Differential Phenolic Accumulation in Two *Hypericum* Species in Response to Inoculation with *Diploceras hypericinum* and *Pseudomonas putida*

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Abstract

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The genus *Hypericum* L. (St. John's-wort, Hypericaceae) has received scientific interest in recent years, because it is a source of a variety of bioactive compounds including the phenolics. We determine whether the typical phenolic constituents of *Hypericum* plants, namely chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine, and quercetine, may be implicated as part of an inducible plant defence response in two St. John's-wort species, *Hypericum perforatum* L. and *Hypericum triquetrifolium* Turra. To achieve this objective, greenhouse-grown plantlets were inoculated with the fungal pathogen *Diploceras hypericinum* and the plant growth promoting bacterium *Pseudonomas putida*. Phenolic compounds levels of the *Hypericum* plantlets increased significantly in response to inoculation with both organisms. So far, little effort has been dedicated to investigate whether phenolic compounds are inducible by pathogen/herbivore attack or if they could play a role in plant defence. Results from the study indicate that the phenolic compounds investigated could be involved in the plant defence system and implicated as part of an inducible plant defence response in both St. John's Wort species.

Keywords: fungal pathogen; *Hypericum perforatum*; *Hypericum triquetrifolium*; phenolic compounds; plant defence; bacterial infection

Hypericum perforatum L. is a well-known traditional medicinal plant that has been used for centuries for the treatment of several diseases, such as skin lesions, eczema, burns and microbial, inflammatory, and psychological disorders (Sanchez-Mateo *et al.* 2002). The crude extract of *H. perforatum* is now widely used in Europe as a drug for the treatment of depression. Proven photodynamic, antiviral, antiretroviral, and antitumor effects of *Hypericum* extracts also suggest using this plant in in the case of Acquired Immune Deficiency Syndrome (AIDS) and cancer treatments (Guedes & Eriksson 2005). *Hypericum triquetrifolium* Turra is another widespread species of *Hypericum* genus. It has traditionally been used in the treatment of burns and gastrointestinal disease in Turkish folk medicine (Baytop 1999). Results from recent studies reporting anti-inflammatory (Ozturk *et al.* 2002), antibacterial (Pistelli *et al.* 2005), antifungal (Fraternalea *et al.* 2006), antioxidant, and cytotoxic (Conforti *et al.* 2007) activities of *H. triquetrifolium* sign out the great potential of this species as a promising medicinal plant.

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Chemistry of *Hypericum* genus has been extensively investigated and it has been shown that methanolic extract from the aerial parts of *Hypericum* plants typically contains hypericins, hyperforins, and several phenolic compounds, altogether good candidates for the activity of the drug (Patocka 2003). Especially phenolics such as flavonoids are well-known antioxidant agents, some of which exert therapeutic antiviral, antiallergenic, antiplatelet, and anti-inflammatory effects (Russo *et al.* 2000). Even though two of the principal ingredients in *Hypericum* extracts are hypericin and hyperforin, it is postulated that the effects of St. John's-wort are a result of the synergistic actions of its many constituents (Gastpar & Zeller 2005). Some of the biological activities, attributed to phenolic compounds of *Hypericum* plants have also been reported to be related with the plant defence mechanisms (Conceicao *et al.* 2006).

Plants have developed a broad range of strategies to protect themselves against biotic and abiotic stress (Carrasco *et al.* 2001). The first step of defence is based on the activation of pre-existing components, resulting in overproduction of toxic compounds. Likewise, induction of secondary metabolites from different classes has been documented in response to biotic challenges in a number of plant species and most phenolics have been considered to be involved in the chemical defence arsenal of plants against herbivores and plant pathogens (Arnason *et al.* 1983; Bennett & Wallsgrove 1994). In previous studies, several phenolics as mangiferin, mangostin, luteolin, hyperoside, quercetine, isoquercitrine, and their derivatives were reported to enhance in *H. perforatum* in response to challenge by some chemical elicitors such as methyl jasmonate and salicylic acid or the biotic factor *Colletotrichum gloesporioidies*, a plant pathogen, causing an anthracnose disease on many crops (Conceicao *et al.* 2006). Total hypericin levels were also reported to increase significantly in the case of artificial contamination with *Phytophthora capsici* and *Diploceras hypericinum* in greenhouse-grown *H. perforatum* and *H. pruinatum* Boiss. & Bal. planlets (Çirak *et al.* 2005).

In the present study, we aimed to determine whether the phenolic compounds, namely chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine, and quercetine may be implicated as a part of inducible plant defence response in two St. John's-wort species, namely *H. perforatum* and *H. triquetrifolium*. To achieve this objective, we used fungal pathogen *Diploceras hypericinum* and plant growth promoting bacterium *Pseudonomas putida*.

MATERIAL AND METHODS

Brief description of plant materials. *H. perforatum* and *H. triquetrifolium* plantlets were established by 5-month-old seeds, collected from the plants, growing wild in Samsun and Tokat provinces of Turkey respectively, in the greenhouse. Plant samples were identified by Dr. Hasan Korkmaz, Department of Biology, University of Ondokuz Mayis, Samsun, Turkey. Seeds were germinated in float system, commonly used for seedling production of broad-leaves tobaccos 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*. Fungus was isolated from diseased tissues of *H. perforatum* plants which were cut into small pieces and surface-sterilised with 1% NaOCl for 2 min, then 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 2–3 days. Mycelium tips from edge of the growing colony were transferred onto PDA in Petri plates and the plates were incubated at 24–25°C for one week.

