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Environmental Factors Affecting Productivity, Indican Content, and Indigo Yield in *Polygonum tinctorium* Ait., a Subtropical Crop Grown under Temperate Conditions

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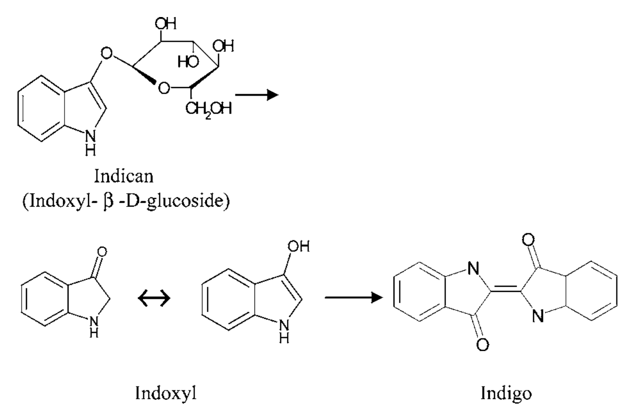
Polygonum tinctorium Ait. is a herbaceous subtropical annual plant, belonging to the family Polygonaceae. Within the cells of its leaves *P. tinctorium* accumulates large amounts of a colorless glycoside, indican (indoxyl *β*-D-glucoside), from which the blue dye indigo is synthesized. *P. tinctorium* is well-known in Japan, where it had been cultivated to produce natural indigo for textile dyeing, whereas it represents a potentially interesting new crop in Europe. To better understand the effects of environmental parameters on *P. tinctorium* crop production and indigo yield, field experiments were carried out in central Italy under temperate climate. Three lines were tested during the 2001 and 2002 growing seasons, and plant/leaf yields as well as indican contents were evaluated. The results showed that *P. tinctorium* grown in temperate climate conditions can be harvested three times a year. Yields of 82 and 120 t ha^{-1} of fresh plant yield were obtained in 2001 and 2002, respectively. The contrasting weather conditions between the two years significantly affected biomass production, which was higher in the 2002 season, characterized by wet weather conditions. The cycle length from sowing to the last harvest was accomplished in 229−238 days when plants had accumulated 2017−2018 °C. Green leaves accounted for 40−45% by weight of fresh plant tissue and contained 11−20 g kg^{-1} indican. The three lines did not significantly differ in the main productive parameters or in fresh leaf indican content (14.1 g kg^{-1} mean value). Photosynthetic active radiation influences indican leaf production according to the model $y = 0.0004x + 8.566$ ($P < 0.01$, correlation coefficient = 0.818). Indican content ranged from 12 to 25 g kg^{-1} of fresh leaves with PAR daily values from 10000 to 40000 mEinstein m^{-2} (recorded in May and at the end of July−beginning of August, respectively). The results indicate that in nonlimiting rainfall conditions a very high indican content and a potentially high indigo yield can be obtained by cultivating *P. tinctorium* in this pioneer geographical area.

KEYWORDS: **Indican; *Polygonum tinctorium*; leaves; yield; indigo; photosynthetically active radiation**

INTRODUCTION

Polygonum tinctorium (Ait.), also known as "Dyer's knotweed", is a herbaceous subtropical annual plant, belonging to the family Polygonaceae. It had been grown in Japan and China to obtain natural indigo (*1*−*4*), the compound commonly used as a blue dye. The source for indigo production is indican (indoxyl *β*-D-glucoside), a colorless glucoside representing a major leaf secondary metabolite present in the vacuoles of the leaves (*5*). It is water-extracted from leaves and degraded to indoxyl and glucose by the action of a native *β*-glucosidase. Dimerization of indoxyl by air oxidation will follow, leading to the formation of indigo, as fully characterized by Minami et al. (*5*).

For textile dying, indigo used to be the only natural blue dye until the end of the 19th century, when synthetic indigo almost completely displaced the natural one (*6*). Indigo-producing plants ceased to be cultivated on a wide scale and, consequently, the development of agronomic techniques and dye extraction procedures has been suppressed considerably (*7*, *8*).



Recently, the European Commission (EC) has been funding a number of research projects aiming to reintroduce indigo-

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delivering crops into European agriculture, to provide a source of natural indigo as an alternative to the synthetic one. Research studies have been undertaken to investigate the agronomic aspects of *P. tinctorium* cultivation in different European countries, and preliminary results have shown that environmental growth conditions play a crucial role in influencing the yield of both plant material and indigo. *P. tinctorium* adapted well to growing in Central Europe and in Mediterranean countries, but it is intolerant to drought, whereas indican content is positively influenced by sunlight intensity (*9*).

In Italy *P. tinctorium* could represent a valuable source of natural indigo. Sensitive techniques, such as high-performance liquid chromatography (HPLC) coupled to an evaporative light scattering detector (ELSD), have been developed to quantify the indigo precursor indican (*9*). Nevertheless, a considerable demand for research aimed to optimize *P. tinctorium* plant cultivation and indigo production in temperate areas is still required.

