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Sowing date, transplanting, plant density and nitrogen fertilization affect indigo production from *Isatis* species in a Mediterranean region of Spain

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Abstract

The increasing interest in natural products from a renewable source has encouraged growers to reintroduce indigo-producing crops into the European agriculture. We studied agronomic conditions (sowing date, plant density, nitrogen fertilization, irrigation rate, seedling transplanting) influencing production of the blue pigment indigo, from *Isatis tinctoria* and *I. indigotica* crops in a Mediterranean region of Spain (Valencia). *I. tinctoria* was more suitable for cultivation in our climate conditions than *I. indigotica*. Indigo yield from Spanish *I. tinctoria* trials was greater than in Northern and Central Europe. Furthermore, indigo production was maintained when water and nitrogen supplies were significantly restricted, showing that *I. tinctoria* is not a high-demanding crop.

Keywords: Indigo yield; Indigotin; *Isatis indigotica*; *Isatis tinctoria*; Natural dyes; Woad

1. Introduction

The blue pigment indigo (indigotin) is one of the oldest natural dyes known to man. For centuries, indigo has been obtained from a wide variety of plant sources such as *Indigofera* spp. (Africa, Asia, South America), *Polygonum tinctorium* (China, Ko-

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rea) and *Isatis tinctoria* (Europe). In the middle ages, *I. tinctoria*-derived indigo was the basis for a large industry in Europe that started declining after the 17th century due to competition from imported indigo, obtained from tropical *Indigofera* species (Kokubun et al., 1998). Presently, in Europe, there is an increasing interest in natural dyes that fulfill ecological standards and that could be produced from a renewable source (Bechtold et al., 2002). Natural plant-derived indigo provides an ideal starting point for the reintroduction of naturally derived compounds into the market place since (1) there is already an existing demand for the final product, mainly for dyeing cotton yarn for the production of denim, and (2) natural indigo is chemically indistinguishable from its synthetic counterpart (Gilbert and Cooke, 2001). However, as a result of the historical disappearance of plant indigo-producing farming in Europe, there is a considerable lack of knowledge regarding the basic cultivation of these plants and its indigo production (Bechtold et al., 2002).

Indigo is not found as a native compound in plants, but as a product of a secondary metabolite named indoxyl. This metabolite is present in most of the indigo-producing plants as indican (indoxyl-β-D-glucoside). In *Isatis* species, however, isatan B (indoxyl-β-ketogluconate) is the major indigo precursor (Maier et al., 1990). Since the biosynthetic pathway of indigo production was not completely understood, the purity and quality of the natural product could not be controlled, being it one of the main reasons for their replacement by synthetic indigo at the end of the 19th century (Gilbert and Cooke, 2001). Indigo-producing plants contain some metabolites previously considered as "impurities" such as *cis*-indigo (blue), *cis*-indirubin (isoindirubin, red), indigo brown (isoindigo), indigo gluten, indigo yellow and traces of flavonoids. Indirubin is a by-product of indigo biosynthesis, originating from the condensation of indoxyl with isatin, which is generated from indoxyl in an oxygen-rich environment as a side reaction. Some of these indigo by-products are being investigated for their possible pharmacological applications (Kimoto et al., 2001; Molina et al., 2001; Danz et al., 2001, 2002; Mak et al., 2004). In addition, new technologies to process indigo products have recently been developed. A method for the extraction of natural indigo from woad, based on a technique used to extract indigo from *Indigofera* spp., was developed by Stoker et al. (1998a). Advances on indigo extraction from *P. tinctorium* were also reported by Maugard et al. (2002). Other investigations deal with biotechnological approaches in relation to indigo synthesis by bacteria (O'Connor and Hartmans, 1998; Itoh et al., 1999; Bhushan et al., 2000) and plant cell or tissue cultures (Shim et al., 1998; Young-Am et al., 2000). All these facts encourage and emphasize the importance of the reintroduction of indigo-producing plants into European farming.

The genus *Isatis* (Brassicaceae) encompasses about 30 species, most of them producing indigo. *I. tinctoria* ("woad" in U.K., "añil" in Spain) is the earliest known source of indigo. It is a biennial and mainly outbreeding species, probably indigenous to southeastern Asia. *I. tinctoria* seedlings form a rosette of leaves in the first year of their cycle, and an erect stem bearing yellow flowers on short racemes in the following spring. Woad has not been grown in Europe as a source of indigo since the 17th century, although it continued to be cultivated in the U.K. until the 19th century as a fermentation aid for the woad vat. *I. indigotica* (Chinese woad or tein-cheing) is very closely related to woad (Gilbert et al., 2002), although there are several differences from the latter: the leaf surfaces are glaucous as opposed to shiny. It appears to have a more upright habit, contains more indigo precursors and the leaves tend not to be pubescent. Although it is not known whether it has been used for indigo production, *I. indigotica* has been widely used in traditional Chinese medicine as an antiviral agent.