The cultures of the fungus were identified to the morphology of conidia by using light microscope at 400×. Conidia of *D. hypericinum* were cylindrical, a little curved, 3-septate with two shoots 15.4 × 3.5 µm out of the ends.

Isolation and identification of *Pseudomonas putida*. A total of 40 soil samples were collected from tomato greenhouses in Samsun province. Relative to the size of greenhouse, 3–12 samples were randomly collected from 0–20 cm depth and then mixed. A sub-sample of 1 kg of soil was then taken per greenhouse and stored in sterile, polyethylene bags at 4°C for 2–3 days before processing.

Stored soil samples were used to isolate fluorescent *Pseudomonas* isolates. Firstly, each soil sample was sieved through a 1-mm diameter mesh sieve, mixed at a ratio of 1 : 10 with sterile, distilled water, shaken thoroughly on a rotary shaker at 150 *g* at 24–26°C for 60 min, and serial dilutions (10-2-10-4) were prepared. Diluted samples were placed on King B Agar (KBA) and incubated at 24–26°C for 24–48 hours. Identification of bacterial isolates was based on colony morphology and fluorescent character, according to the standard diagnostic methods (Lelliott & Stead 1987; Kiewnick & Sands 2001). One of the fluorescent *Pseudomonas* isolates, FPDa5, was selected

for this study after a preliminary study. It was later identified by using the computer assisted microbial identification system (MIS) which employs gas-liquid chromatographic analysis of bacterial fatty acids.

The bacterial isolate was also re-isolated from the *H. perforatum*. For this purpose, the plant tissues were surface sterilised in 1% sodium hypochlorite (NaOCl), and the samples were then washed three times in sterile distilled water, and transferred aseptically into a sterile mortar and macerated with a sterile pestle. The macerate was placed in 10 ml of sterile distilled water. The suspensions were then diluted to 10^4 and 10^6. The dilutions were streaked on nutrient King B medium and the plates were incubated at 24–26°C for 24–48 hours. This pure isolate was later identified using MIS and appeared to be identical to the isolate described previously.

Inoculation of organisms. For conidia production of *Diploceras hypericinum*, 5 mm diameter disks were transferred from the margin of an advancing culture of pathogen onto PDA in 9 cm diameter Petri plates. The plates were incubated at 24–26°C under continuous 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.

For inoculation, 5 ml inoculums of *Diploceras hypericinum* and *Pseudomonas putida* at 1×10^2, 1×10^4, 1×10^6, and 1×10^8 spores per ml were applied to each 3-month-old plantlet of *H. perforatum* and *H. triquetrifolium* 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 with 16 h light : 8 h dark cycle. Beginning the 5^{th} day after inoculation, seedlings were evaluated for development of lesions on stems and leaves, caused by *Diploceras hypericinum*. Aerial parts of five pots for each dose of inoculums were harvested at days 4, 8, and 12 after inoculation. The plant material was assayed for chemical contents by HPLC after drying at room temperature.

Preparation of plant extracts and HPLC analysis. Air-dried plant material was mechanically ground with a laboratory mill to obtain a homogeneous drug powder. Samples of about 0.5 g (weighed with 0.0001 g precision) were extracted in 50 ml of 100% methanol by ultrasonication at 40°C for 30 min in a Sonorex Super model RK 225H ultrasonic bath (Bandelin Electronic GmbH, Berlin, Germany). The prepared extracts were filtered through a membrane filter with the pore size of 0.22 µm (Carl Roth GmbH, Karlsruhe, Germany) and kept in a refrigerator until analysis no longer than for 3 hours.

A Shimadzu Prominence LC-20A (Shimadzu Europa GmbH, Duisburg, Germany) chromatographic system equipped with two LC-20AD model pumps, a SIL-20AC auto-injector, a CTO-20AC thermostat, and a SPD-M20A detector was used for the HPLC analysis. Separation of all compounds was carried out using an YMC Pack Pro-C18 (YMC Europe GmbH, Dinslaken, Germany) column (150 mm × 4 mm *i.d.*; 3 µm particle sizes) with 10 mm guard-precolumn. The mobile phase consists of solvent A (water containing 0.1% trifluoroacetic acid – TFA) and solvent B (acetonitrile containing 0.1% TFA). The following binary gradient elution program was used: 0–1 min (B 5 → 5%), 1–14 min (B 5 → 20%), 14–20 min (B 20 → 80%), 20–30 min (B 80 → 100%), 30–39 min (B 100 → 100%), 39–39.5 min (B 100 → 5%), 39.5–45 min (B 5 → 5%). The mobile phase was delivered with a flow rate of 1.0 ml/min; volume of extract injected was 10 µl. Detection was performed at 210–790 nm wave length range with a constant column temperature of 40°C.

The determination of eluted compounds was performed according to a modified method for *Hyperici herba* described in European Pharmacopoeia (2010) monograph. Phenolic compounds were identified on the basis of their retention time by comparison with retention time of reference standards and also confirmed with UV spectra of reference standards in the wavelength range of 210–790 nm. The HPLC chromatogram of *H. perforatum* methanolic extract is shown in Figure 1.

