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Differences in leaf yield and indigo precursors production in woad (*Isatis tinctoria* L.) and Chinese woad (*Isatis indigotica* Fort.) genotypes

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Abstract

Isatis tinctoria L. (woad) is one of the earliest known sources of indigo in Europe where it was cultivated since the Middle Ages. *Isatis indigotica* Fort. (Chinese woad), widely distributed in China, had been used from ancient times as indigo-producing plant and medicinal plant. Both species produce indigo precursors indican (indoxyl β-D glucoside) and isatan B (indoxyl ketogluconate) in their leaves. In order to identify new suitable crops for indigo production in Italy, 17 woad lines were studied under field conditions in Central Italy (Pisa, 43°40′N, 10°19′E) from 2001 to 2003. We analyzed the effects of year, genotype, and harvest times together with their reciprocal interactions on leaf yield and indigo precursors production. Woad lines were then compared with seven *I. indigotica* lines in a field crop experiment set up in 2003. Extraction and quantification of indigo precursors were accomplished by HPLC-ELSD. Isatan B and indican content, as well as equivalent indigo and fresh/dry leaf yield, were compared between species and among genotypes.

In *I. tinctoria* wide variations in phytochemical and agronomic traits were observed among genotypes, with significant differences in isatan B (1–2 g kg^{-1} FW), indican (0.3–0.7 g kg^{-1} FW) and leaf yield *per* harvest (11–22 t FW ha^{-1}). In *I. indigotica* significant differences were observed in indican (0.3–0.6 g kg^{-1} FW) and fresh leaf yield *per* harvest (10–20 t FW ha^{-1}). Chinese woad showed higher isatan B than woad (4.9 and 1.5 g kg^{-1} FW, respectively). In both species isatan B represented the major precursor, particularly in *I. indigotica*. The ratio indican:isatan B recorded was 1:5 in woad against 1:14 in Chinese woad, leading to significantly higher +55% equivalent indigo in the latter. Interestingly, *I. tinctoria* showed good adaptation to Mediterranean climate conditions with high re-growth capacity after harvest and elevated biomass production. Conversely, *I. indigotica*, although its higher indigo precursors content/leaf weigh, appeared to be more affected by climate conditions and produced −25% leaf yield per hectare per season. The present work identified high indigo yielding genotypes that may be used for genetic improvement in order to re-introduce *Isatis* species in the agricultural systems of Mediterranean regions.

Keywords: *Isatis tinctoria*; *Isatis indigotica*; Genotypes; Leaf yield; Indigo yield; Indigo precursors; Isatan B; Indican; Indigo-producing crops

1. Introduction

Natural indigo (indigotin) is obtained by chemical synthesis from leaf-produced indossilic precursors (Schunck, 1855; Beijerinck, 1900; Epstein and Nabors, 1967; Maugard et al., 2001; Angelini et al., 2003; Oberthür et al., 2004a,b; Gilbert et al., 2004) from a wide range of plants belonging to different species, genera and families. One of the earliest known sources of indigo is *Isatis tinctoria* L. (woad), a biennial member of the family Cruciferae cultivated in Europe since the Middle Ages (Hurry, 1930; Balfour-Paul, 1998). Leaves can be harvested several times in the first year of growth, when plants are at the rosette stage. Although widely used to produce indigo, woad has not been subjected to any formal breeding programme. Hence, it expresses a variety of phenotypes which differ for morphological and phytochemical characteristics. Recently, selective crosses for high-yielding strains led to obtain lines with phenotypical homogeneity and other positive traits (Angelini, 1997, 1999; Gilbert and Cooke, 2001; Durante et al., 2003; John, 2004).

Isatis indigotica Fort. (Chinese woad) also has been used as dye-plant as well as medicinal plant in traditional Chinese medicine (Wu et al., 1997). This biennial herbaceous plant, widely distributed in the Changjiang River valley, was first described by Fortune in 1846. *I. indigotica*, although closely related to its European counterpart *I. tinctoria*, features several morphological, genetic and physiological differences. Both species develop a rosette in the first year of their cycle, with *I.*

