BIOLOGIA PLANTARUM 52 (2): 391-395, 2008

BRIEF COMMUNICATION

Effect of wounding on chalcone synthase and pathogenesis related *PR-10* gene expression and content of phenolic compounds in bilberry leaves

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Abstract

The influence of artificial wounding on biosynthesis of flavonoids and hydroxycinnamic acids was studied in bilberry leaves using two separate wounding experiments. In the first experiment bilberry leaves were wounded by cutting. The expression of the first gene from flavonoid pathway, chalcone synthase (*CHS*) and a wound induced pathogenesis related *PR-10* gene was analysed from samples collected immediately and after 3, 6, 24 h and 4 d from the wounding treatment. In the second experiment annual shoots were removed. Proanthocyanidins, flavonol glycosides and hydroxycinnamic acids were quantified in leaf samples after 0 - 5 d (experiment 1) and 5 weeks (experiment 2) from the treatment. In the first experiment, no change was observed in the expression of *CHS* whereas increase in expression of *PR-10* gene was detected after 6 h of wounding treatment. In both experiments, the contents of flavonol glycosides and hydroxycinnamic acids were not influenced by the wounding treatment and the contents of proanthocyanidins were decreased.

Additional key words: flavonoids, hydroxycinnamic acids, simulated herbivory.

Wounding is caused by herbivory, parasitism or by mechanical injury and it results to the retardation of plant growth and offer the easy penetration of pathogens into the plant tissues. Plants respond to wounding by different strategies. The biochemical changes and the activation of genes to produce defence related proteins have been detected in many studies (Bowles 1990, Liu *et al*. 2003). The wound-inducible proteins include pathogenesis related (PR) proteins, proteinase inhibitors and enzymes involved in phenylpropanoid metabolism (Dixon and Paiva 1995, Liu *et al.* 2003).

Flavonoid compounds are involved in a wide range of functions in plants, including the protection against biotic and abiotic stresses. Flavonoids are synthetized *via* the phenylpropanoid pathway starting with the conversion of phenylalanine to cinnamate by phenylalanine ammonia-lyase (PAL) enzyme. The condensation of one molecule of 4-coumaroyl CoA with three molecules of malonyl CoA, the first committed step in flavonoid biosynthesis branch, is catalysed by chalcone synthase (CHS). It is generally accepted that flavonoids along with other phenolic compounds play a role in protecting plants from both insect and mammal herbivory (Dixon and Paiva 1995, Harborne and Williams 2000). The responses can, however, vary remarkable between the different plant species.

Bilberry (*Vaccinium myrtillus* L.) is a characteristic field layer species in boreal forests and an important forage plant for herbivores, such as reindeer, microtine rodents, birds and insect caterpillars. Bilberry leaves contain high amounts of phenolic compounds, especially proanthocyanidins and flavonols from flavonoids, in addition to hydroxycinnamic acids (Witzell *et al.* 2003, Jaakola *et al.* 2004). The role of these compounds in the

Received 20 September 2006, *accepted* 19 January 2007.
Abbreviations: CTAB - hexadecyltrimethylammonium bromide; CHS - chalcone synthase; DAD - diode-array detection; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; PR-10 - pathogenesis related protein 10.
Acknowledgements: We wish to express our gratitude to Eeva-Liisa Palkispää for her help in sample preparations in the quantification of phenolic compounds. This study was a part of the co-operation program of the University of Oulu and the Kuusamo town and was supported financially by EU (ESF), Regional council of Northern Ostrobothnia and Kuusamo town, by the Academy of Finland/SUNARE project (grant no. 52741), and by the Emil Aaltonen Foundation.
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defence against herbivores in bilberry is presently unknown.

The aim of our work was to investigate whether bilberry has ability to increase the production of phenolic compounds for the protection against herbivory, or whether the species constantly keeps the content of phenolics at a high level. In this study, we carried out two experiments that simulate insect or rodent herbivory. Firstly, we detected short-term responses of leaf wounding by setting an experiment where the expression of the first gene of the flavonoid pathway, chalcone synthase (*CHS*) and a wound induced *PR-10* gene, was analysed immediately and after 3, 6, 24 hours and four days of the experimental wounding treatment. The flavonoids and hydroxycinnamic acids were quantified from the samples, along with untreated controls. In the second experiment, we wanted to clear out, if the removal of the new shoots will effect on the contents of phenolic compounds in the bilberry leaves.

Bilberry plants growing at test field of the Botanical Gardens, University of Oulu, Finland, were used as test plants. For the wounding treatments, both the treated and the control plants represented the same tissue cultured clones originated from Oulu (Jaakola *et al.* 2001a).