The quantification of detected compounds was achieved by using the external standard method at the maximal absorption on the UV spectra of corresponding compounds: chlorogenic acid (325 nm wavelength), rutin (353 nm), hyperoside (353 nm), isoquercetine (353 nm), quercitrine (347 nm), and quercetine (368 nm). A six-point calibration curves were obtained with pure standards dissolved in MeOH in the concentration range of 0.2–110 µg/ml. All calibration cures showed good linear correlation ($r^2 > 0.999$) within the test range. All solvents and standards of reference substances were of HPLC grade and were purchased from Roth Chemical Company (Karlsruhe, Germany). The analyses were done in triplicate for each sample.

Data analysis. The data for chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine, and quercetine contents of plant materials inoculated with *Diploceras hypericinum* and *Pseudomonas putida* in

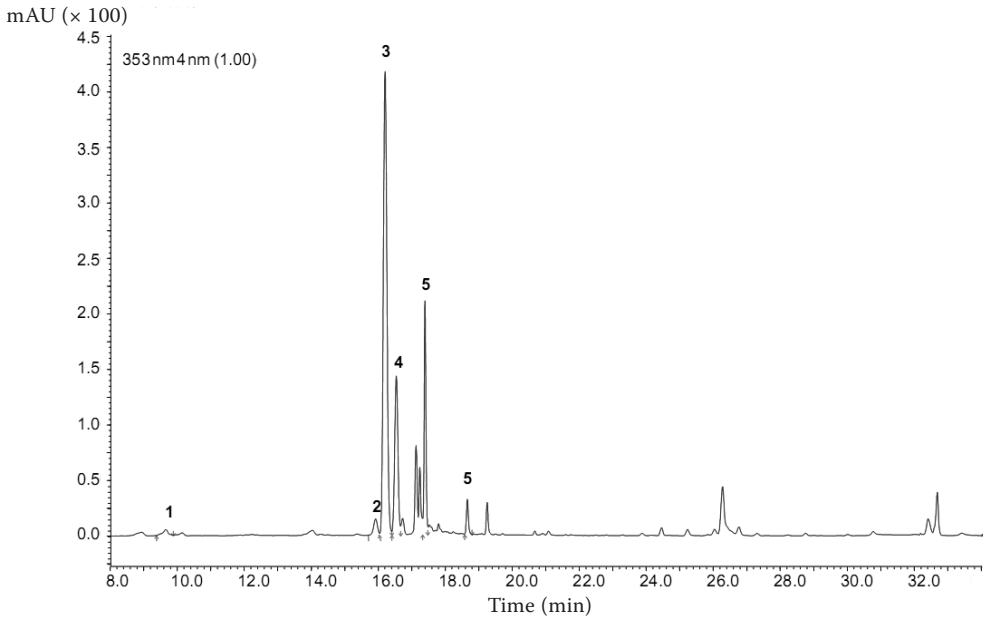

Figure 1. HPLC chromatogram of methanolic extract of *Hypericum perforatum* infected with *Diploceras hypericinum*

Peaks identified at UV wave length 353 nm: 1 – chlorogenic acid (Rt 9.636 min); 2 – rutin (Rt 15.911 min); 3 – hyperoside (Rt 16.180 min); 4 – isoquercetin (Rt 16.516 min); 5 – quercitrin (Rt 17.371 min); 6 – quercetin (Rt 18.634 min)

different levels were objected to ANOVA, separately for each species and organism. Differences among treatments were tested using Duncan Multiple Range Test (significance level $P < 0.01$).

RESULTS

Symptoms of pathogenicity in response to infections by *Diploceras hypericinum* began to appear within five days of inoculation and were similar for both *Hypericum* species (Figure 2). At the beginning of inoculation, there were many of circular and expanding brown lesions on leaves and stems. Increased stem dieback accompanied higher doses of inoculum and plant mortality was observed at the dose of 1×10^8 spores per ml in several pots of *H. perforatum*. Likewise this plant pathogen was reported to cause leaf blight and stem dieback on *H. perforatum* (Putnam 2000) and to be widespread in Turkey (Aksoy & Çirak 2005).

Challenge by *Diploceras hypericinum* resulted in a significant increase in phenolic contents of both *H. perforatum* and *H. triquetrifolium* ($P < 0.01$). Phenolic contents of plantlets also increased significantly with advancing of infection and the plantlets, harvested 12 days after inoculation, produced higher