As part of a European Union-funded research project, in this work, we studied agronomic factors optimizing *I. tinctoria* and *I. indigotica* cultivation in the south-east of Spain. In addition, we compared the indigo production of these two species. These low technology-demanding crops could be an alternative to the traditionally cultivated vegetables in this region. In southern countries of Europe, moderate temperatures could allow successive harvests during an extended growing season (from May to November) that could increase indigo yields. On the other hand, it has been suggested that indigo precursor contents in *I. tinctoria* plants increase under intense light regimes (Stoker et al., 1998b). Under these premises, indigo production is expected to be higher in the Mediterranean regions than in Northern and Central Europe. We have established optimal conditions for *Isatis* crops in a Mediterranean region.

2. Material and methods

2.1. Field trials

Field studies were conducted during two consecutive years (from August 2001 to September 2003) at the Center for Agricultural Research of Fundación Ruralcaja Valencia, Spain. Plots were located in Bolbaite and Paiporta, Valencia (39°03′ N, 0°25′ W and 39°16′ N, 0°14′W, respectively). Soil in Bolbaite has a medium-clay texture, pH 8.3, 0.13% N, 0.006% P, 0.022% K and 0.66% of organic carbon. Crops in Paiporta were established on a sandy medium soil with pH 8.6, 0.09% N, 0.005% P, 0.021% K and 0.85% of organic carbon.

Seeds of *I. tinctoria* L., from the U.K., named as Hill variety, were kindly provided by Mr. Bateson (Go Botanical Ltd., U.K.). Seeds of *I. indigotica* Fort (Chinese woad) were provided by Dr. Gilbert from the University of Bristol, U.K. In each experiment, treatments were arranged in a randomized complete block design with three replications. Individual plots consisted of 10 m^2 areas with 15 rows, 5 m long with a row spacing of approximately 30 cm.

Agronomic factors studied were: sowing date, plant density, nitrogen fertilization, seedling transplanting (Bolbaite's trials) and irrigation rate (Paiporta's trial). Unless otherwise specified, standard plant density was 10 plants m^{-2} and soil watering was done by flooding. Seeding dates for the 2002 trials were August 6th, September 4th, December 14th (2001), January 24th, February 28th and March 27th. The 2003 trial seeding dates were August 4th, September 12th, December 16th (2002), January 24th, February 28th and March 27th. August and September dates were tested only for seed production. Seeding rates were 0.5 g of siliqua m^{-2} and germination was evaluated 2 months later. Assessed plant densities (February sowing) were 5, 10 and 20 plants m^{-2}. The standard nitrogen fertilization [as $(NH_4)_2SO_4$] consisted of 100 kg N ha^{-1} before sowing and 50 kg N ha^{-1} after each harvest. Assessed N rates were 0 + 0 (low), 100 + 50 (standard) and 200 + 100 kg N ha^{-1} (high), before sowing and after each harvest, respectively. Plots corresponding to the nitrogen fertilization and transplanting trials were included in the March sowing. In the transplanting experiment, seeds were first germinated in peat moss compost under greenhouse conditions. After 3 weeks, plantlets were transferred to the field. Irrigation experiments were done with a drip irrigation system. The plots were seeded in February and the assayed irrigation rates were calculated from the reference crop evapotranspiration (ET_o) as follows: $ET_o - 30\%$, ET_o and $ET_o + 30\%$. Total water supplies were 6474, 9158 and 11902 m^3 ha^{-1} in 2002 (March–November) and 4770, 7542 and 10059 m^3 ha^{-1} in 2003 (March–September).

Plants were harvested by cutting the rosette leaves 10 cm from the ground every time they reached 25–30 cm high. The above ground biomass production was determined as t ha^{-1} (fresh and dry weight). From each harvested plot, 15 medium-size leaves were sampled for indigo content determinations (g kg^{-1}). Leaves were maintained at 4 °C in plastic bags until indigo extraction (2–3 h later). Values of biomass production and leaf indigo content were used to estimate the final indigo production (kg ha^{-1}) for each plot and harvest. Since the number of harvests performed depended on plant development and climate conditions, results are presented as the sum of the total production of each trial. Plants from the August and September sowings were allowed to flower after winter and seeds were collected in the following June.

