Enhanced Hypericin Production in *Hypericum perforatum* and *Hypericum pruinatum* in Response to Inoculation with Two Fungal Pathogens

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Abstract

ÇIRAK C., AKSOY H. M., AYAN A. K., SAĞLAM B., KEVSEROĞLU K. (2005): **Enhanced hypericin production in** *Hypericum perforatum* **and** *Hypericum pruinatum* **in response to inoculation with two fungal pathogens**. Plant Protect. Sci., **41**: 109–114.

Recent years has seen increasing interest in the genus *Hypericum* because it is the source of a variety of compounds and the biological activities of the genus are mainly derived from its hypericin content. The present study was conducted to determine whether this compound may be implicated as part of an inducible plant defense response in *H. perforatum* and *H. pruinatum*. Greenhouse-grown plants were inoculated with the plant pathogens *Phytophthora capsici* and *Diploceras hypericinum*. Hypericin levels of the *Hypericum* species increased significantly in response to inoculation with both pathogens. While up to now little effort has been made to determine whether hypericin is inducible by pathogen/herbivore attack or if it could play a role in plant defense, the present study indicates that hypericin is a component in inducible plant defense response of *H. perforatum* and *H. pruinatum*.

Keywords: *Hypericum perforatum*; *H. pruinatum*; *Phytophthora capsici*; *Diploceras hypericinum*; hypericin; plant-pathogen interaction

Hypericum species have been of great interest over many centuries and found use as healing agents due to their various medicinal properties (DIAS *et al.* 1998). The genus *Hypericum* of the Guttiferae is represented in Turkey by 89 species of which 43 are endemic and widespread, with the most abundant and well known being *H. perforatum* L. (DAVIS 1988). Commonly known as St. John's Wort, it is a herbaceous perennial plant that has received considerable interest worldwide. The chemical constituents of *H. perforatum* have been extensively investigated and it has been shown that the methanolic extract from the aerial parts of the plant typically contains hypericins, hyperforins and phenolic compounds, altogether good candidates for the activity of the drug. Of all *Hypericum* species, only a few have been reported to contain hypericin, one of which is *H. pruinatum* Boiss. and Bal. growing wild on rocky igneous slopes at very high altitudes in Turkey (AYAN *et al.* 2004).

The induction of secondary metabolites from different classes in response to biotic challenges has been documented in a number of plant species, and most phytotoxins, like hypericin, have

been considered to be involved in the chemical defense arsenal of plants against herbivores and plant pathogens (Arnason *et al.* 1983). In previous studies, hypericin was reported to increase in *H. perforatum* in response to challenge by some chemical elicitors such as mannan (Kirakosyan *et al.* 2000), cork pieces (Kirakosyan *et al.* 2001), jasmoninic acid (Walker *et al.* 2002) or the biotic factors *Colletotrichum gloesporioidies*, a plant pathogen causing an anthracnose disease on many crops (Sirvent & Gibson 2002), and *Spilosoma virginica, S. congrua* and *Spodoptera exigua*, generalist beetles for many plant species (Sirvent *et al.* 2003). In the present study, we aimed to determine whether this compound may be implicated as part of an inducible plant defense response in both *H. perforatum* and *H. pruinatum*. To achieve this objective, we used the fungal pathogens *Diploceras hypericinum* and *Phytophthora capsici. Diploceras hypericinum* was reported to cause leaf blight and stem dieback on *H. perforatum* (Putnam 2000), while *Phytophthora capsici* causes *Phytophthora* blight disease on many plants including cucumbers, squash, pumpkins, peppers, eggplants and tomatoes.

MATERIALS AND METHODS

Brief description of plant materials. *H. perforatum* and *H. pruinatum* plantlets were established in the greenhouse from 5 month old seeds collected on plants growing wild in the Gümüş district of Amasya, Turkey. Plant samples were identified by Dr. Hasan Korkmaz, Department of Biology, University of 19 Mayıs, Samsun, Turkey. Seeds were germinated in a float system, commonly used for seedling production of broad-leaves tobacco Burley and Flue-Cured-Virginia under a 16 h light:8 h dark cycle. Newly emerged seedlings were transferred to pots 30 cm in diameter and watered daily until they reached maturity, then three times a week.