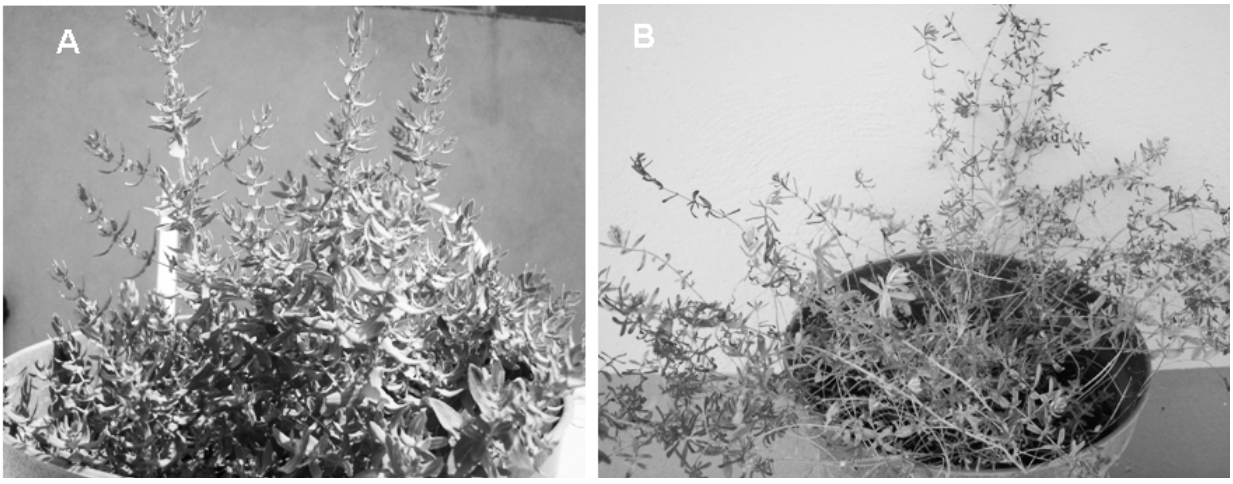

Figure 2. A view of control (A) and infected by *Diploceras hypericinum* at dose of 1×10^8 (B) *Hypericum perforatum* plantlets

contents of detected chemicals at all tried doses of spores (Figures 3 and 4). For *H. perforatum*, in all doses of spores the chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine, and quercetine contents increased significantly during infection when compared to non-inoculated control and the plantlets, harvested on day 12 after inoculation, produced the highest value of all evaluated phenolics. The highest values were observed in plantlets inoculated with 1×10^4 spores per ml for chlorogenic acid (6.565 mg/g dry weight – DW), rutin (0.24 mg/g DW), and quercitrine (1.55 mg/g DW), 1×10^8 spores per ml for hyperoside (10.42 mg/g DW), isoquercetine (9.24 mg/g DW), and quercetine (2.13 mg/g DW) on day 12 after inoculation, while moderate quantities of this component were established in plantlets, inoculated with other doses. In contrast to *H. perforatum*, induction with *Diploceras hypericinum* did not overwhelm plant defence and no plant mortality was observed in the tried doses in *H. triquetrifolium*. Phenolic contents of the plantlets elevated linearly with pathogen attack in increasing doses during the course of infection. Plantlets, inoculated with 1×10^4 spores per ml produced the highest content of chlorogenic acid (6.31 mg/g DW) and rutin (1.09 mg/g DW) while the highest hyperoside (1.67 mg/g DW), isoquercetine (7.52 mg/g DW), quercitrine (7.81 mg/g DW), and quercetine (2.01 mg/g DW) contents were produced by the plantlets inoculated with 1×10^8 spores per ml on day 12 after inoculation.

In a similar way of fungal pathogenic infection, levels of the phenolic compounds tested increased significantly in response to inoculation with *Pseudomonas putida* during the course of infection in both *Hypericum* species ($P < 0.01$). Plantlets of *H. perforatum*, inoculated with 1×10^4 spores per ml, produced the highest content of rutin (0.27 mg/g DW) and quercitrine (1.46 mg/g DW) while the highest accumulation

