Physiological and Molecular Plant Pathology 72 (2008) 146–150

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

Artificial infection of *Vaccinium vitis-idaea* L. and defence responses to *Exobasidium* species

Tuija Pehkonen [a], Janne Koskimäki [a], Kaisu Riihinen [b], Anna Maria Pirttilä [a], Anja Hohtola [a], Laura Jaakola [a], Anne Tolvanen [c,*]

[a] Department of Biology, University of Oulu, FIN-90014, Oulu, Finland
[b] Department of Biosciences, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland
[c] Finnish Forest Research Institute, Muhos Research Unit, Kirkkosaarentie 7, 91500 Muhos, Finland

ARTICLE INFO

Article history:
Accepted 15 August 2008

Keywords:
Boreal
Exobasidium splendidum
Exobasidium vaccinii
Flavonoids
Inoculation
Lingonberry
Subarctic
Vaccinium vitis-idaea

ABSTRACT

An inoculation method for *Exobasidium splendidum* and *Exobasidium vaccinii* was developed on the dwarf shrub *Vaccinium vitis-idaea*. Using inoculated ramets, we investigated whether there are differences between *V. vitis-idaea* populations in the susceptibility to *Exobasidium* infections and whether the defence reaction of *V. vitis-idaea* is visible at a molecular level. Sixteen *V. vitis-idaea* clones from four populations were propagated in tissue cultures and the ramets were inoculated with *E. splendidum* or *E. vaccinii* fungi. The expression of three flavonoid biosynthetic genes (chalcone synthase, dihydroflavonol 4-reductase and anthocyanidin synthase) and the accumulation of flavonoids and hydroxycinnamic acids were determined in response to *E. splendidum* infection. A pathogenesis-related (PR 4) gene was isolated and its expression was studied in host ramet leaves. To our knowledge, this was the first successful artificial infection reported with *E. splendidum*. Disease frequencies of the inoculated ramets were between 32% and 47% for *E. splendidum* and 33% for *E. vaccinii*, but below 10% in uninoculated control ramets. There were no differences in disease frequencies between *V. vitis-idaea* populations. Both symptomatic leaves and healthy leaves of diseased ramets showed activation of flavonoid biosynthesis at the gene level, whereas expression of PR 4 was observed only in symptomatic leaves. The increase of flavonoid biosynthesis in healthy leaves of diseased ramets may represent a general response to stress or a role in defence against the pathogen *E. splendidium*. Ability of *V. vitis-idaea* to defend chemically against *Exobasidium* fungi and the heterogeneity of genotypes, age, size, and growth rates in host plant populations might be reasons for the low infection incidence of *Exobasidia* in nature.

1. Introduction

Both environmental and genetic factors are known to influence the susceptibility of plants to pathogen infections [1–3]. When the pathogen infections are scattered, however, it is difficult to investigate which factors have an impact on the disease frequencies on plants in nature. Experimental work and pathogen inoculations are therefore needed.

In this study, a dominant boreal-subarctic dwarf shrub *Vaccinium vitis-idaea* L. and its two highly host and tissue specific biotrophic pathogens, *Exobasidium splendidum* Nannf. and *Exobasidium vaccinii* Woron (Basidiomycotina) were investigated. *E. splendidum* is a monocyclic fungus, i.e. it reproduces only once during the growing season [2]. Dormant spores overwinter on vegetative buds and infect the newly developing shoots in spring. Infections by *E. vaccinii* are found from early until late season, which indicate that the species reproduces several times during the growing season. *E. splendidum* is restricted to high latitudes and altitudes, whereas *E. vaccinii* follows its host in most of its area except the highest latitudes and altitudes [2]. Environmental factors are essential in determining the frequency of *Exobasidium* infections in *V. vitis-idaea* [1,4,5], whereas there is no information on genetic differences between *V. vitis-idaea* populations with respect to their susceptibility to *Exobasidium* infections. Since the natural infection rate is less than 5% for *E. splendidum* and less than 10% for *E. vaccinii* [4], other factors than environment probably also limit their distribution. One of these factors may be the ability of *V. vitis-idaea* to defend chemically against the pathogen.