Isolation and identification of *Diploceras hypericinum.* Diseased tissues from *H. perforatum* plants growing wild in the campus area of Ondokuz Mayıs University, Samsun, Turkey, were cut into small pieces, surfaced-sterilised with 1% NaOCl for 2 min and placed on potato dextrose agar (PDA) in Petri plates. The plates were incubated at 24–25°C under 16 h of fluorescent light and 8 h darkness for 2 to 3 days. Mycelial tips from the edge of a colony were transferred onto PDA

in Petri plates and the plates were incubated at 24–25°C for one week.

Identification of *Diploceras hypericinum* was based on the morphology of conidia under a light microscope at 400×. Conidia of *D. hypericinum* were cylindrical, a little curved, 3-septate with two shoots out of the ends, 15.4 × 3.5 μm.

Inoculation of plants. For conidia production of *Diploceras hypericinum*, 5mm-diameter disks were transferred from the margin of an advancing culture of the pathogen onto PDA in 9 cm-diameter Petri plates. The plates were incubated at 24–25°C under continuous white fluorescent light for one week. Conidial suspensions were prepared by adding sterile distilled water to each plate and the conidia were dislodged using a soft brush. The same procedure was followed to produce zoospore of *Phytophthora capsici* (kindly provided by the Ankara Agricultural Control Institute, Turkey, strain number Aksu 11) except for using of carrot agar as growing medium. Sporangial suspensions were prepared by adding sterile distilled water to each plate and the sporangia were dislodged using a soft brush. The suspensions were incubated at 5°C for 30 min to induce the release of zoospore from sporangia.

For inoculation, 5 ml inocula of both pathogens at 1×10^2, 1×10^4, 1×10^6 and 1×10^8 spores per ml were applied to three month old plantlets of *H. perforatum* and *H. pruinatum* using a custom-made spray tower with six replicates per dose and two independent replications. Control plantlets were treated with only sterile distilled water. The pots were incubated at 24–25°C, 90% humidity and a 16 h light:8 h dark cycle. Beginning on the 5th day after inoculation, seedlings were evaluated for development of lesions on stems and leaves.

Extraction and spectrophotometry of hypericin. A method described by Cellarova *et al.* (1994) was used to determine the "total hypericin" content of plant materials including whole plants, stems, leaves and reproductive parts for each species. Briefly, dried and homogenised plant material (1 g) was accurately weighed into a thimble and extracted with chloroform (100 ml) in a sonicator to remove their chlorophyll contents until samples appeared colourless and filtered. The repeated and prolonged extraction of the samples with chloroform was used to remove the chlorophyll since it interferes with the UV assay. The thimble was dried in vacuo (8 h) and re-extracted with methanol (100 ml) and the filtrate allowed

to evaporate in a water bath. This was followed by an addition of chloroform (10 ml), and the mixture was shaken. After drying in vacuo, the final samples were centrifuged (5000 rpm), the supernatants were discarded and the solid phase, containing hypericin and its derivatives, was dissolved in methanol and filtered.

Hypericin concentrations were determined in methanol extracts using the extinction coefficient of 7.18×10^4 at 592 nm (Kaya 1998). UV analysis was performed on a spectrophotometer (Shimadzu UV 3000). Three determinations were done for each sample and the mean value was calculated.