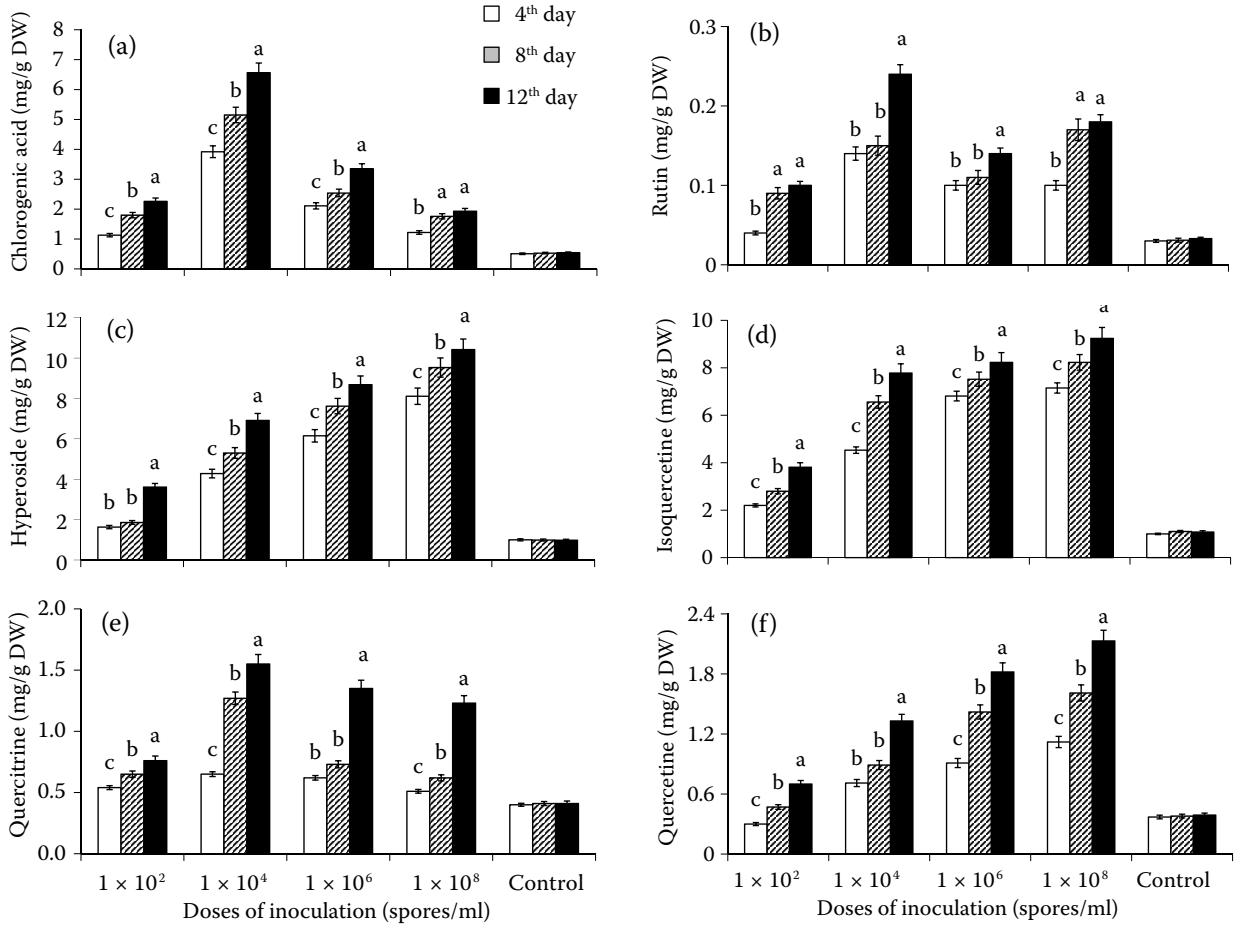

Figure 3. Chlorogenic acid (a), rutin (b), hyperoside (c), isoquercetine (d), quercitrine (e), and quercetine (f) contents of *Hypericum perforatum* plantlets inoculated with *Diploceras hypericinum* at different doses of spores on days 4, 8, and 12 after inoculation

[a–c]values with different small letters within columns for each dose of inoculum differ significantly at the level of $P < 0.01$; bars are ± SE