2.2. Indigo content determination

A calibration curve was prepared by dissolving indigo standard (Sigma, purity 99%) in ethyl acetate as follows: 2.4 mg of indigo was successively boiled in 100 ml distilled water, alkalinised with $Ca(OH)_2$ and acidified with 50% HCl, before extraction with 400 ml of ethyl acetate to obtain a stock solution of 6 mg l^{-1}. Aliquots of this stock solution were taken to obtain several dilutions of 4.5, 3 and 1.5 mg l^{-1}. The absorbance of these solutions was measured at 600 nm (three replicates), and the following algorithm was obtained: indigo concentration = $0.065 + 25.485 \times Abs_{600}$ ($r = 99.9\%$).

Indigo content of leaves was extracted according to the protocol described by Stoker et al. (1998a), with some modifications. From each plot, 15 medium-size leaves were randomly chosen. One disc (1 cm diameter) was taken from each leaf, and a bulked sample with 15 leaf discs (0.25–0.5 g) was subsequently prepared. Once weighed, discs were immersed in 10 ml of distilled water, boiled for 5 min in a water bath and cooled rapidly in ice water. The discs were removed

and 200 μl of a saturated solution of $Ca(OH)_2$ were added. Samples were then aerated for 30 s and allowed to stand at room temperature for at least for 1 h, before acidification with 50 μl of 50% HCl. After standing for 30 min, an aliquot of 500 μl was extracted with 3–5 ml of ethyl acetate. The indigo concentration of these extracts was determined from their absorbance at 600 nm by using the algorithm previously obtained.

2.3. Statistical analyses

Significance of treatment effects on total fresh biomass production, mean indigo content of plants along the growing season and estimated total indigo production was determined using analysis of variance. When appropriate, variations among treatment means were analyzed using Tukey (1953). Since analyses of results concerning dry mass determinations corroborated conclusions presented here, only data from fresh mass determinations are shown.

3. Results

3.1. Effect of sowing date

Germination rates were scored in plots 2 months after sowing, and no significant differences were observed for both species in the four seeding dates (data not shown, mean = 17%). Plants from December and January sowings were first harvested in the 2nd–3rd week of May (160–162 and 117–121 days after seeding, respectively), while plants from February and March sowings were first harvested in the 1st–2nd week of June (after 89–97 and 75–83 days, respectively). Crops sown in early winter were harvested one more time than those sown in spring. Harvests were usually done every 4 weeks, except from mid June to mid August, where plants were harvested every 2–3 weeks. *I. indigotica* showed a limited regrowth capability, and crops could only be harvested three to four times. In contrast, the regrowth of axillary leaves of *I. tinctoria*

Table 1
Effect of the species and sowing date on biomass production and indigo yield from *Isatis* spp. plots growing in Bolbaite

Seeding date	2002			2003		
	Biomass (t FW ha^{-1})[a]	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass (t FW ha^{-1})[a]	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})[b]
Isatis indigotica						
December	11.4 (4)	0.8	9.5	9.7 (4)	0.7	5.3d
January	30.6 (5)	0.8	24.5	8.5 (4)	0.7	5.3d
February	11.9 (3)	0.6	7.9	9.1 (4)	0.6	5.0d
March	14.7 (3)	0.5	7.4	8.0 (3)	0.6	5.0d
Isatis tinctoria						
December	75.4 (7)	0.8	54.7	66.4 (5)	0.4	26.2a
January	105.7 (7)	0.6	62.8	46.2 (5)	0.7	18.7ab
February	66.3 (6)	0.6	42.6	32.8 (4)	0.5	16.8bc
March	68.2 (6)	0.6	38.3	33.1 (4)	0.3	10.4cd

Source of variation	g.l.	2002			2003		
		Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})
Analysis of variance, mean squares							
Species (A)	1	22872.6**	0.008 NS	8327.5**	7679.6**	0.10 NS	991.7**
Sowing (B)	3	1095.8**	0.069**	522.2**	401.0**	0.06 NS	66.1**
A × B	3	152.2 NS	0.014 NS	55.7 NS	351.7**	0.02 NS	61.4**
Error	16	105.5	0.013	55.5	25.3	0.42	7.9

Data are means of three replications. NS, non-significant.
[a] Data in parenthesis indicate the number of harvests taken during the growing season.
[b] For each species and entry, means followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$).
** Significant at $p \leq 0.01$.

plants was favoured after the first cutting. Thus, up to seven harvests could be obtained from the *I. tinctoria* crops.

