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The *Ex Situ* Comparison of Two Improved St. John's wort (*Hypericum perforatum*) Cultivars with an Iranian Wild Population

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Abstract

This research is part of an ongoing selection and breeding effort to target Iranian genotypes of *Hypericum perforatum* with the potential to produce higher amounts of desired secondary metabolites and greater resistance to fungal pathogens. There is a significant interest in the development of such cultivars to supply materials to the local pharmaceutical industries. For this reason, two improved cultivars of *H. perforatum* ("Gold" and "Veperikon") were compared with a wild Iranian population (Ardabile population) under common garden conditions in Iran. Plants were cultivated from seed in a greenhouse and seedlings were transplanted after one month to the field plots. The statistical design of this study was a Randomized Complete Block Design with three replications. During the period of full flowering, selected phenological (number of days to flowering), morphological (plant height, mean leaf area, number of black glands/leaf) and chemical (hypericin and pseudohypericin content) characteristics were assessed. Our observations were that the "Veperikon" cultivar is very sensitive to soil-borne diseases. All transplanted seedlings were infected by the plant pathogenic fungus *Colletotrichum gloeosporioides*, which caused necrosis of the whole plant. Both the "Gold" cultivar and plants from the wild population persisted despite mild infections with *C. gloeosporioides* and produced flowering shoots at both the first and second years after cultivation. The "Gold" cultivar was superior to the Ardabile population in terms of phenological and morphological characteristics. The average naphthodianthrone content (% dry weight of tissue) for the wild Iranian population was 0.04(±0.01)%, but for the "Gold" cultivar, 0.33(±0.43)%. These data indicate that selection and directed cultivation of Iranian *H. perforatum* plants can result in plants with improved morphological, phenological and chemical characteristics.

Keywords

Hypericaceae; breeding lines; phenology; morphology; naphthodianthrone; hypericin; *Colletotrichum gloeosporioides*

INTRODUCTION

Hypericum perforatum L. (Common St. John's wort) is an important medicinal herb with a long history of traditional use. The aerial flowering portions of the plant are prepared as a decoction or infusion and taken internally for sedative or tonic purposes or applied externally as a poultice. Flowers and fruits are prepared as an oil-infusion for the treatment of sciatica, neuralgia and to speed wound-healing (Müller, 2005). Formulations based on the

flowers are currently sold to treat mild to moderate depression, particularly in Western Europe and North America. A tea made from the leaves and flowers is used by the Turkmen ethnic group in the Golestan and Khorasan Provinces in Iran as a sedative and relaxant (Ghorbani, 2005). Components that are thought to contribute to the antidepressant and relaxant activity include naphthodianthrone (e.g. hypericin and pseudohypericin), flavonol glycosides (e.g. isoquercitrin and hyperoside), biflavonoids (e.g. amentoflavone), and phloroglucinol derivatives (e.g. hyperforin and adhyperforin) (Fig. 1) (Nahrstedt and Butterweck, 1997; Hölzl and Petersen, 2003; Avato, 2005; Rammal *et al.*, 2009). In *Hypericum*, exudate-containing glands and canals that are found variously on the stems, leaves, sepals, petals and the anther connective appear either translucent (pale yellow to amber in color) or dark (red to nearly black) (Fig. 2).

The steadily increasing market interest in antidepressants is one of the factors that have contributed to the economic value of this plant to the herbal industry. In 2004, *H. perforatum* products accounted for nearly 13% of all European herbal product sales and in Germany were valued at ca. €70 million (Bäcker *et al.*, 2006). It was ranked among the top ten best-selling herbal dietary supplements in the United States in 2008, with sales reaching ca. 8.2 million USD (ABC, 2008). Two of the top-selling St. John's wort supplements in Iran are the products Hypiran® (liquid formulation, produced by the Pursina Pharmaceutical Company, Karaj, Iran) and Perforan® (tablet formulation, produced by the Goldaru Company, Isfahan, Iran).

The economic value of this plant has stimulated the development of programs aimed toward the selection, breeding and cultivation of improved cultivars of *H. perforatum* in Europe and North America (Gaudin *et al.*, 2003). The primary objective of such breeding programs is to target plants with particular genotypes, whose phenotypic expression results in the display of desirable phenological, morphological, phytochemical and pathogenic-resistance characteristics. A secondary goal, but one which has equal importance, is to stabilize the expression of these characteristics under field cultivation conditions. Much work has been conducted in the research area of *in vitro* tissue culture of *H. perforatum*, as well as related species, with the aim of selecting particular genotypes expression higher amounts of bioactive metabolites (ellárová, 2003; Al Rifaae *et al.*, 2010). In addition, field trials have been conducted to examine the effects of drought and fertilizer use on the production of bioactive secondary metabolites. Water deficit has been demonstrated to negatively affects yield but increases hyperforin content, while various nitrogen:phosphorus fertilizer treatments (optimum: 125 Kg nitrogen/hectare:50 Kg P₂O₅/hectare) improved both yield, flavonoid and hypericin content in *H. perforatum* (Azizi and Dias, 2004; Azizi and Omidbaigi, 2002; Zobayed *et al.*, 2005).