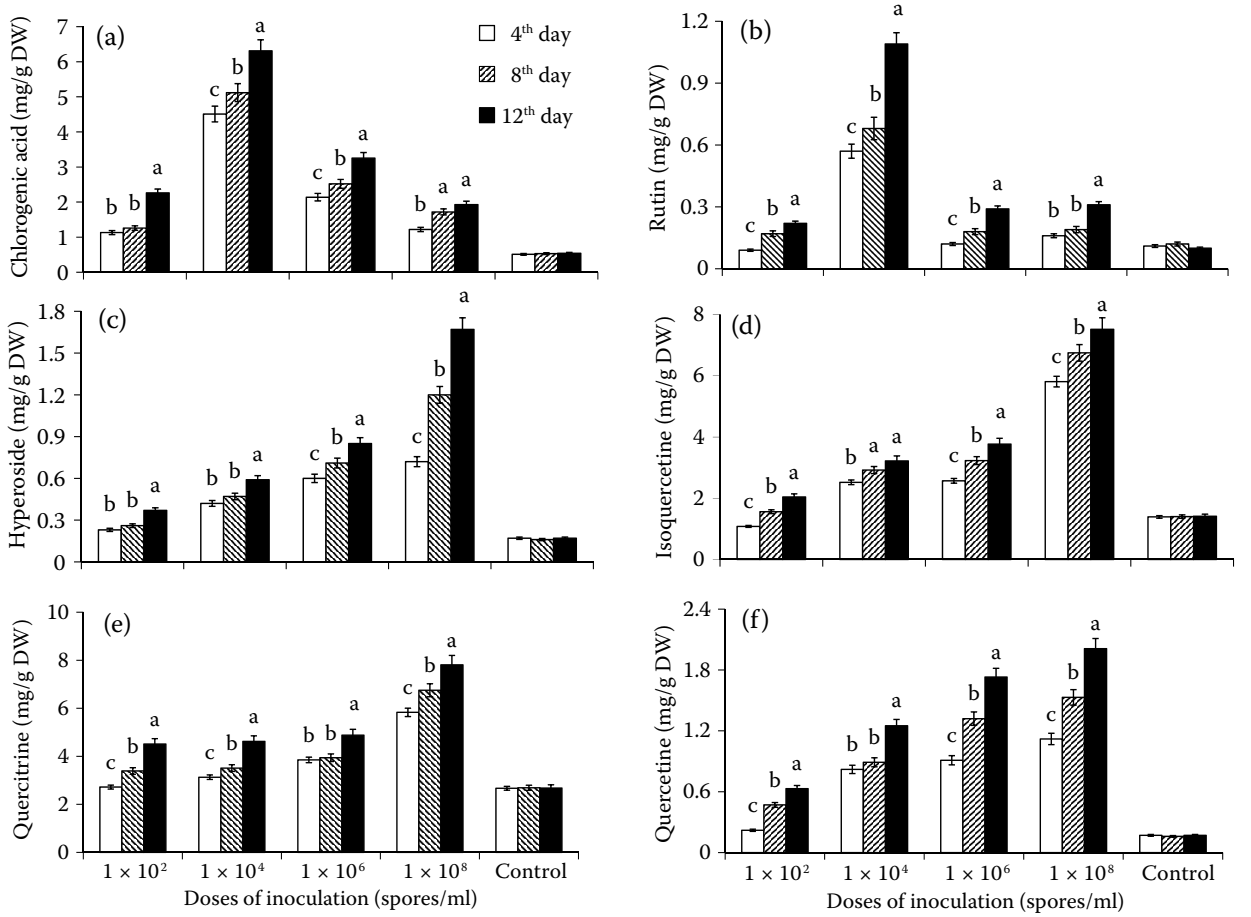

Figure 4. Chlorogenic acid (a), rutin (b), hyperoside (c), isoquercetine (d), quercitrine (e), and quercetine (f) contents of *Hypericum triquetrifolium* plantlets inoculated with *Diploceras hypericinum* at different doses of spores on days 4, 8, and 12 after inoculation

[a–c]values with different small letters within columns for each dose of inoculum differ significantly at the level of $P < 0.01$; bars are ± SE

levels were observed in plantlets inoculated with 1×10^6 spores per ml for chlorogenic acid (4.47 mg/g DW) and isoquercetine (4.62 mg/g DW), 1×10^8 spores per ml for hyperoside (5.52 mg/g DW) and quercetine (2.22 mg/g DW) on day 12 after inoculation (Figure 5). In *H. triquetrifolium*, lower inoculation doses brought about higher accumulation levels of phenolics when compared to *H. perforatum*. Plantlets, inoculated with 1×10^4 spores per ml, produced the highest content of rutin (1.02 mg/g DW), hyperoside (1.32 mg/g DW), and quercitrine (8.47 mg/g DW) while inoculation with 1×10^6 spores per ml resulted in the highest chlorogenic acid (4.12 mg/g DW), isoquercetine (5.39 mg/g DW), and quercetine (2.04 mg/g DW) accumulations on day 12 after inoculation (Figure 6).

DISCUSSION

Plant resistance may increase as a result of prior feeding by herbivores or infection by microbial pathogens (Ajlan & Potter 1990; Bruce & Pickett 2007). This plastic increase of resistance is termed induction, and often involves elevated levels of certain secondary metabolites (Monterio *et al.* 2003; Lozovaya *et al.* 2004). Among the inducible secondary metabolites, plant phenolics have a distinct role in plant defence and their role in resistance against fungi is more dynamic than their role against insects or any other attacking organism (Bennett & Wallsgrove 1994). What has been unclear in the literature to date, however, is whether constitutively expressed secondary metabolites could be induced to higher levels under herbivore or pathogen attack and whether those levels serve defensive roles (Sirvent *et al.* 2003). Conceicao *et al.* (2006) and Gadzovska *et al.* (2007) reported enhancing levels of several phenolics as mangiferin, mangostin, luteolin, hyperoside, quercetine, isoquercitrine, and their derivatives as defensive plant chemicals in response to challenge by some chemical elicitors such as jas-