Early winter sowing dates (December and January) gave higher biomass productions when compared to the spring sowing dates (February and March). In the 2002 experiments, the higher biomass production in both species was obtained in plots sown in January (mean production 68.2 t ha^{-1} in comparison to 43.4, 39.1 and 41.5 t ha^{-1} from December, February and March sowings, respectively). In the 2003 experiments, the sowing date did not influence biomass production of *I. indigotica* (Table 1). In contrast, *I. tinctoria* plots seeded in December produced the greatest yield (Table 1). Early winter sowings also produced more indigo, although a significant effect was only found in 2002 plots (mean indigo contents of 0.8, 0.7, 0.6 and 0.6 g kg^{-1} from December, January, February and March plants, respectively). *I. tinctoria* crops always produced significantly greater biomass rates than *I. indigotica* crops (Table 1). As a result, indigo yield (kg ha^{-1}), estimated from both biomass production and indigo content determinations, was always greater in *I. tinctoria*. Best results were obtained from plots seeded in early winter (January 2002, December or January 2003).

In order to avoid a possible bias in statistical analyses, results from the following experiments were computed separately for each species.

3.2. Effect of plant density

Biomass yield seems to be related to plant density. We found greater biomass yield as a trend in the plots with 10–20 plants m^{-2} as compared to the plots with 5 plants m^{-2} (Table 2). This trend was statistically significant for *I. tinctoria* in the 2003 plots. The increased plant density did not reduce the leaf indigo content (Table 2). Consequently, the total indigo yield

Table 2
Effect of plant density on biomass production and indigo yield from *Isatis tinctoria* and *I. indigotica* plots sown in February in Bolbaite

Plant density (m^{-2})	2002			2003		
	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})
Isatis indigotica						
5	12.9	0.6	8.4	5.6	0.7	3.2
10	11.9	0.6	7.9	9.1	0.6	5.0
20	12.8	0.6	8.0	7.7	0.7	5.6
Isatis tinctoria						
5	54.7	0.5	29.0	21.3b[a]	0.5	10.6
10	66.3	0.6	42.6	32.8a	0.5	16.8
20	84.8	0.5	43.5	35.4a	0.5	17.8

Source of variation	g.l.	2002			2003		
		Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})
Analysis of variance, mean squares							
Isatis indigotica							
Plant density	2	0.8 NS	0.001 NS	0.21 NS	9.3 NS	0.012 NS	4.7 NS
Residual	6	4.3	0.021	8.7	2.4	0.009	1.2
Isatis tinctoria							
Plant density	2	691.3 NS	0.007 NS	196.8 NS	168.3*	0.002 NS	45.6 NS
Residual	6	249.4	0.003	59.2	23.6	0.008	24.9

Data are means of three replications. NS, non-significant.

[a] Effect of plant density on biomass production *in I. tinctoria* plots. Means followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$).

* Significant at $p \leq 0.05$.

Table 3
Effect of nitrogen fertilization on biomass production and indigo yield from *Isatis tinctoria* and *I. indigotica* plots sown in March in Bolbaite

Nitrogen rates[a]	2002[b]			2003[b]		
	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})
Isatis indigotica						
0	8.6c	0.6	5.1c	5.8	0.4	2.3b
100	14.7b	0.5	7.4b	8.0	0.6	5.0a
200	19.1a	0.6	10.9a	6.8	0.7	5.0a
Isatis tinctoria						
0	39.8b	0.6	22.5b	26.2	0.4b	11.4
100	68.2a	0.6	38.3a	33.1	0.3b	10.4
200	75.1a	0.6	40.4a	23.7	0.5a	19.4

Source of variation	g.l.	2002			2003		
		Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})
Analysis of variance, mean squares							
Isatis indigotica							
Nitrogen rate	2	83.0**	0.004 NS	25.7 NS	3.6 NS	0.04 NS	7.4*
Residual	6	2.7	0.03	9.1	2.4	0.01	0.9
Isatis tinctoria							
Nitrogen rate	2	1054.7**	0.0003 NS	286.4**	71.2 NS	0.04**	72.9 NS
Residual	6	95.1	0.004	14.1	51.2	0.006	26.2

Data are means of three replications. NS, non-significant.
[a] Nitrogen rates: 0 = 0 + 0, 100 = 100 + 50, 200 = 200 + 100 kg N ha^{-1} (before sowing + after each harvest).
[b] For each species and entry, means followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$).
* Significant at $p \leq 0.05$.
** Significant at $p = 0.01$.

was also greater in those plots with an increased plant density.