In this paper, we measured plant and leaf yields, leaf indican, and indigo content using three *P. tinctorium* lines grown under natural sunlight conditions in central Italy during two growing seasons. This work was carried out as part of an EC-funded research project (SPINDIGO), with the objective of bringing about new knowledge on indican and biomass variation in this pioneering geographical area of cultivation.

MATERIALS AND METHODS

Field Technique. Three *P. tinctorium* lines (PT1, PT3, and PT4) were studied under field conditions at the Experimental Centre of Rottaia (Pisa, central Italy, 43° 40′ N latitude; 10° 19′ E longitude). Plant lines originated from Japan and seeds were provided by Thüringer Landesanstalt für Landwirtschaft, Dornburg, Germany (PT4), Long Ashton Research Station, Bristol, U.K. (PT3), and the Department of Agronomy, University of Pisa, Italy (PT1).

Seeds were sown in a cold greenhouse in March 2001 and March 2002, and the resulting 4-week-old plants were transplanted to deep silt-loam soil (clay 23%, silt 24%, sand 53%, total nitrogen 1.04‰, pH 8.2, organic matter 1.56%). Single experiments were laid out in a split-plot design with different lines as main plot and different harvests as subplots, with four replications. In 2001, subplot sizes were 12 m^2 area (3 × 4 m), with 13 rows (3 m long), whereas in 2002 subplot sizes were 20 m^2 area (5 × 4 m), with 17 rows (4 m long). Plant density was ~12 plants/m^2, with inter-row and intrarow spacing of 0.3 × 0.3 m.

The soil was a typical Xerofluvent, representative of the lower Arno River plain, and it was characterized by a superficial water table 120 cm deep in the driest conditions. Fields had been previously cultivated with wheat, and soil tillage was done in November of both 2000 and 2001 with deep ploughing and superficial disk harrowing at the beginning of April, to prepare the sowing bed.

Throughout the two experimental periods plants were maintained under identical fertilization conditions. Mineral fertilizer was applied at preplanting at rates of 100/100/100 kg ha^{-1} of N/P/K. After the first and second harvests, 50 kg ha^{-1} of N was supplied. 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 ~80% of field capacity.

Environmental Parameters. Changes in air temperature, rainfall, global radiation, and photosynthetically active radiation (PAR) were recorded throughout by using a weather station, properly equipped to the purpose. Cumulative sums of PAR (mEinstein/m^2; 1 Einstein = 1 mol of photons) and global radiation (kJ/m^2) on hourly and daily bases from sowing to harvesting were calculated by a data logger Campbell CRX10. Sensors were model Rg19 by Silimet Quantum Sensor system. Plant cycle length was measured by growing degree days (GDD) as daily accumulated mean temperature, with a base temperature of 5 °C and a maximum cutoff temperature of 30 °C GDD (NOAA method), similarly to other subtropical plants such as maize. The calculation procedure of the cutoff method assumes that the maximum sustainable *P. tinctorium* growth rate is 30 °C in the absence of other limiting conditions. Higher temperatures do not sustain faster growth rates due to water stress.

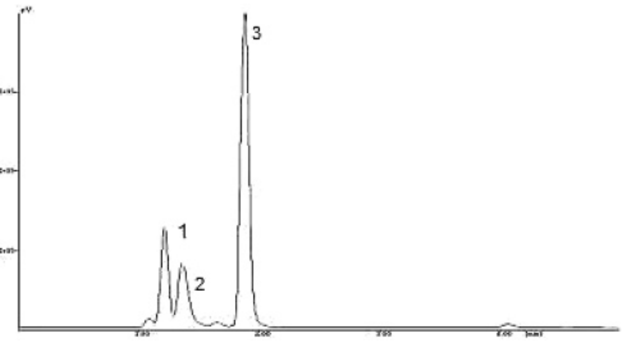

Figure 1. Typical chromatogram of the 100 °C water extract of *P. tinctorium* leaves: 1, unidentified compound; 2, glucose; 3, indican.

Parameters Analyzed. Plants were harvested by cutting stems 10 cm above the soil level at the beginning of the flowering phase when they had reached their maximum height. Subsequent harvests were taken when inter-row closure was complete and maximum height had been regained. Measurements made on individual harvests (leaves, stems, and total fresh and dry plant yields in t ha^{-1}) were summed to estimate crop seasonal yield. Samples were taken from an area of at least 2 m^2 per plot in the middle rows, and a minimum of 24 plants per plot were sampled for each parameter. At subsequent harvesting times indican content (g kg^{-1}) was measured to estimate the potential seasonal yield of indigo from the *P. tinctorium* crop.