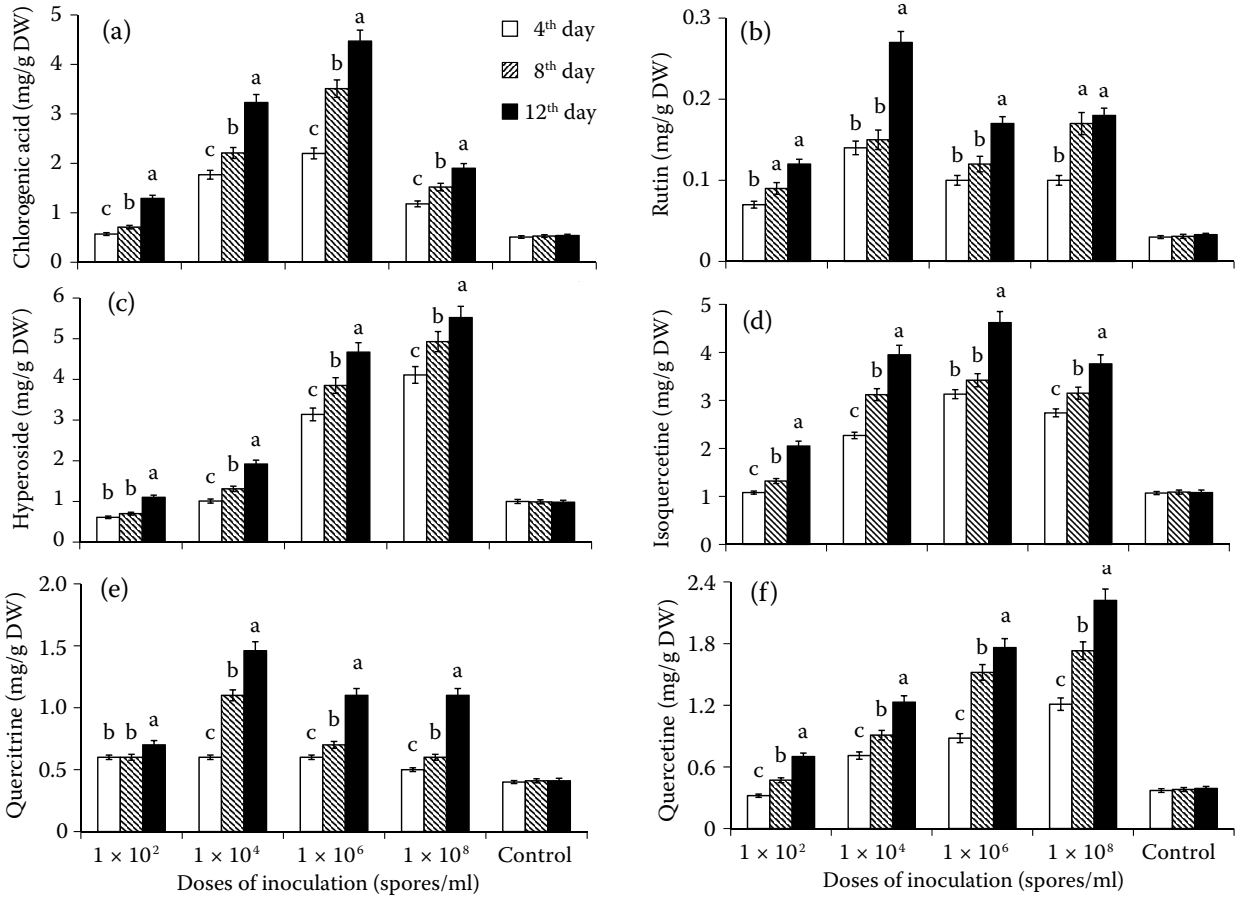

Figure 5. Chlorogenic acid (a), rutin (b), hyperoside (c), isoquercetine (d), quercitrine (e), and quercetine (f) contents of *Hypericum perforatum* plantlets inoculated with *Pseudomonas putida* at different doses of spores on days 4, 8, and 12 after inoculation

[a–c]values with different small letters within columns for each dose of inoculum differ significantly at the level of $P < 0.01$; bars are ± SE

monic acid, methyl-jasmonate, and salicylic acid or the fungal pathogen *Colletotrichum gloesporioidies*. For *Hypericum* plants, Crockett and Boeve (2011) reported a distinct role of several flavonoid glycosides as rutin, hyperoside, isoquercitrine, and quercitrine in plant defence and enhancing levels of the phenolic compounds in wild growing *H. perforatum* and *H. hirsutum* plants in response to feeding by larvae of the sawfly *Tenthredo zonula*. Germ *et al.* (2010) pointed out defensive roles of flavonoids and tannins in plant metabolism by observing the increased leaf concentrations of flavonoids in *H. perforatum* plants exposed to high level of UV-B radiation.

In the current study, we assessed whether the fungal pathogen *Diploceras hypericinum* could affect levels of phenolics as chlorogenic acid, rutin, hyperoside, quercetine, isoquercetine, and quercitrine in *H. perforatum* and *H. triquetrifolium*. It was observed that challenge by the fungal pathogen significantly enhanced phenolic accumulation, especially at the higher doses of inoculum in both *Hypericum* species. Similarly, Soylu (2006) examined interactions in leaves of *Arabidopsis* ecotype Col-5 during compatible and incompatible interactions with isogenic pairs of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and pv. *phaseolicola* (*Pph*) 1448A strains. The author reported that accumulation of phenolics may be involved in resistance against bacterial pathogens. Petkovsek *et al.* (2009) reported 7.6 times more hydroxycinnamic acids, 2.6 times more flavan-3-ols, and up to 2.9 times higher values of flavanols in leaf and fruit tissues infected with fungal pathogen *Venturia inaequalis* when compared to healthy tissues in two apple cultivars. Besides, the content level of total phenolics in the infected tissue was 1.3–2.4 times higher than in the healthy leaves and fruit. Lopez-Gresa *et al.* (2011) reported a rapid accumulation of the flavonoid rutin and the phenylpropane chlorogenic acid as well as phytoalexin hydroxycinnamic acid amides (HCAA) of noradrenaline and octopamine