The first wounding experiment was carried out on 18 cloned patches of bilberry plants. Two patches were randomly marked as 'initial state', 4 patches as untreated controls and 4 patches as wounded plants. Wounding was carried out by clipping every leaf alongside the leaf central vein from all plants, which coarsely simulates herbivory caused by insect caterpillars. In this way approximately 1/3 of the leaf area was removed. Control plants were left untreated. Leaf samples were collected immediately from two patches, and these were called initial state samples. The rest of the samples were collected after 3, 6, 24 h and 4 d from the wounding. The wounding experiment was repeated in controlled conditions (16-h photoperiod, irradiance of 75 µmol m^{-2} s^{-1}, temperature 22 °C) for the real time PCR analyses and the samples were collected at the same points of time. For the quantification of phenolic compounds, samples were gathered 5 d after the treatment along with the controls.

In the second wounding experiment, 7 patches of bilberry plants were randomly marked as controls and 7 patches as wounded plants. Wounding was carried out by removing all new annual shoots at the beginning of June, which simulates damage caused by microtine rodents (Tolvanen and Laine 1997). The collected shoots were named as initial state and stored for later analyses. After five weeks, new annual shoots grown after the wounding were collected along with the untreated controls and stored in -80 °C for the chemical analysis.

The cDNA fragment of *CHS* gene was isolated from bilberry using gene-specific primers in PCR, subcloned and sequenced as described in Jaakola *et al*. (2002). The cDNA of *PR-10* was isolated from bilberry using degenerated primers planned according to *PR-10* gene sequences from other related species. The achieved fragment was subcloned and sequenced by *AB 3730* DNA analyzer (*Applied Biosciences*, Warrington, UK) and the similarity to the corresponding *PR-10* genes from other species was confirmed by aligning the sequence in the Genbank using the *BLASTN* program (Koskimäki *et al*., unpublished results). Total RNA was isolated from leaf samples with the CTAB based method (Jaakola *et al.* 2001b). The expression of *CHS* gene was firstly studied with cDNA blotting method (Jaakola *et al*. 2001c).

The real-time PCR analyses were performed using *LightCycler* instrument (*Roche Molecular Biochemicals*, Mannheim, Germany) and *DyNAmoTM SYBR® Green qPCR* kit (*Finnzymes*, Espoo, Finland). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s (ramp rate 20 °C s^{-1}), 60 °C for 20 s and 72 °C for 10 s. Melting curve was measured in one cycle of 95 °C for 0 s and 57 °C for 15 s, each with a temperature transition rate of 20 °C s^{-1}, and then ramping to 98 °C for 0 s at 0.1 °C s^{-1}. The primers for the *CHS* gene were 5'-CCAAGGCCA TCAAGGAATG-3' and 5'-TGATACATCATGAGT CGCTTCAC-3'. For the *PR-10* gene the primers were 5'-CCATCAAGCTCACCACTTTC-3' and 5'-GTGAGC TTCAACAGCCTTG-3'. The primers 5'-CAAACTGTC TTGCCCCACTT-3' and 5'-CAGGCAACACCTTACCA ACA-3' were designed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used for the relative quantification of PCR products. The efficiency of the primers was tested in preliminary experiment with dilutions of bilberry leaf cDNA sample which was also used as normalizing sample in each experiment to which the expression of the same gene in other samples was compared. PCR products were analysed using melting curves, and agarose gel electrophoresis to ensure single product amplification. Quantification of PCR products was performed via calibration curve procedure using GAPDH as an internal standard. The equations were performed by *LightCycler* software using standard formulas for real time PCR.

The leaves were ground to a fine powder using mortar and pestle in liquid nitrogen. The powdered leaf samples (0.25 g) were suspended in acidified (0.6 M HCl) methanol. Samples (1 cm^3) were taken for the quantification of flavonols and hydroxycinnamic acids and the rest of the suspension was refluxed for 2 h (60 - 70 ºC) for the quantification of proanthocyanidins. Heating in acid converts procyanidin and prodelphinidin units to corresponding anthocyanidins and enables their estimated quantification (Määttä *et al*. 2001). All samples were filtered and analysed with reversed-phased high-performance liquid chromatography combined to diode-array detection (DAD). The chromatographic conditions (column and gradient systems) and the device used were the same as described earlier (Määttä *et al*. 2001). UV-visible spectral identification and quantification of phenolic compounds in extracts and acid hydrolysate was described in detail previously (Määttä *et al*. 2001, Jaakola *et al*. 2004). Flavonol glycosides and conjugated forms of hydroxycinnamic acids in extracts were quantified for the mass of the phenolic unit in the molecule using quercetin

glucose-rhamnoside (rutin), *p*-coumaric acid and caffeic acid as representative standards (Määttä *et al*. 2001).