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indigotica leaves showing a glaucous instead of a shiny surface, being rarely pubescent with a greater thickness and a more upright habit. Differences in vernalisation requirement are also apparent. In particular, few cool nights are sufficient to accelerate the reproductive development of *I. indigotica* grown under temperate climate, thus suggesting a late spring planting (Angelini, unpublished results). The two species both produce indican (indoxyl β-D glucoside) and isatan B (indoxyl ketogluconate), as indigo precursors. The precursors are extracted from the plant fresh leaves and treated to make indigo. Methods to identify indican and isatan B have been devised (Fischer et al., 1990; Wouters and Verhecken, 1991; Gilbert et al., 2000; Minami, 2001). Indican is a stable compound and can be easily identified and quantified (Minami, 2001; Gilbert et al., 2004) while isatan B is unstable and difficult to quantify. More recently other isatans have been proposed by Maugard et al. (2001), Oberthür et al. (2004a,b). The indossilic precursors are thought to be derived, in the same way as indoles, from the shikimic acid pathway, either via tryptophan or indole-3-pyruvate (Xia and Zenk, 1992). *I. indigotica* is characterized by higher indigo production capacity than *I. tinctoria* (Stoker, 1997; Tozzi et al., 2005). A high degree of genetic diversity in *Isatis* spp. has been assessed by amplified fragment length polymorphism (AFLP) that revealed *I. indigotica* to possess a fingerprint entirely different from *I. tinctoria* and more closely related to other *Isatis* species (Gilbert et al., 2002). Such genetic differences could be associated to biochemical traits, but research on *Isatis* spp. is still in its infancy. Interestingly, light wavelength has been shown to influence indigo precursor production and seed germination in *I. tinctoria* and *I. indigotica* (Tozzi et al., 2005). Quantitative determination of the active principles is a prerequisite for plant selection, breeding and optimization of indigo precursor extraction. In consideration of a possible development for *Isatis* spp. as renewable sources of natural indigo in the European agriculture, aims of this work are: (i) to evaluate differences in indigo precursors and leaf yield among woad genotypes grown in a field experiment from 2001 to 2003; (ii) to further investigate the role played on indigo precursors and leaf yield by environmental conditions, including year of growth and harvest time; (iii) to compare woad and Chinese woad genotypes for indigo precursors and leaf yield under Mediterranean climate; (iv) to identify potential high yield plants to be included in further breeding programmes.

2. Materials and methods

2.1. Field techniques

Seventeen *I. tinctoria* lines were studied under field conditions at the Experimental Center of Department of Agronomy of Pisa University (Pisa, Italy, 43°40′N latitude; 10°19′E longitude) from 2001 to 2003. In the 2003 field trial, seven *I. indigotica* lines were cultivated under the same field conditions. Lines of *I. tinctoria* originated from Europe, lines of *I. indigotica* from China. Seeds were provided in 1992 by different European public institutions (Table 1). Woad and Chinese woad are predominantly obligate out-breeders with high genetic variability (Gilbert et al., 2002) and therefore plants underwent several rounds of selection (Angelini, 1997). Plants showing positive traits were grown and multiplied under isolation conditions leading to genotypes characterized by a high degree of phenotypical homogeneity. These genotypes have been used in this experimental work and compared for indigo precursors content and leaf yield.

Table 1
I. tinctoria and *I. indigotica* genotypes analyzed

Species	Genotypes code	Origin
Isatis tinctoria L.	IS 4	Italy
	IS 5	Germany
	IS 6	Germany
	IS 7	Germany
	IS 9	Germany
	IS 12	Austria
	IS 14	Italy
	IS 17	Italy
	IS 18	Finland
	IS 19	Italy
	IS 20	UK
	IS 22	USA
	IS 23	Italy
	IS 24	Germany
	IS 25	France
	IS 26	France
	IS 27	Italy
Isatis indigotica Fort.	II1	China
	II1 A	China
	II1 B	China
	II2 A	China
	II2 B	China
	II3	China/UK
	II4	China

Country of origin is shown.

Single experiments were laid out in a randomized block design and replicated four times. In 2001 plot size was 3 m^2 (1.5 m × 2 m), in 2002 it was 6 m^2 (3 m × 2 m) and in 2003 3.75 m^2 (2.5 m × 1.5 m). Plant density was about 12 plants/m^2, with inter-row and intra-row spacing of 0.3 m × 0.3 m.

Seeds were sown in March in a cold greenhouse from which 3-week-old plants were transplanted to deep silt-loam soil. The soil was a typical Xerofluvent, representative of the low Arno river plain; it was characterized by a superficial water table 120 cm deep in the driest conditions (Table 2).

The experimental fields had previously been cultivated with wheat. Soil tillage was done in November 2000, 2001 and 2002 with deep ploughing and superficial disk harrowing at the beginning of April, to prepare the sowing bed.

Plants were maintained under identical fertilizer regimes throughout. Mineral fertilizer was applied at pre-planting at rates of 100/100/100 kg ha^{-1} of N/P/K. After harvesting, 50 kg ha^{-1} of N were supplied. Water (20 m^3 ha^{-1}) was supplied to all plots to facilitate post-transplanting recovery. Thereafter plants were irrigated by a trickle line-source lateral irrigation system and the volume of water for daily irrigation maintained the soil water content in the root zone at around