3.3. Effect of nitrogen fertilization

Results from 2002 experiments showed that both biomass and total indigo production in *I. indigotica* and *I. tinctoria* plots increased along with N fertilization (Table 3), but the leaf indigo content was not significantly influenced by this factor. Note, however, that in *I. tinctoria*, there were no significant differences when either the standard or the high-nitrogen levels were applied, suggesting that this species is not a strong nitrogen-demanding plant. These results were partially confirmed in 2003 experiments, where the significant influence of N fertilization was only evident for total indigo production of *I. indigotica* (Table 3). This positive effect was due in part to an increase of the indigo content of leaves with increasing nitrogen fertilization. The same effect of nitrogen fertilization on indigo leaf content was observed in *I. tinctoria*, although this increase did not significantly affect the total indigo production (Table 3).

3.4. Effect of irrigation rate

The irrigation experiments were performed in Paiporta, where plots were established with a drip irrigation system. Under these conditions, *I. tinctoria* and *I. indigotica* plants showed a faster development (first harvests 76–82 days after sowing) as compared to Bolbaite crops. Irrespective of the species and year, irrigation rates tested did not significantly affect biomass production or leaf indigo content (Table 4). Note, however, that at the 2002 experiment, a significantly reduced indigo production was found in *I. indigotica* plots when an excess of water was applied.

Table 4
Effect of irrigation rate on biomass production and indigo yield from *Isatis tinctoria* and *I. indigotica* plots growing in Paiporta

Irrigation rate[a]	2002			2003		
	Biomass[b] (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass[b] (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})
Isatis indigotica						
R1	30.8 (5)	0.6	16.8a[c]	26.3 (3)	0.3	9.3
R2	29.2 (5)	0.6	16.8a	26.2 (3)	0.4	10.4
R3	28.5 (5)	0.5	11.8b	33.1 (3)	0.3	10.2
Isatis tinctoria						
R1	74.6 (6)	0.4	32.5	90.7 (6)	0.4	28.0
R2	78.2 (6)	0.5	38.0	93.7 (6)	0.4	39.3
R3	71.4 (6)	0.5	30.0	96.0 (6)	0.4	32.2

Source of variation	g.l.	2002			2003		
		Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})
Analysis of variance, mean squares							
Isatis indigotica							
Irrigation	2	4.1 NS	0.010 NS	24.7*	47.4 NS	0.007 NS	0.9 NS
Residual	6	22.2	0.005	1.7	22.0	0.005	4.9
Isatis tinctoria							
Irrigation	2	35.4 NS	0.002 NS	49.9 NS	21.1 NS	0.005 NS	97.0 NS
Residual	6	89.3	0.003	29.5	76.6	0.002	98.8

Data are means of three replications. NS, non-significant.
[a] Irrigation rates: R1 = 70% ET_o, R2 = ET_o, R3 = 130% ET_o.
[b] Data in parenthesis indicate the number of harvests taken during the growing season.
[c] Effect of irrigation rate on indigo production in *I. indigotica* plots. Means followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$).
* Significant at $p \leq 0.05$.

3.5. Effect of transplanting

We compared indigo production in plots established by seeding and by transplanting greenhouse-germinated seedlings. To avoid bolting, these experiments were done only in March, but still 10–20% of the transplanted *I. indigotica* plants flowered in April, probably as a consequence of the last winter cold days. The behavior of the tropical species *I. indigotica* contrasts to that observed in the temperate Hill variety of *I. tinctoria,* which did not flower. Production of transplanted plots was compared to that obtained from the corresponding sowing (March) as well as to those seeded plots producing the greatest indigo yields (January in 2002 and December in 2003). As shown in Table 5, transplanting significantly increased biomass production and indigo yield of *I. indigotica* compared to the corresponding March seeding. Furthermore, both parameters were similar or slightly greater than those obtained from early sowing in winter. For *I. tinctoria,* transplanting also produced significantly more biomass and indigo yield than the corresponding 2003 sowing experiment (Table 5). Although statistically not significant, a similar trend was observed in the 2002 experiments. Again, results obtained from the transplanting trials were comparable to those from seeded plots producing the highest indigo yield.