Statistical analyses were performed only in the second wounding experiment (n = 7), since the number of replicates was too low (n = 2) to be tested in the first experiment. The changes in the contents of hydroxycinnamic acids, proanthocyanidins and flavonol glycosides caused by sampling occasion were tested by comparing the initial state between the control plants sampled 5 weeks later (*t*-test, df = 2). The differences in the contents caused by wounding were tested by comparing wounded and control plants sampled 5 weeks after the treatment (*t*-test, df = 12).

In the first experiment, no change was observed in the expression of *CHS* gene, after leaf wounding treatment in the blotting analysis (Fig. 1). Also, in the repeated experiment with real time PCR, the same results were detected for the expression of CHS gene (Fig. 2*A*). However, activation of wound-induced PR-10 gene was detected after 6 h of wounding treatments in the same samples (Fig. 2*B*). Moreover, the results from HPLC-DAD analysis showed that the contents of flavonol glycosides and proanthocyanidins were not influenced by the treatment although there was a slight decrease in the concentration of hydroxycinnamic acids as a consequence of leaf wounding (Table 1).

In the second experiment, after five weeks from the wounding treatment, the concentrations of hydroxycinnamic acids and flavonol glycosides were unaffected by both sampling occasion and wounding. The concentrations of proanthocyanidins increased as a response to time, but decreased as a response to the wounding treatment (Table 1).

The results from the gene expression and chemical analysis indicated that wounding did not induce biosynthesis of flavonoids in bilberry leaves. Hence our results support earlier studies, which showed that the concentration of total phenolics in bilberry leaves was not affected by microtine grazing (Laine and Henttonen 1987).

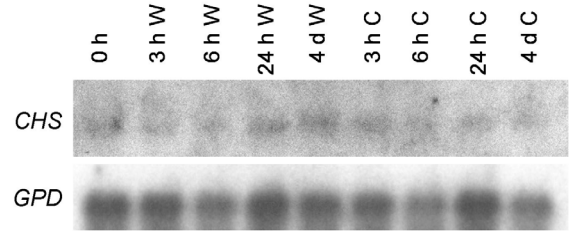

Fig. 1. Expression of chalcone synthase (*CHS*) gene after 0, 3, 6, and 24 h and 4 d of wounding (W) treatment in experiment 1. The same membrane was rehybridized with a glyceraldehydes-3-phosphate dehydrogenase (GAPDH) probe, used as a reference gene (C - controls).

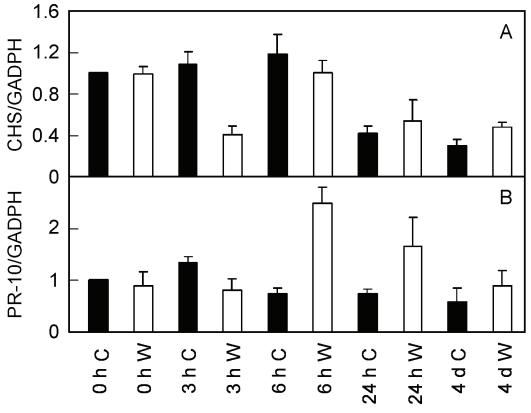

Fig. 2. Relative expression of chalcone synthase (*CHS*) gene (*A*) and pathogenesis related *PR-10* gene (*B*) after 0, 3, 6, and 24 h and 4 d of wounding treatment in control (C) and sample (W) leaves. Expression was determined by *Lightcycler* real-time PCR and is shown relative to expression of GAPDH in each sample. All data is presented as mean of four replicates.

Table 1. Contents of hydrocycinnamic acids, proanthocyanidins and flavonol glycosides [mg g^{-1}(d.m.)] in bilberry leaves in the two wounding experiments. Means ± SE, n = 7. Different letters indicate significant differences caused by sampling occasion (*t*-test, df = 12, $P \leq 0.001$). *Asterisks* indicate significant differences between wounded (W) and control (C) plants (*t*-test, df = 12, $P \leq 0.005$). Results in the first experiment were not statistically tested as n = 2.