Statistical analysis. The data were objected to ANOVA, separately for each species, and differences between treatments were tested by the Duncan Multiple Range Test (Level of significance $P < 0.01$).
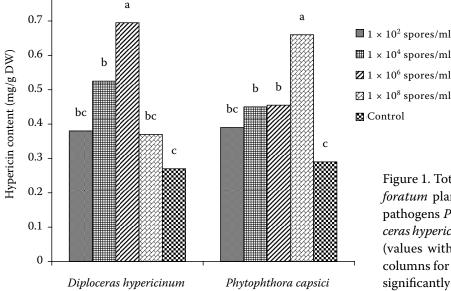
RESULTS

Symptoms of infections by *Phytophthora capsici* and *Diploceras hypericinum* began to appear within 7 days after inoculation and were similar for both *Hypericum* species. At the beginning of infection, *Phytophthora capsici* caused small, irregular to round, and water-soaked spots on leaves and stems. With time, the spots, from dark brown to black, enlarged and turned to leaf blotch. Although plants were infected by all doses of inoculum of both pathogens, plant mortality was observed depending on the concentration of spores. In *Diploceras hypericinum* there were many circular and expanding brown lesions on leaves and stems. Increased stem dieback accompanied higher doses of inoculum, and plant mortality was also observed with lower doses of inoculum when compared to inoculation with the other pathogen. Plantlets were harvested at 12 days after inoculation and assayed for hypericin content.

According to the results of ANOVA, inoculation with both pathogens had a significant effect on hypericin induction in the two *Hypericum* species ($P < 0.01$) (Figures 1 and 2). Challenge by *Phytophthora capsici* resulted in an increase in hypericin contents of both *H. perforatum* and *H. pruinatum* plantlets. It is interesting to note that the highest dose of spores 1×10^8 overwhelmed the plant defense of *H. pruinatum,* whereas the same dose brought about the highest hypericin level in *H. perforatum*, twice the level found in the non-inoculated control. In *H. pruinatum*, all doses of spores increased the hypericin content significantly when compared to the non-inoculated control, but the highest value (0.775 mg/g dry weight) was observed in plantlets inoculated with 1×10^6 spores per ml while moderate quantities of this component were detected in plantlets inoculated with other doses. In contrast to *H. pruinatum*, infection with *Phytophthora capsici* did not cause plant mortality at the tried doses in *H. perforatum* and the hypericin contents of the plantlets rose linearly as the level of infection rose with increasing concentration of the inoculum

Figure 1. Total hypericin contents of *H. perforatum* plants inoculated with the fungal pathogens *Phytophthora capsici* and *Diploceras hypericinum* at different doses of spores (values with different small letters within columns for each pathogen treatment differ significantly at the level of $P < 0.01$)
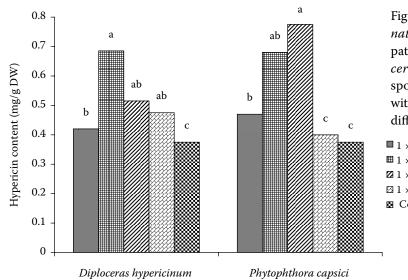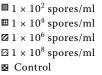
Figure 2. Total hypericin contents of *H. pruinatum* (b) plants inoculated with the fungal pathogens *Phytophthora capsici* and *Diploceras hypericinum* at different doses of spores (values with different small letters within columns for each pathogen treatment differ significantly at the level of $P < 0.01$)

- 1×10^2 spores/ml
- 1×10^4 spores/ml
- 1×10^6 spores/ml
- 1×10^8 spores/ml
- Control

(0.390, 0.450, 0.455 and 0.660 mg/g dry weight for 1×10^2, 1×10^4 1×10^6 and 1×10^8 spores per ml, respectively).