3.6. Seed production

I. tinctoria and *I. indigotica* plants grown from seeds sown in either August or September started flowering at the end of February. *I. tinctoria* and *I. indigotica* plants harvested in June produced a seed yield average of 0.33 and 0.16 kg m^{-2}, respectively. Some plots of *I. tinctoria* from February and March sowings were

Table 5
Effect of transplanting on biomass production and indigo yield of *Isatis indigotica* and *I. tinctoria* plots from different origin

Origin	2002[b]			2003[b]		
	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})
Isatis indigotica						
Early winter seeding[a]	30.6a	0.8	24.5a	9.7b	0.7a	5.3b
March, seeding	14.7b	0.5	7.4b	8.0b	0.6b	5.0b
March, transplanting	26.4a	0.6	17.2a	20.3a	0.6b	10.6a
Isatis tinctoria						
Early winter, seeding[a]	105.7a	0.6	62.8	66.4a	0.4	26.2b
March, seeding	68.2b	0.6	38.3	33.1b	0.3	10.4c
March, transplanting	83.4b	0.6	47.3	61.9a	0.5	36.8a

Source of variation	g.l.	2002			2003		
		Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})	Biomass (t FW ha^{-1})	Indigo (g kg^{-1} FW)	Indigo (kg ha^{-1})
Analysis of variance, mean squares							
Isatis indigotica							
Origin	2	203.7*	0.052 NS	228.0*	132.8*	0.022*	29.8*
Residual	6	9.9	0.023	19.8	9.5	0.004	3.0
Isatis tinctoria							
Origin	2	1063.3*	0.001 NS	461.6 NS	960.1*	0.016 NS	529.3*
Residual	6	121.7	0.012	115.6	64.8	0.005	26.4

NS, non-significant.
[a] January and December sowings in 2002 and 2003, respectively.
[b] For each species and entry, means followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$).
* Significant at $p \leq 0.05$.

also allowed to flower. In these plots, seed production was similar to that obtained in the summer sowings (0.28 and 0.19 kg m^{-2} for *I. tinctoria* and *I. indigotica*, respectively). Therefore, sowing date had no effect on seed production of *I. tinctoria* and *I. indigotica* plots.

4. Discussion

The objective of the field plot experiments carried out in this study was to examine the effect of some crop parameters (sowing date, plant density, nitrogen fertilization, water supply, transplanting) on indigo yield of two *Isatis* species in a Mediterranean region of Spain. The aim was to (1) determine which one of these species could be best suited for cultivation in our climate and soil conditions, and (2) to design field practices for high yields of natural indigo.

Results herein demonstrate that *I. tinctoria* always produced more indigo than *I. indigotica*. This was not due to a higher leaf indigo yield of the former, but to their higher biomass production. This species had better regrowth ability after harvesting and was less sensitive to the attacks of insects, particularly the cabbage webworm, *Hellula undalis* and nematodes (*Meloidogyne* spp.). Consequently, *I. tinctoria* seems to be more suitable for cultivation in the Valencian region than *I. indigotica*. In the past, *I. tinctoria* was the species traditionally cultivated in Spain for indigo production, as it was in Southern Italy (Guarino et al., 2000). Since the Hill variety employed in our experiments had been selected for cultivation in the U.K., results herein may be improved by breeding programs aimed at enhancing indigo content and regrowth vigour after harvest.

Although biomass production was improved when *Isatis* crops were established at the high-plant density (Table 2), indigo yields could not be improved by in-

creasing this parameter from 10 to 20 plants m^{-2}. Plant densities assayed in this study did not influence leaf indigo content, suggesting that variation on radiation absorption by the canopy did not affect biosynthesis of indoxyl precursors. Thus, light incidence should not be a limiting factor in our climate conditions. Because of this, an optimal plant density could be determined (ranging from 10 to 20 plants m^{-2}) depending on soil fertility and water availability.

The biomass and indigo production of *I. indigotica* increased along with nitrogen fertilization (Table 3). In contrast, the high-nitrogen fertilization (200 kg N ha^{-1}) did not significantly increase biomass or indigo yield of *I. tinctoria* in the experiments performed. These results indicate that *I. tinctoria* is less nitrogen demanding than *I. indigotica* and that greater indigo yields from *I. tinctoria* can be obtained with standard fertilizations in our Mediterranean region. Note that only in the 2003 experiments, the addition of 200 kg N ha^{-1} increased the leaf indigo content of this species (Table 3). Climate conditions could explain the differences between the 2 years. The 2003 summer was dryer and hotter (T_m = 24.4 °C, P = 98 mm from May to August) than the 2002 summer (T_m = 21.4 °C, P = 223 mm in the same period, see Fig. 1a). Thus, we suggest that limited plant development under high-temperature regimes resulted in an increased indigo content.