Numerous breeding lines of *H. perforatum* have been developed during the past thirty years, of which "Hyperimed," "Elixir" and "Topas" are perhaps the best known, and which were selected on the basis of their lush growth, abundance of flowers, and more or less stable production of desired secondary metabolites (particularly hypericin). "Topas," formerly the most widely cultivated variety of *H. perforatum* displayed sensitivity to the fungal plant pathogen *Colletotrichum gloeosporioides* Penz. (anthracnose), which emerged as a significant problem for growers as the frequency and density of *H. perforatum* plantings in European countries increased, and recently new cultivars with less anthracnose susceptibility have emerged on the European market (Gaudin *et al.*, 2003).

These types of programs are still at a comparably early stage in Iran, but a significant interest exists in the development of breeding lines with lower susceptibility to plant pathogens, including *C. gloeosporioides*, and higher levels of the naphthodianthrone. Towards this goal, the morphological, phenological and chemical characteristics of two

improved cultivars of *H. perforatum* L. (“Gold” and “Veperikon”) were compared with those of a wild Iranian population (Ardabile population), grown under common garden conditions in Iran.

MATERIALS AND METHODS

Plant Materials, Experimental Design and Treatments

St. John’s wort (*H. perforatum*) seeds of “Gold”, “Veperikon” and an Iranian native population (Ardabile) were soaked in tap water overnight, then sown on moistened potting soil at the Department of Horticulture in Mashhad, Iran. Vouchers from these plants have been deposited at the herbarium of the Ferdowsi University of Mashhad (FUMH 2529). Seed germination was observed after 10–12 days, and plants were allowed to attain a height of 20–25 cm before being transplanted to a field plot, spaced 30×40 cm apart. The statistical design of this study was a Randomized Complete Block Design with three replications. During the flowering period, characters of plant height, days from transplanting to shoot formation, mean leaf area, and number of black glands/leaf were measured for 12 plants (3 replicates of 4 plants per sample) per population. The upper 25 cm of the flowering stem was harvested and dried at 25°C in the dark before being prepared for phytochemical analysis.

Analysis for Naphthodianthrone

1 g powdered plant material per sample was extracted with 10 ml methanol by sonication for 10 min at the Department of Pharmacognosy in Graz, Austria. After centrifugation, the extracts were dried down using a rotovapor and stored at –20°C until analysis. Approximately 10 mg of each extract (see Table 2 for exact weights) was placed in a volumetric flask, brought up to 5 ml, 1 ml of which was then filtered through a 0.45 µm Nylaflo filter (Gelman, Ann Arbor, MI, USA) prior to injection in triplicate (5 µl sample injection). An amount of 1.50 mg of each standard (hypericin and pseudohypericin) was placed in a 2 ml volumetric flask and dissolved in methanol to form stock solutions. Six additional calibration levels were prepared by diluting this solution with methanol. Within the range of concentrations injected (750–2.3 µg/ml) the detector response was linear (correlation coefficient $R^2 = 0.999$ for hypericin and 0.998 for pseudohypericin). High performance liquid chromatography (HPLC) was performed using a Agilent 1100 Separations Module (Agilent Technologies, USA) and a Synergi MAX-RP 80 Å column (150×4.6 mm, 4-µm particle size) from Phenomenex (Torrance, CA, USA), coupled to a UV detector (detection at 270 nm) with a flow rate of 1 ml/min and controlled column temperature of 40°C. The mobile phase used was a 10 mM ammonium acetate buffer (pH 5.0) (A) and an acetonitrile/methanol mixture (B) with a gradient program of 87A/13B in 10 min to 83A/17B, then in 25 min to 100B (run time = 35 min; equilibration = 10 min) (Ganzera *et al.*, 2001; Crockett *et al.*, 2005). All solvents and chemicals used were HPLC grade and purchased from Carl Roth GmbH (Karlsruhe, Germany). Quantification of total naphthodianthrone (hypericin plus pseudohypericin) was performed by comparison with response of hypericin (Merck, Darmstadt, Germany). Peaks were assigned by spiking the samples with the standards hypericin and pseudohypericin, and comparison of the UV spectra and retention times.