Sample Collection and Extraction Procedure. Ten leaf samples from each field experimental plot were taken before harvesting. Leaf disks (1 cm in diameter) were obtained from the central part of the leaf (excluding veins) and immediately transferred into a glass tube with deionized water in a 1:10 weight/volume ratio. Indican water extraction was carried out at 100 °C in a boiling bath for 7 min.

Indican Determination by HPLC-ELSD Method. Leaf water extracts were diluted 1:10 (v/v) with water and 20 μL aliquots injected into the HPLC system (Jasco PU980) coupled with an evaporative light scattering detector (ELSD 2000, Alltech), according to the method of Angelini et al. (*9*). Indican was separated on an X-Terra RP18 3.5 μm Waters column. Analysis was performed in a solution containing 68% H_2O, 32% acetonitrile, and 1‰ formic acid, in isocratic conditions with a flow rate of 0.8 mL/min. The detector program was set to a drift tube temperature of 107 °C, gas flow of 2.7 SLPM, attenuation 2/5, and impactor off. Chromatograms were acquired online, and data were collected via a Jasco interface (Hercules 2000) and analyzed using a Jasco-Borwing 2000 data system. The indican peak was identified using as a marker synthetic indican from Sigma (95% purity), dissolved in water and quantified by a calibration curve (indican concentration vs peak area) obtained by using serial dilutions from a 100 μg mL^{-1} stock solution. The theoretical indigo amount obtainable from the complete reaction of indoxyl was predicted by stoichiometric calculations. In the typical *P. tinctorium* chromatogram (**Figure 1**), the indican peak (3) was the main one, with a retention time (RT) of 1.9 min. A further peak (2) with RT of 1.3 min was identified as glucose by comparison with standard glucose, whereas peak 1 (RT of 1.1) had not been identified yet. The method, fully described by Angelini et al. (*9*), allowed a sensitive and reproducible resolution of samples in a short running time (5 min).

Statistical Analysis. All variables were analyzed by ANOVA using a standard split-plot experimental design to test the significance of differences associated with growth season (2001, 2002), lines (PT1, PT3, PT4), and harvesting time (C1, C2, C3) and to estimate their reciprocal interactions. Significantly different means were separated at the 0.05 probability level by the least significant difference (LSD) test (*10*).

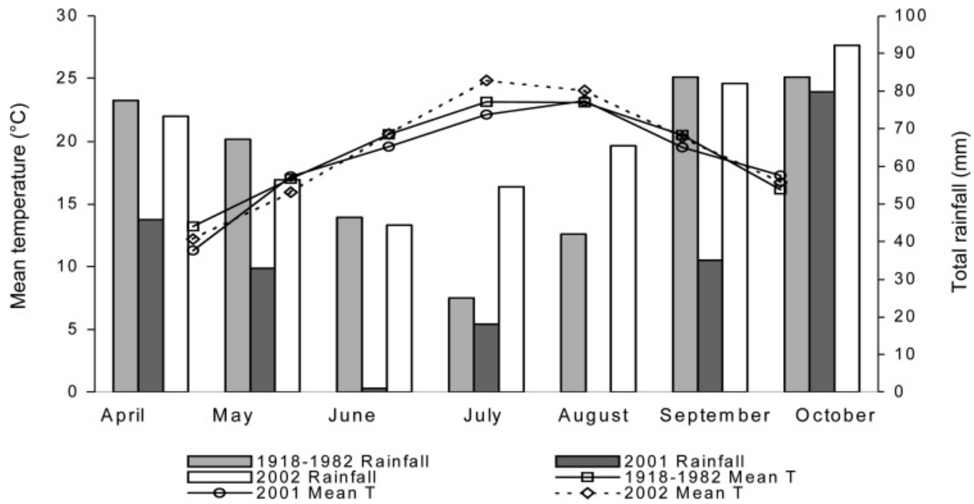

Figure 2. Total rainfall (mm) and mean air temperature (°C) from April to October in 2001 and 2002 growing seasons in comparison with long-term 1918–1982 data for the same site.