We found no significant variation on biomass production of *Isatis* crops over a wide range of irrigation (Table 4). In the 2002 rainy summer (see Fig. 1b), plots irrigated with 30% over ET_o showed a decrease in indigo production, although it was only significant for *I. indigotica* plants. Both *I. indigotica* and *I. tinctoria* are

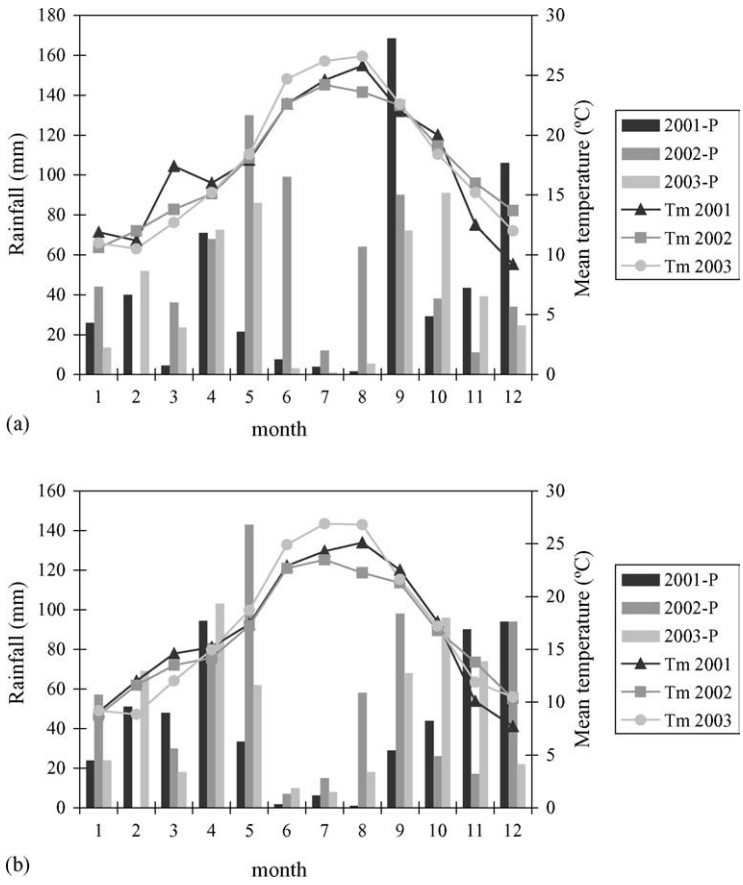

Fig. 1. Rainfall (P, in mm) and mean temperatures (T_m, in °C) in Bolbaite (a) and Paiporta (b) along the 2001–2003 years.

not high water-demanding crops, since production was not significantly affected when water supply was limited to a 70% of ET_o. Accordingly, *Isatis* crops showed the greatest water efficiency at the lesser irrigation level $ET_o - 30\%$ (mean indigo production of 2.3 and 6.5 g m^{-3} of water for *I. indigotica* and *I. tinctoria*, respectively) as compared to the ET_o and $ET_o + 30\%$ levels (1.6 and 1.0 for *I. indigotica* and 4.7 and 2.9 g m^{-3} for *I. tinctoria*, respectively). This is an important point, since water is a limiting resource in our region.

In our experiments, the greatest indigo yields were estimated from crops of *I. tinctoria* established by sowing in early winter (26.2–62.8 kg ha^{-1}) or by transplanting 3-week-old plants in March (36.8–47.3 kg ha^{-1}), under standard nitrogen fertilization. Although further studies are needed to determine optimal crop conditions for this species over a wide range of locations, these yields are better than those found in U.K. for the same species (up to 20 kg of indigo ha^{-1}; Stoker et al., 1998a). On the other hand, transplanting may be considered as an alternative to early sowing, allowing rotation with a winter crop that could increase the final income of growers. Finally, natural indigo production in the Mediterranean region from *I. tinctoria* crops could be substantially improved by breeding programs providing varieties with greater yields in our climate and soil conditions. Since there is a demanding market for natural dyes, indigo-producing plants could be an alternative crop for some agricultural regions of Europe.

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References

Bechtold, T., Turcanu, A., Geissler, S., Ganglberger, E., 2002. Process balance and product quality in the production of natural indigo from *Polygonum tinctorium* Ait. applying low-technology methods. Bioresour. Technol. 81, 171–177.