RESULTS AND DISCUSSION

Morphological and Phenological Characteristics of the Plants

The cultivars “Gold” and “Veperikon,” as well as the wild Iranian population (Ardabile) were grown under common garden conditions in Iran. All plants were cultivated from seed in a greenhouse and seedlings were transplanted after one month to the field plots. This field

plot was located at a latitude of $19^{\circ}36'$ and altitude of 980 m. The relative air humidity was 50%, annual irrigation was 256.3 mm, measured evapotranspiration was 1840.6 mm, and plants experienced a temperature range of $7.2\text{--}21.2^{\circ}\text{C}$. During the course of the investigation, all transplanted seedlings became infected with the plant pathogenic fungus *Colletotrichum gloeosporioides*, which caused either selective necrosis of particular organs and tissues, or necrosis of the whole plant (Fig. 3). The “Veperikon” cultivar was highly sensitive to this soil-borne disease, and did not persist long enough to allow morphological and phenological characteristics to be compared with those of the “Gold” cultivar and Ardabile plants. Both the “Gold” cultivar and plants from the wild population, however, persisted despite displaying the symptoms of mild infections with *C. gloeosporioides* and produced flowering shoots during both the first and second years of cultivation. During the period of full flowering (Year 1), selected phenological (number of days to flowering) and morphological (plant height, mean leaf area, number of black glands/leaf) characteristics were assessed for these plants, and the analysis of variance calculated for this data (Table 1). The “Gold” cultivar plants displayed a higher leaf area and number of black glands/leaf than plants of the Ardabile population.

Chemical Characteristics of Plants

Hypericin and pseudohypericin content for the three pooled samples (4 plants per sample per population) from the “Gold” cultivar and Ardabile population collected during Year 1 of cultivation are shown in Table 2. The average naphthodianthrone content (% dry weight of tissue) for the wild Iranian population was $0.012(\pm 0.002)\%$, but for the “Gold” cultivar, $0.091(\pm 0.001)\%$, a nearly 10-fold difference. Previous studies with plants of *H. perforatum* grown in Canada have shown that higher gland density positively correlates with higher total naphthodianthrone content, and our results also support this finding (Zobayed *et al.*, 2006). This research supports the hypothesis that selection and directed cultivation of Iranian *H. perforatum* plants can result in the development of cultivars with improved morphological, phenological and chemical characteristics and indicates that the “Gold” cultivar could be further exploited for the purposes of providing raw material with high naphthodianthrone content to the Iranian pharmaceutical industries. Further research concerning the stability of naphthodianthrone production by this cultivar, when plants are placed under a variety of environmental stressors, is ongoing.

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Figure 1.
Some biologically active constituents of *Hypericum perforatum*.

Figure 2.
Examples of the dark (e.g. naphthodianthrone-containing) glands and canals found on petals (left) and the anther connective (right) in *Hypericum perforatum* (Photos: M. Azizi).

Figure 3. Examples of leaf (left) and stem tissue of *Hypericum perforatum* plants infected by the pathogenic fungus *Colletotrichum gloeosporioides*, which causes necrosis (arrows indicate necrotic tissue) (Photos: M. Azizi).

Table 1

Analysis of variance for evaluated morphological characters, mean square.

Number of black glands/leaf	Leaf Area (cm ²)	Plant height (cm)	Time from transplanting to shoot formation (days)	Degree of freedom	Source of variation
324.14**	0.88**	1109.76**	1249.19**	1	Cultivar
3.17	0.01	56.74	49.23	4	Error

Table 2

Naphthodianthrone content from analyzed *Hypericum perforatum* samples.

Population	Extract Obtained (mg from 1 g tissue)	Extract Prepared for Analysis (mg)	PS ($\mu\text{g/ml}$) ^A	Average PS ($\mu\text{g/ml}$)	Average % PS in Tissue (w/w)	HY ($\mu\text{g/ml}$) ^A	Average HY ($\mu\text{g/ml}$)	Average % HY in Tissue (w/w)	Total Naphthodianthrone % w/w
Aradibile 1	145	9.8	3.1	2.6 (± 0.6)	0.003 (± 0.001)%	9.4	9.6 (± 1.1)	0.010 (± 0.003)%	0.012 (± 0.002)%
Aradibile 2	114	9.8	2.0			8.6			
Aradibile 3	205	10.3	2.6			10.8			
"Gold" 1	177	10.3	30.0	29.9 (± 0.1)	0.030 (± 0.001)%	71.3	61.3 (± 0.1)%	0.061 (± 0.001)%	0.091 (± 0.001)%
"Gold" 2	144	9.7	30.8			54.2			
						58.4			