Exobasidium species infect immature, vegetative and reproductive plant tissues [1,2], which die after the sporulation at the end of the growing season. Symptoms of *E. splendidum* are slightly elongated shoots and moderately enlarged, bright red leaves. *E. vaccinii*

* Corresponding author. Tel.: +358 10 211 3782; fax: +358 10 211 3701.
E-mail addresses: tuija.pehkonen@inari.fi (T. Pehkonen), janne.koskimaki@oulu.fi (J. Koskimäki), kaisu.riihinen@uku.fi (K. Riihinen), am.pirttila@oulu.fi (A.M. Pirttilä), anja.hohtola@oulu.fi (A. Hohtola), laura.jaakola@oulu.fi (L. Jaakola), anne.tolvanen@metla.fi (A. Tolvanen).

0885-5765/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.pmpp.2008.08.002

causes conspicuous and thickened leaf-concavities, bright red above, and pinkish red beneath. Induction of pink or bright red colouration after the infection by *Exobasidium* fungi indicates biosynthesis of anthocyanins, a flavonoid subclass.

Flavonoids are phenolic compounds that are involved in many biological functions in plants, including the pigmentation of flowers and fruits or protection against UV, drought or damage caused by pathogen attack. Flavonoids are produced via the phenyl propanoid pathway. Increase in the production of key enzymes from general phenyl propanoid or flavonoid pathway has been detected in plant tissues as a response to pathogen attacks [6,7]. In addition, the expression of pathogenesis-related (*PR*) genes is usually associated with plant pathogen infections and related abiotic stresses. PR proteins belong to a large group of genes and are divided by their structural and functional properties, up to 17 different families identified so far [8]. Functions of PR families range from signal transduction to cell wall strengthening and antimicrobial activity. Chitinases are especially interesting because their substrates originate from fungal cell walls and the exoskeleton of arthropods [9]. PR 4 proteins are classified as endochitinases that are shown to have antifungal properties in a range of studies [10].

Due to low disease frequencies, it is difficult to investigate plant–pathogen interactions between *V. vitis-idaea* and *Exobasidium* spp. in nature. An inoculation method for two *Exobasidium* species on *V. vitis-idaea* was developed in the present study, which allowed to investigate, 1) whether there are differences between *V. vitis-idaea* populations with respect to the susceptibility to *Exobasidium* infections, and 2) whether the defence reaction of *V. vitis-idaea* is detectable at a molecular level. To our knowledge, inoculation with *E. splendidum* resulted in the first successful artificial infection reported. Quantities of anthocyanins, proanthocyanidins, flavonols and hydroxycinnamic acids, in addition to the expression of flavonoid pathway and PR 4 genes were analyzed in healthy ramets and ramets infected by *E. splendidum*.

2. Material and methods

2.1. Experimental design and inoculation method

V. vitis-idaea clonal fragments containing 5–8 interconnected ramets, each with several actively growing shoot tips, were obtained from four different populations in Finland, during autumn 1999 and spring 2000. The populations were 1) subarctic Kevo, 69°45′N, 27°01′E, 110 m a.s.l., 2) northern boreal Rovaniemi, 66°26′N, 25°39′E, 110 m a.s.l., 3) northern boreal Kuusamo, 66°20′N, 29°20′E, 190 m a.s.l., and 4) northern boreal Ranua, 65°54′N, 26°36′E, 150 m a.s.l.. In each population, 5–10 clonal fragments were obtained. Sites were relatively dry heath forests dominated by *Pinus sylvestris* L. *V. vitis-idaea* is the dominant species of the understorey layer in these forests. Each fragment was taken at least 100 m away from the next to ensure that they were separate genotypes. In Sweden, genotypes stretching as far as between 20 m and 30 m have been identified in certain *V. vitis-idaea* populations [11]. Plants were kept moist and cool and transferred quickly to be tissue cultured at the Botanical Gardens of the University of Oulu. Plants were micropropagated on a modified Murashige and Skoog medium [12] and planted in pots containing *Sphagnum* peat, vermiculite and sand (2:1:1) [12,13]. The rooted micro shoots were grown for 2–4 months at the green house before transplantation. The final number of genotypes reduced to four per population, 16 genotypes in total. To prevent the infection from background inoculum, protective plywood walls (30 cm high, 5 mm thick) were placed around all experimental plants in each treatment.