Bhushan, B., Samantha, S.K., Jain, R.K., 2000. Indigo production by naphtalene-degrading bacteria. Lett. Appl. Microbiol. 31, 5–9.

Danz, H., Stoyanova, S., Wippich, P., Brattstrom, A., Hamburger, M., 2001. Identification and isolation of the cyclooxygenase-2 inhibitory principle in *Isatis tinctoria*. Planta Med. 67, 411–416.

Danz, H., Stoyanova, S., Thomet, O.A.R., Simon, H.U., Dannhardt, G., Ulbrich, H., Hamburger, M., 2002. Inhibitory activity of tryptanthrin on prostaglandin and leukotrene synthesis. Planta Med. 68, 875–880.

Gilbert, K.G., Cooke, D.T., 2001. Dyes from plants: past usage, present understanding and potential. Plant Growth Regul. 34, 57–69.

Gilbert, K.G., Garton, S., Karam, M.A., Arnold, G.M., Karp, A., Edwards, J., Cooke, D.T., Barker, J.H.A., 2002. A high degree of genetic diversity is revealed in *Isatis* spp. (dyers woad) by amplified fragment length polymorphism (AFLP). Theor. Appl. Genet. 104, 1150–1156.

Guarino, C., Casoria, P., Menale, B., 2000. Cultivation and use of *Isatis tinctoria* L. (Brassicaceae) in Southern Italy. Econ. Bot. 54, 395–400.

Kimoto, T., Himo, K., Koya-Miyata, S., Yamamoto, Y., Takenchi, M., Nishizaki, Y., Micallef, M.J., Ushio, S., Iwaki, K., Ikeda, M., Kurimoto, M., 2001. Cell differentiation and apoptosis of monocytic and promyelocytic leukemia cells (U-937 and HL-60) by tryptanthrin, an active ingredient of *Polygonum tinctorium* Lour. Pathol. Intern. 51, 315–325.

Kokubun, T., Edmonds, J., John, P., 1998. Indoxyl derivatives in woad in relation to medieval indigo production. Phytochemistry 49, 79–87.

Itoh, K., Aoki, S., Yatome, C., 1999. Production of indigo related pigments by *Nocardia glomerula*. J. Soc. Dyes Colour 115, 233–235.

Maier, W., Schumann, B., Gröger, D., 1990. Biosynthesis of indoxyl derivatives in *Isatis tinctoria* and *Polygonum tinctorium*. Phytochemistry 29, 817–819.

Mak, N.K., Leung, C.Y., Wei, X.Y., Shen, X.L., Wong, R.N.S., Leung, K.N., Fung, M.C., 2004. Inhibition of RANTES expression by indirubin in influenza virus-infected human bronchial epithelial cells. Biochem. Pharm. 67, 167–174.

Maugard, T., Enaud, E., de la Sayette, A., Choisy, P., Legoy, M.D., 2002. beta-glucosidase-catalyzed hydrolisis of indican from leaves of *Polygonum tinctorium*. Biotech. Prog. 18, 1104–1108.

Molina, P., Tárraga, A., González-Tejero, A., Rioja, I., Úbeda, A., Terencio, M.C., Alcaraz, M.J., 2001. Inhibition of leukocyte functions by the alkaloid isaindigotine from *Isatis indigotica* and some new synthetic derivatives. J. Natl. Prod. 64, 1297–1300.

O'Connor, K.E., Hartmans, S., 1998. Indigo formation by aromatic hydrocarbon-degrading bacteria. Biotechnol. Lett. 20, 219–223.

Shim, J., Chang, Y., Kim, S., 1998. Indigo and indirubin derivatives form indoles in *Polygonum tinctorium* tissue cultures. Biotechnol. Lett. 20, 1139–1143.

Stoker, K.G., Cooke, D.T., Hill, D.J., 1998a. An improved method for the large-scale processing of woad (*Isatis tinctoria*) for possible

commercial production of woad indigo. J. Agric. Eng. Res. 71, 315–320.
Stoker, K.G., Cooke, D.T., Hill, D.J., 1998b. Influence of light on natural indigo production from woad (*Isatis tinctoria*). Plant Growth Regul. 25, 181–185.
Tukey, J.W., 1953. Some selected quick and easy methods for statistical analysis. Trans. N Y Acad. Sci. Ser. II 16, 88–97.
Young-Am, C., Yu, H., Song, J., Chun, H., Park, S., 2000. Indigo production in hairy root cultures of *Polygonum tinctorium* Lour. Biotechnol. Lett. 22, 1527–1530.