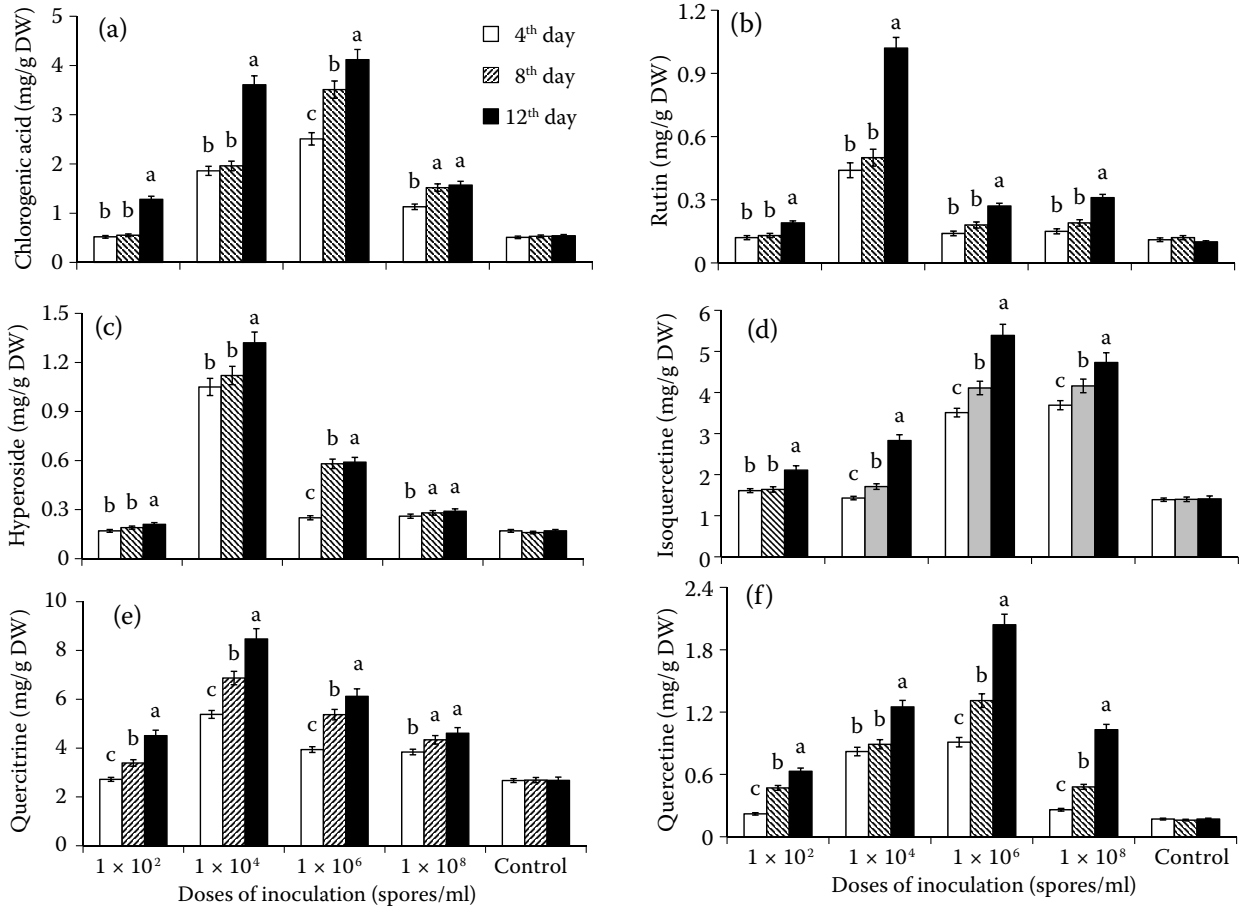

Figure 6. Chlorogenic acid (a), rutin (b), hyperoside (c), isoquercetine (d), quercitrine (e), and quercetine (f) contents of *Hypericum triquetrifolium* plantlets inoculated with *Pseudomonas putida* at different doses of spores on days 4, 8, and 12 after inoculation

[a–c]values with different small letters within columns for each dose of inoculum differ significantly at the level of $P < 0.01$; bars are ± SE

in tomato plants infected with the bacterial pathogen *Pseudomonas syringae*. Kröner *et al.* (2012) treated five potato cultivars spanning a range of quantitative resistance with a concentrated culture filtrate (CCF) of the fungal pathogen *Phytophthora infestans* and reported that CCF induced differential accumulation of major phenolics, namely chlorogenic acid, phenolamides, rutin, and nicotiflorin among cultivars. Based on the results, authors concluded that phenolics are inducible chemicals in response to pathogen attacks and have important role in plant defence system.

Pseudomonas putida is a gram negative, rod-shaped, saprophytic soil bacterium (Anzai *et al.* 2000). The bacterium has been considered as one of the plant growth-promoting organisms. It can stimulate plant growth by enhancing the plant's photosynthetic capacity by increasing tolerance to abiotic stress or by suppressing plant diseases (van der Ent *et al.* 2009). *Pseudomonas putida* has the demonstrated potential bio control properties, as an effective antagonist of damping off diseases such as *Pythium* and *Fusarium* (Amer & Utkhede 2000; Validov *et al.* 2007). The disease suppressive activity of plant growth-promoting organisms including *Pseudomonas putida* is exerted by eliciting a plant-mediated systemic resistance response. The systemic resistance triggered by beneficial microorganisms is generally related to overproduction of plant secondary metabolites from different classes and endows a broad-spectrum resistance to host plants that is effective against different types of attackers (van Wees *et al.* 2008). Our results in the present study confirmed this phenomenon. Inoculation with *Pseudomonas putida* especially in moderate doses of spores resulted in a significant increase in phenolic contents of both *H. perforatum* and *H. triquetrifolium*.

In conclusion, the phenolic constituents of *Hypericum* plants, namely chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine, and quercetine

were strongly induced by inoculation with both the fungal pathogen *Diploceras hypericinum* and plant growth promoting bacterium *Pseudonomas putida* in two St. John's-wort species, *H. perforatum* and *H. triquetrifolium*. The chemicals could be involved in the plant defence system and implicated as part of an inducible plant defence response. The results suggest that the phenolic compounds are not phytoalexin but phytoanticipin, antimicrobial compounds that are present in moderate quantities in plant tissues and are also induced by pathogen or herbivore attack. Further studies are needed to validate these conclusions.

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