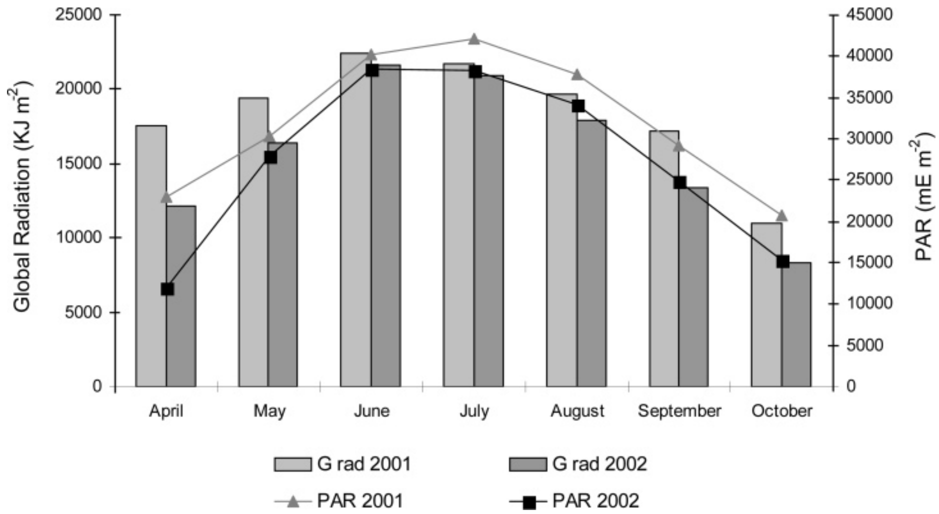

Figure 3. Mean monthly PAR (mEinstein m^{-2}) and global radiation (kJ m^{-2}) measured from April to October.

RESULTS AND DISCUSSION

Total rainfall per month and mean air temperatures were measured in 2001 and 2002 and compared to the long-term trend (**Figure 2**). Considerable variability in rainfall amount and distribution was observed between the two years (213 and 468 mm from April to October in 2001 and 2002, respectively), with the 2002 value falling closer to the long-term average (426 mm from April to October). In 2001 rainfall was significantly lower than in 2002 and lower than the long-term trend, with a very dry spring (7.1, 61.6, and 110 mm from the second half of May to the 10th of July, respectively) and a dry summer. In 2002, rainfall was evenly distributed until the end of May, whereas June, July, and August were particularly rainy, with a total rainfall of 164.4 mm versus 19 mm in 2001 and 113.4 mm in the long-term period.

Mean air temperatures recorded in the two experimental seasons showed the typical long-term trend, with an increase from sowing to July. From April to July mean daily air temperatures ranged from 10 to 26 °C (maximum temperatures from 16 to 32 °C). The highest values were recorded at the end of July, with a decreasing trend observed thereafter. The highest mean daily air temperature in 2002 was 29 °C versus 27 °C in 2001. In particular, higher mean temperatures recorded in the summer correlated to higher maximum temperatures.

Cumulative daily PAR (mean values per month in mEinstein m^{-2}) and global radiation (mean values per month in kJ m^{-2}) are reported in **Figure 3**. Values recorded in 2002 were lower than in 2001. Furthermore, 2002 weather conditions were unstable throughout the summer, with no sustained periods of high irradiance. In fact, the 2002 summer daily total PAR was ~35000 mEinstein m^{-2} day^{-1}, slightly below the 2001 value (40000 mEinstein m^{-2} day^{-1}).

The contrasting climatic conditions between the two growing seasons under study significantly affected biomass production, which was higher in 2002 (**Table 1**). We found that *P. tinctorium* growth requirements were for high air temperature, with a minimum temperature of 10 °C as typical for warm-season crops such as maize. The life cycle from sowing to the last harvest was accomplished in 229 (year 2002) and 238 (year 2001) days when the crop had accumulated 2017 °C (year 2001) and 2018 °C (year 2002) GDD (**Table 1**). Three harvests per year, from July to November, were possible in the years 2001 and 2002. Seasonal whole plant yield was significantly higher in 2002, due to higher rainfall as well as higher air temperatures. Amounts of fresh leaves, representing the actual economic yield, were also greater in 2002, and the indican content was significantly higher, with a potential indigo yield of up to 326 kg ha^{-1}. Dry yield was not statistically different between the two years, reaching an average of 15.6 t ha^{-1} (with dry leaves

Table 1. Cycle Length, Seasonal Crop and Leaf Yields, Mean Indican Content, and Indigo Yield on Fresh and Dry Weight (FW and DW) in the 2 Years of Trial[a]

	cycle length		crop yield, $t\ ha^{-1}$ FW	leaves, $t\ ha^{-1}$ FW	indican, $g\ kg^{-1}$ FW	crop yield, $t\ ha^{-1}$ DW	leaves, $t\ ha^{-1}$ DW	indican, $g\ kg^{-1}$ DW	indigo yield	
	days	GDD,[b] °C							$kg\ ha^{-1}$ FW	$kg\ ha^{-1}$ DW
2001	238	2017	81.9 b	37.1 b	12.9 b	15.3	6.88	48.6 b	214.3 b	156.1 b
2002	229	2018	118.5 a	47.3 a	15.3 a	15.9	6.83	79.2 a	325.5 a	247.2 a
signif	ns	ns	*	*	*	ns	ns	**	**	**

[a] Mean values of the three *P. tinctorium* lines examined. [b] Growing degree days as accumulated daily mean temperature with a base temperature of 10 °C and a maximum cutoff temperature of 30 °C (NOAA method).