The ramets were transplanted to experimental sites at subarctic Kevo (69°45′N, 27°01′E) and northern boreal Kuusamo (66°20′N, 29°20′E), in August 2000. Ramets were about 5 cm tall and grew as single shoots. At subarctic Kevo, 64 ramets were planted. Of these, 32 ramets (two replicates per genotype) were inoculated with basidiospores of *E. splendidum* while 32 ramets were left as uninoculated controls. At northern boreal Kuusamo, 96 *V. vitis-idaea* ramets were planted. They were divided into three treatments, 32 ramets in each, with two replicate ramets per genotype. Treatments were: 1) inoculation with *E. splendidum*, 2) inoculation with *E. vaccinii*, and 3) uninoculated controls. Inoculation of *E. vaccinii* was not carried out in Kevo due to the lack of plant material.

Ramets were inoculated two times with basidiospores of *E. splendidum* in 2001; Aug. 30th, and Sept. 10th in Kevo, and Aug. 29th and Sept. 4th in Kuusamo. Inoculation was carried out three times with *E. vaccinii* in Kuusamo, dates being Aug. 10th, Aug. 17th and Aug. 29th. Inoculation times varied depending on the pathogen species, as the timing of their basidiospore maturation differs. Pathogen material was gathered from wild *V. vitis-idaea* ramets infected by *Exobasidium* fungi in the vicinity of the experimental sites. Spore suspensions for the inoculations were prepared and the inoculations were carried out the same day the pathogen material was collected. Spores from the surface of the symptomatic plant parts were scraped with the help of a knife and added to sterilized water. A 10 μl drop of spore suspension was administered using a pipette on each apical bud of each experimental ramet. The number of buds per ramet was on average 4.4 ± 0.2 (mean ± SE). The amount of spores in the suspensions ranged from 1.0×10^6/ml to 1.5×10^6/ml in *E. splendidum*, and from $1.2 \times 1.5 \times 10^5$/ml to $1.5 \times 1.5 \times 10^5$/ml in *E. vaccinii*. Immediately after inoculation, transparent plastic cover of approximately 1 mm in thickness was stretched above the plywood walls for 24 h to ensure that the spores were sticking on leaf surfaces and that the plants were protected from external factors such as rainfall and wind. Sheets were also assumed to maintain suitable temperature and humidity conditions for spore germination.

Disease frequencies were recorded in ramets during the following spring in 2002. In each ramet, the number of symptomatic and healthy shoots was recorded. Ramets showing symptoms of *Exobasidium* infections were classified as diseased ramets. To investigate the induction of resistance of *V. vitis-idaea* to the fungi, the treated ramets were reinoculated in autumn 2002. Reinoculation with *E. splendidum* was carried out on Aug. 31st and Sept. 8th in Kevo and on Aug. 30th and Sept. 5th in Kuusamo. Reinoculation with *E. vaccinii* was carried out on Aug. 9th, Aug. 17th and Aug. 29th. Healthy uninoculated control ramets were used as controls. Disease frequencies of the reinoculated ramets were recorded in spring 2003.

2.2. Gene expression analysis

For gene expression analyses, leaves pooled from ramets from Rovaniemi population were used in order to get enough material. Three plant samples were pooled for each analysis. The relative expression levels of flavonoid biosynthetic genes and the quantities of flavonoids and hydroxycinnamic acids were analyzed from three groups of leaves: 1) symptomatic leaves, i.e. leaves carrying symptoms of *E. splendidum* (SL), 2) healthy leaves of diseased ramets (HL), and 3) healthy leaves of uninoculated control ramets (C). Total RNA was isolated from leaf samples with the CTAB based method [14]. The expression of flavonoid biosynthesis genes, chalcone synthase (*CHS*), dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*) was studied with cDNA blotting method [15,16]. In addition, a fragment of a pathogenesis-related (PR 4) gene was isolated from *V. vitis-idaea* and its expression was studied in the same samples. The experiments were repeated over time. The same membrane used for the flavonoid and PR 4 gene expression was rehybridized with the GPD (glyceraldehyde-3-phosphate dehydrogenase) probe.