12 $\pm$ 1.46	20 $\pm$ 2.02	25 $\pm$ 3.78	70	60	50
	50	2 $\pm$ 0.10	4 $\pm$ 0.68	10 $\pm$ 2.10	95	92	80
<i>Trichoderma viridae</i>	control	21 $\pm$ 2.05	50	50			
	20	4 $\pm$ 0.90	6 $\pm$ 1.08	29 $\pm$ 3.05	81	88	42
	50	1 $\pm$ 0.2	2 $\pm$ 0.75	20 $\pm$ 1.86	95	96	60