Table 2. Plant Height, Seasonal Crop Yield, Leaf Yield, Harvest Index, Mean Indican Content, and Indigo Yield in Three *P. tinctorium* Lines[a]

	height, cm	crop yield, $t\ ha^{-1}$ FW	leaves, $t\ ha^{-1}$ FW	indican, $g\ kg^{-1}$ FW	crop yield, $t\ ha^{-1}$ DW	leaves, $t\ ha^{-1}$ DW	harvest index, %	indican, $g\ kg^{-1}$ FW	indigo yield $kg\ ha^{-1}$ FW	indigo yield $kg\ ha^{-1}$ DW
PT 1	65.43	104.5 a	41.7	14.8	15.4	6.58	42.8 b	66.0	283.7	202.5
PT 3	66.63	105.7 a	43.1	13.8	16.3	6.78	41.7 b	61.8	266.6	195.6
PT 4	56.43	90.5 b	41.8	13.7	15.2	7.20	47.5 a	63.7	259.4	211.6
signif	ns	*	ns	ns	ns	ns	*	ns	ns	ns

[a] Mean values of the two growing seasons. Mean values followed by the same letter are not significantly different according to the LSD test from ANOVA analysis; * and ns indicate significance at $P \leq 0.05$ and nonsignificance, respectively

Table 3. Total Fresh and Dry Crop Yield, Leaves, Mean Indican Content, and Indigo Yield on Fresh Weight in Three *P. tinctorium* Lines Tested in 2001 and 2002[a]

		total crop yield, $t\ ha^{-1}$ FW	total leaves, $t\ ha^{-1}$ FW	indican, $g\ kg^{-1}$ FW	total crop yield, $t\ ha^{-1}$ DW	total leaves, $t\ ha^{-1}$ DW	indican, $g\ kg^{-1}$ DW	indigo yield $kg\ ha^{-1}$ FW	indigo yield $kg\ ha^{-1}$ DW
2001	PT 1	85.4 a	37 ab	13.55	14.85	6.57	50.67	223.2 ab	155.3
	PT 3	90.2 a	39.2 a	13.33	16.46	6.96	49.58	234.0 a	159.2
	PT 4	70.1 b	35.1 b	11.86	14.63	7.10	45.42	185.6 b	153.9
	signif	*	*	ns	ns	ns	ns	*	ns
2002	PT 1	123.6	46.46	16.09	15.94	6.59 b	81.39	344.0	249.7
	PT 3	121.1	46.98	14.29	16.1	6.61 b	74.10	299.2	222.5
	PT 4	110.9	48.53	15.46	15.67	7.28 a	81.99	333.2	269.2
	signif	ns	ns	ns	ns	*	ns	ns	ns

[a] Values followed by the same letter are not significantly different according to the LSD test from ANOVA analysis; * and ns indicate significance at $P \leq 0.05$ and nonsignificance, respectively.

accounting for 44% of weight). *P. tinctorium* being a subtropical species, the high rainfall recorded in summer 2002 coupled with high temperatures positively affected both biomass yield and indican production (**Table 1**).

By comparing three different *P. tinctorium* lines (PT1, PT3, and PT4), high crop yield and indican production stability were observed in all cases (**Table 2**). Despite their origin from different European environments, the three lines showed no significant differences in the productive parameters or in the indican content (mean value = 14.1 g kg^{-1} fresh leaves) and estimated seasonal indigo yield (mean value = 270 kg ha^{-1}). The only exception was represented by PT4, which showed a significantly lower crop fresh yield. PT1 and PT3 are pink-flowered lines, whereas PT4 has white flowers. PT4 also showed lower plant height and larger leaves with shorter internodal stem length when compared to the other two lines. Those plant features influenced both total plant and leaf biomass ratios, giving significantly higher harvest index values.

Notably, dry yield was not significantly different among the three lines. In fact, the difference in rainfall during the two years of trial affected only fresh yield, measured as specific leaf weight.

None of the parameters analyzed was significantly affected by the interaction year × lines. Data from each year submitted separately to variance analysis showed that PT4 gave significantly lower biomass and indigo production in 2001, whereas in wet conditions (year 2002) PT4 indigo yield was comparable to that of the other lines (**Table 3**).

To assess the effect of harvesting time on the different lines, data were analyzed by ANOVA test (**Table 4**). In 2001 and 2002 three harvests were obtained, with comparable growth cycles. The first harvest was in July, ~12 weeks after transplanting. Plants were ready for the second harvest after 7 weeks (end of August) and for a third harvest after a further 8 weeks (end of October−beginning of November). As a general trend, greatest crop and leaf yields were achieved with summer harvests that contributed to over 77 and 70% of the yearly plant and fresh leaf production, respectively. Similarly, the first and second harvests contributed 82% of the plant yield and 75% of dry leaf yield obtained in the year (**Table 4**). In 2001, line PT4 showed significantly different dry crop production in all three harvests in comparison with the other lines, whereas in 2002, under wet weather conditions, no significant differences were observed among the three lines, either in fresh or in dry yield. In 2001 the very long dry spell, which occurred between the 20th of May and the 10th of July, negatively affected plant and leaf fresh yields mainly in PT4, which appeared to be more sensitive to drought.