Similarly, levels of hypericin increased in response to inoculation with *Diploceras hypericinum* in both *Hypericum* species. Plantlets inoculated with 1×10^4 spores per ml gave the highest hypericin content in *H. pruinatum* (0.685 mg/g dry weight) and higher doses caused plant mortality similar to *Phytophthora capsici* which was mortal at the highest dose. Nevertheless, the hypericin contents of plantlets inoculated with the doses up to 1×10^4 spores per ml were higher than those of the non-inoculated control (0.515, 0.475 and 0.420 mg/g dry weight for 1×10^6, 1×10^8 and 1×10^2 spores per ml, respectively). For *H. perforatum,* 1×10^8 spores per ml was the only dose overwhelming plant defense and inoculation with this fungal pathogen in increasing doses elevated hypericin contents of plantlets significantly (0.695, 0.525 and 0.380 mg/g dry weight for 1×10^6, 1×10^4 and 1×10^2 spores per ml, respectively). The increase in hypericin content was more evident in *H. perforatum.*

DISCUSSION

Plant resistance may increase as a result of prior feeding by herbivores or infection by microbial pathogens (Karban & Myers 1989; Ajlan & Potter 1990). This plastic increase in resistance is termed induction, and often involves elevated levels of certain secondary metabolites (Monteroi *et al.* 2003; Lozovaya *et al.* 2004). What has been

unclear in the literature to date, however, is whether constitutively expressed secondary metabolites can be induced to higher levels under herbivore or pathogen attack and whether those levels serve defensive roles (Sirvent *et al.* 2003).

Enhanced levels of hypericin in *H. perforatum* in response to biotic challenge by plant pathogen (Sirvent & Gibson 2002) and herbivors (Sirvent *et al.* 2003), and the defensive role of hypericin in plant metabolism were reported in those two previous studies, but there is no third study. In the current study, we assessed whether the fungal pathogens *Phytophthora capsici* and *Diploceras hypericinum* could affect hypericin levels in *H. perforatum* and *H. pruinatum*. We found that the challenge by both pathogens caused the same trend in elevating levels of hypericin at the higher doses of inoculum in both *Hypericum* species. The results are in accordance with those of the two previous studies (Sirvent & Gibson 2002; Sirvent *et al.* 2003).

Although, to the authors knowledge, there is no published report on *H. pruinatum* to date, the chemistry of *H. perforatum* has been extensively investigated and it is known to contain a number of phytomedicinals including hypericin. Yet little effort has been dedicated to investigate whether this compound is inducible by pathogen/herbivore attack or if it could play a role in plant defense. Results from the present study indicate that hypericin is a component of inducible plant defense response of *H. perforatum* and *H. pruinatum,* and are supportive of the general acceptance for hypericin being not a phytoalexin but a phytoanticipin,

i.e. one of the antimicrobial compounds present in low quantities in plant tissues but also induced by pathogen or herbivor attack.

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Souhrn

Çirak C., Aksoy H. M., Ayan A. K., Sağlam B., Kevseroğlu K. (2005): **Zvýšená tvorba hypericinu rostlinami** *Hypericum perforatum* **a** *Hypericum pruinatum* **jako reakce na inokulaci dvěma houbovými patogeny**. Plant Protect. Sci., **41**: 109–114.

V posledních letech vzrůstal zájem o rod *Hypericum*, protože je zdrojem různých sloučenin. Biologické aktivity tohoto rodu jsou odvozovány hlavně z obsahu hypericinu. Cílem práce bylo zjistit, zda tato látka může být zahrnuta mezi indukované obranné reakce *H. perforatum* a *H. pruinatum*. Rostliny vypěstované ve skleníku byly inokulovány rostlinnými patogeny *Phytophthora capsici* a *Diploceras hypericinum*. V reakci na napadení oběma patogeny se obsah hypericinu v druzích rodu *Hypericum* významně zvýšil. Dosud bylo jen málo úsilí věnováno

zkoumání, zda tvorba hypericinu je indukována napadením patogeny či škůdci, nebo zda hyperisin může hrát určitou roli v obranných reakcích. Práce ukazuje, že hypericin je součástí indukovaných obranných reakcí *H. perforatum* a *H. pruinatum*.

Klíčová slova: *Hypericum perforatum*; *H. pruinatum*; *Phytophthora capsici*; *Diploceras hypericinum*; hypericin, interakce rostlina–patogen

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