		Hydroxycinnamic acids	Proanthocyanidins	Flavonol glycosides
1st exp.	0 h	18.27 ± 0.26	1.05 ± 0.03	6.80 ± 0.19
	5 d C	18.56 ± 0.93	0.90 ± 0.05	7.87 ± 0.52
	5 d W	14.43 ± 0.21	0.95 ± 0.03	6.08 ± 0.02
2nd exp.	0 h	15.84 ± 1.07	0.66 ± 0.02[a]	5.62 ± 0.48
	5 d C	14.48 ± 0.76	1.11 ± 0.03[b]*	6.09 ± 0.38
	5 d W	15.43 ± 0.73	0.90 ± 0. 04*	4.96 ± 0.49

Activation of wound-induced *PR-10* gene was detected in the bilberry leaf samples as a response to mechanical wounding. Pathogenesis related proteins belonging to PR-10 class have structural similarity with ribonucleases. *PR-10* genes are related to defence mechanism of plants but they have also been found to express differentially in different organs of plants (Pinto *et al.* 2005). The expression of *PR-10* gene in bilberry leaves was highest after 6 h of the wounding treatment decreasing close to the level of the control samples in 4 d. Similar pattern of expression of *PR-10* gene has been detected earlier in soybean leaves as a response to mechanical wounding (Graham *et al.* 2003). In western white pine needles the activation in the expression of *PR-10* gene was detected 8 h following the mechanical wounding treatment (Liu *et al.* 2003).

The role of phenolic compounds preventing herbivory is not clear. The activation of *CHS* gene as a response to wounding has been found earlier in *Medicago sativa* leaves (Sallaud *et al*. 1995), *Picea glauca* needles (Richard *et al.* 2000), and in *Phaseolus vulgaris* hypo-

cotyls (Lawton and Lamb 1987) in addition to some other plant species (Dixon and Paiva 1995, De Bruxelles and Roberts 2001). On the other hand, Haukioja (2003) found no clear correlation between the accumulation of phenolic compounds and the consumption of mountain birch leaves by herbivores. In our related study a clear activation of *CHS* gene was detected after 12 and 24 h of infection of bilberry leaves with two different fungal species (Koskimäki *et al.* unpublished results).

The contents of hydroxycinnamic acids or flavonols in bilberry leaves did not show any response to wounding treatment in the present study. In potato (*Solanum tuberosum*), accumulation of ferulic acid esters from hydroxycinnamic acids was detected 3 - 7 d after wounding (Bernards and Lewis 1992). Housti *et al*. (2002) detected the increase in hydroxycinnamic acid contents after wounding of *Thunbergia alata* leaves. However, compared to bilberry, the levels of hydroxycinnamic acids were still notably lower in *Thunbergia alata* leaves. The investigations of the roles of flavonols as response to wounding and herbivory have shown contradictory results. Vogt *et al*. (1994) detected the increase in the content of flavonol kaempferol in petunia stigmas 24 h after the artificial wounding. On the other hand, quercetin, the main flavonol in bilberry leaves, has also been suggested to act as an attractant for insects. Roda *et al.* (2003) found reduced amounts of quercetin from young *Nicotiana attenuata* leaves after herbivore and methyl jasmonate treatment. In addition, they also noticed that quercetin sprayed tobacco leaf surface attracted more mirids than untreated control plants.

In the present study, the contents of proanthocyanidins were detected to decrease as a response to wounding treatments. Proanthocyanidins or condensed tannins are bitter tasting compounds that have been proved to prevent the feeding of plant tissues (Marles *et al*. 2003). There is evidence that inducible proanthocyanidin accumulation may present a defence mechanism of some seeds or leaves against insect and mammalian herbivory (Ceballos *et al.* 2002, Dixon *et al.* 2005). However, the importance of proanthocyanidins in protection against herbivory has also been questioned (Briggs 1990). The contents of proanthocyanidins and other measured phenols are initially high in bilberry leaves and therefore, an increase of the concentration would possibly not bring further protection to leaves. Overall, the roles of proanthocyanidins in leaves are not clear. One assumption is that as polymeric compounds, they could act as storage forms to other flavonoids. In our earlier study on the effect of increased light conditions on bilberry leaves, the contents of other flavonoids and hydroxycinnamic acids increased with the exception of polymeric proanthocyanidins (Jaakola *et al*. 2004) which could indicate their role as storage compounds.

Earlier studies have considered the food quality of bilberry as a factor that might explain changes in herbivore populations (Laine and Henttonen 1987, Selas 2000, 2001, Fernández-Calvo and Obeso 2004). It has not been clear, whether the quality of this species as food for herbivores is determined by its nitrogen concentration or by the concentration of phenolic compounds, since the total phenolic content follows opposite patterns to nitrogen content (Laine and Henttonen 1987). Instead of increasing the biosynthesis of phenolic compounds, the production of new meristems seems to be the principal strategy, in which the bilberry responds to herbivory, a result that is supported by earlier field studies (Tolvanen 1994, Tolvanen and Laine 1997).

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