Indican contents in fresh leaves from the three lines measured separately for the three harvests are reported in **Figure 4a**. In 2001, with dry weather conditions, leaves contained 15% less indican than in 2002. Indican content did not vary among the

Table 4. Fresh and Dry Mean Values of Crop and Leaf Yields in the Three *P. tinctorium* Lines from First (C1) to Second (C2) and Third (C3) Harvests[a]

		C1				C2				C3			
year	line	crop yield, t FW ha^{-1}	leaves, t FW ha^{-1}	crop yield, t DW ha^{-1}	leaves, t DW ha^{-1}	crop yield, t FW ha^{-1}	leaves, t FW ha^{-1}	crop yield, t DW ha^{-1}	leaves, t DW ha^{-1}	crop yield, t FW ha^{-1}	leaves, t FW ha^{-1}	crop yield, t DW ha^{-1}	leaves, t DW ha^{-1}
2001	PT 1	27.1 a	11.79	6.58 ab	2.64	35.7	13.4	5.61 b	2.26 b	22.6	11.85	2.65 a	1.68 a
	PT 3	30.7 a	13.27	7.64 a	2.88	34.8	13.5	5.85 ab	2.25 b	24.7	12.41	2.96 a	1.82 a
	PT 4	18.2 b	9.91	5.49 b	2.88	32.2	13.9	7.08 a	2.97 a	19.7	11.25	2.06 b	1.24 b
	signif	*	ns	*	ns	ns	ns	*	*	ns	ns	*	*
	mean	**25.31**	**11.66**	**6.57**	**2.80**	**34.23**	**13.58**	**6.18**	**2.49**	**22.3**	**11.84**	**2.56**	**1.58**
2002	PT 1	57.45	16.97	8.20	2.75	47.3 a	18.56	5.31	2.34	18.87	10.93	2.44	1.51 b
	PT 3	50.86	15.49	7.55	2.46	44.8 a	17.43	5.35	2.34	25.5	14.06	3.16	1.81 ab
	PT 4	47.2	15.93	7.90	2.87	38.8 b	17.15	4.45	2.23	24.97	15.46	3.31	2.18 a
	signif	ns	ns	ns	ns	5.90	ns	ns	ns	ns	ns	ns	*
	mean	**51.84**	**16.13**	**7.88**	**2.69**	**43.6**	**17.71**	**5.04**	**2.30**	**23.11**	**13.48**	**2.97**	**1.83**

[a] Mean values of each harvest followed by the same letter are not significantly different according to the LSD test from ANOVA analysis; * and ns indicate significance at $P \leq 0.05$ and nonsignificance, respectively.

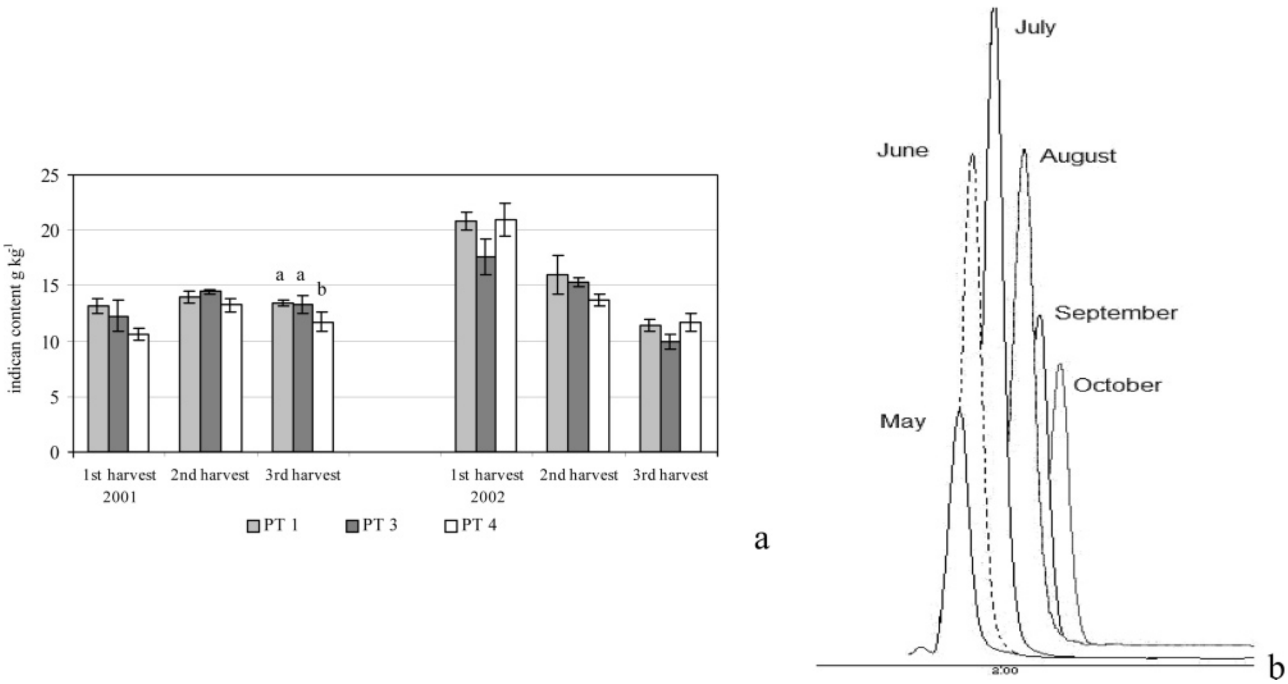

Figure 4. (**a**) Indican content in the three *P. tinctorium* tested lines during the growing seasons 2001 and 2002. Only the third harvest in 2001 gave a significantly different production of indican among the lines, whereas in all other harvests no significant differences are observed. (**b**) Indican peaks of *P. tinctorium* PT4 fresh leaf water extracts from May to October 2002.

three lines either in the first or in the second harvest, whereas PT4 showed a significantly lower indican content in the October harvest. In 2002 a significant regular trend of decrease was observed in all lines from the first to the third harvest. Just before the first harvest (July) the production of indigo precursor was enhanced by high temperatures associated with high rainfall and PAR intensity. In October, PAR decrease combined with a reduced ability to regrow negatively affected indican production as documented by significantly lower mean indican values. Indeed, *P. tinctorium* is a short-day plant, and after the second harvest, due to longer nights, plants switched rapidly to the flowering phase, leading to reduced leaf production. Indican peaks from the chromatograms of water extract of PT4 leaves analyzed from May to October 2002 were overlaid in **Figure 4b**. The indican content increased from May to July and then started to decrease until October, confirming the trend presented before.

Fresh and dry mean yields in plants and leaves and mean indican contents relative to the three harvests in 2001 and 2002 are presented in **Figure 5**. In 2001 the highest plant production (fresh and dry) was gained with the second harvest. Indeed, from transplanting to the first harvest plants were negatively affected by dry weather conditions, which did not allow the expected yield. In 2002, rain was evenly distributed from transplanting to the first harvest, which gave the highest plant and leaf yields. Similarly, indican content was highest in leaves harvested at the end of August 2001 and at the end of July 2002. In both 2001 and 2002 a significant decrease of plant and leaf yields was observed with the third harvest in autumn (**Figure 5**).

To test the hypothesis that active photosynthetic radiation can influence indican leaf production, indican content was correlated to seasonal changes by bulking data and plotting them against PAR total daily values (**Figure 6**). Indican content in leaves was positively affected by PAR intensity according to the model $y = 0.0004x + 8.566$, with $R^2 = 0.67$ and Pearson product moment correlation coefficient = 0.818 ($P < 0.01$). Indican content ranged from 12 to 25 g kg^{-1} of fresh leaves, with values of daily PAR from 10000 to 40000 mEinstein m^{-2} (recorded in May and at the end of July−beginning of August, respec-

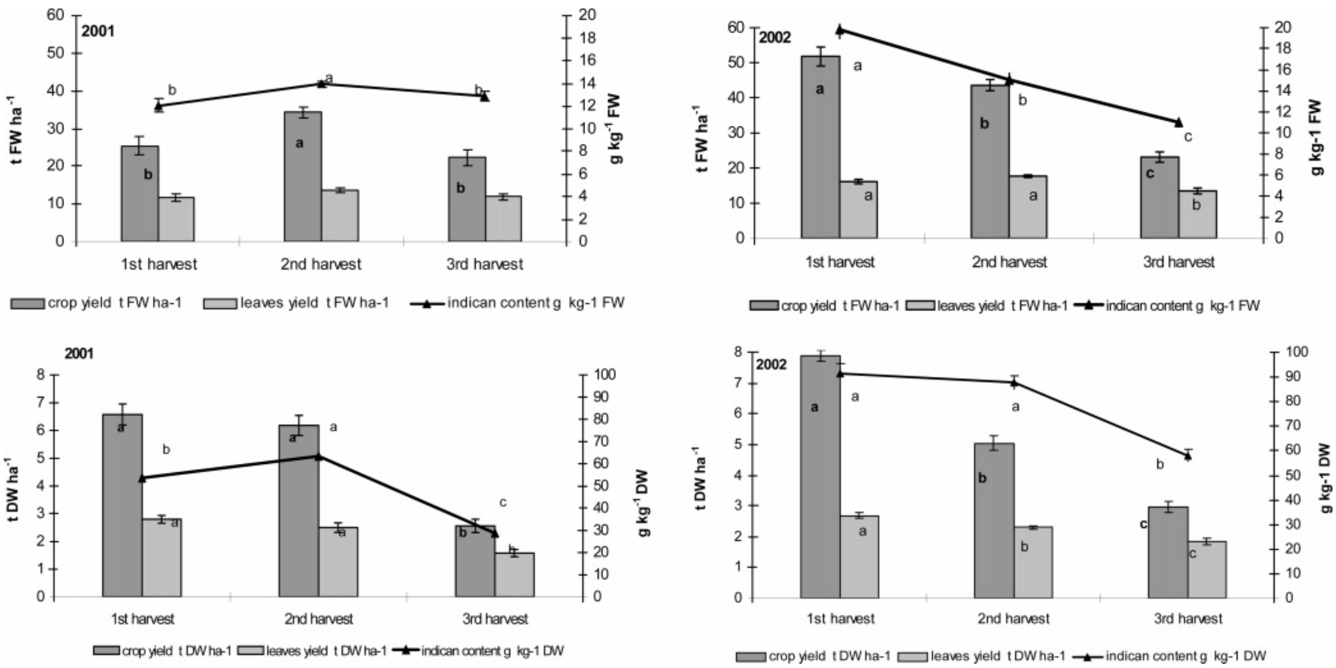

Figure 5. Fresh and dry plant and leaf yields (t ha^{-1}) and mean indican content relative to fresh and dry weight in three *P. tinctorium* lines: first harvest (July); second harvest (August); third harvest (October–November). Different letters show significantly different results according to the LSD test from ANOVA analysis.

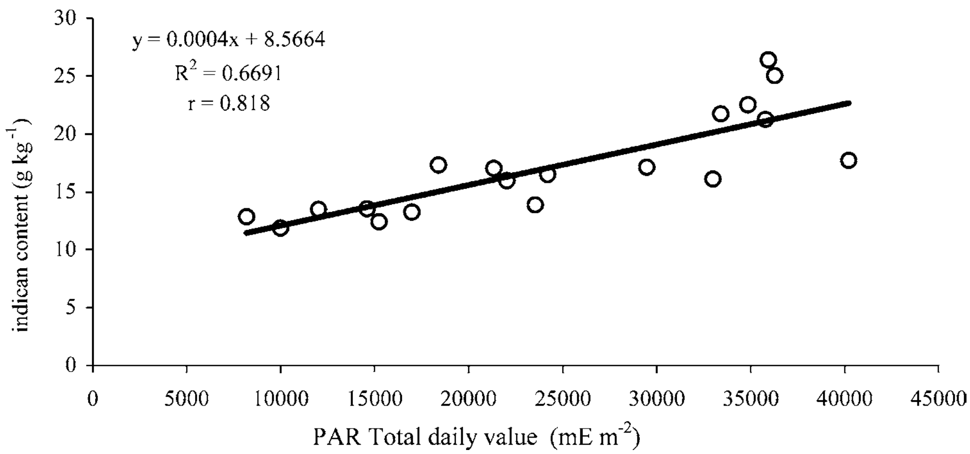

Figure 6. Relationship between indican, leaf content in *P. tinctorium* leaves, and total daily PAR in mEinstein m^{-2}. Fully expanded mature leaves from the upper part of the plants were sampled every 15 days from May to November. Data collected in the two years were bulked.

tively). Whenever rainfall does not represent a limiting factor, a very high indican content, with a potentially high indigo yield, can be obtained in the geographical area of investigation.

Indican is a secondary metabolite the physiological significance of which in *P. tinctorium* leaves is still uncertain. The observation that indican amount appears to be positively affected by light intensity suggests that light can be crucial in influencing the production of indigo. To increase profitability, the *P. tinctorium* growing cycle on field scale should be timed to obtain a growth peak in June−July when the maximum solar radiation level occurs. Furthermore, soil water availability in nonlimiting conditions should be guaranteed during the summer, to allow high accumulation of indican in the leaves. In such conditions indigo yields of up to 300 kg ha^{-1} can be achieved.

Although *P. tinctorium* grows in subtropical habitats, it was possible to cultivate it successfully in central Italy under temperate climate conditions. *P. tinctorium* behaved like a spring−summer annual crop, giving three harvests from July to October. Leaf yield and indican production were, respectively, 50 and 40% higher than those found in Central Europe by Biertümpfel and Vetter (*11*), where only two harvests were possible during growing season.

Further studies are needed to improve indigo production on a farm scale. According to Bechtold et al. (*6*), all steps from plant harvesting to the extraction of indigo precursors have to be carefully evaluated, because the indigo yield is influenced by all of these aspects. The present work gives a contribution to elucidate the role that environmental factors play in influencing leaf indican content and leaf production of *P. tinctorium* grown under temperate climate. This is an original research on a novel crop that has received little or no attention in relation to its potential importance as a nonfood crop for southern Europe.

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