

Glucobrassicin enhancement in woad (*Isatis tinctoria*) leaves by chemical and physical treatments

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Abstract: Woad (*Isatis tinctoria*), a long-known dye plant, is a noticeable source of indolic compounds, bioactive molecules exploitable as fine chemicals. Among these, glucobrassicin and its derivatives seem to play an antitumoral role, especially against mammary cancer. Since different *Brassicaceae*, such as broccoli and cauliflower, which are present in the human diet, contain glucobrassicin, it would be interesting to study its metabolic pathway following the fate of the pure compound *in vivo*. At present such studies are prevented by the difficulties encountered in the purification, mainly due to the lack of a rich vegetable source. Synthetic production is complicated and expensive. This study aimed to assess the possibility of enhancing glucobrassicin in woad leaves through artificial wounding and fertilisation, in the greenhouse and open field, in order to obtain high levels of the compound suitable for its purification. Jasmonic acid treatment on young woad leaves of the 'Casolavalsenio' accession is confirmed to be highly effective in the enhancement of glucobrassicin content, especially in combination with N–S fertilisation, under greenhouse conditions. For large scale production in the open field, where the use of jasmonic acid would be economically prohibitive, an alternative method of stimulation could be advantageously represented by artificial wounding that is able to provoke a remarkable increase of the compound, giving more than 1% d.w., which would allow its purification.

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Keywords: glucobrassicin; *Isatis tinctoria*; jasmonic acid; woad

INTRODUCTION

Woad (*Isatis tinctoria* L.), which belongs to *Brassicaceae* family, has been known since the Neolithic age as a dye plant. Ancient Britons and Celts used woad to colour their faces and bodies with the blue dye extracted from its leaves in order to frighten their enemies. In the Middle Ages the trade of this precious blue dye became a basis of the European economy. Its importance began to decline during the 16th century, when a cheaper blue dye derived from *Indigofera tinctoria* L. was imported from the Far East. By the beginning of 20th century, the cultivation of woad had almost disappeared in Europe, due to the more convenient industrial production of the synthetic indigo.

Woad is also a noticeable source of indole glucosinolates (GLs), a class of highly reactive compounds. GLs, secondary metabolites particularly abundant in *Brassicaceae*, are thioglucosidic compounds with a common structure and a varying aglycon side chain. According to the type of this chain they are classified into aliphatic, aromatic and indole GLs. Among the latter, glucobrassicin (GBS), together with other indol-type GLs, was isolated from *Isatis tinctoria*.^{1–3} In plant cells, GLs co-occur with the endogenous enzyme myrosinase (β -thioglucoside glucohydrolase, EC 3.2.1.147),

being spatially separated. They play a defensive role which is activated after a tissue has been damaged: when cells are crushed, compartmentalisation is disrupted and GLs can be degraded by myrosinase-catalysed hydrolysis mainly into isothiocyanates and/or nitriles, which are bioactive molecules effective against phytopathogenic fungi, nematodes, weeds and also against human tumoural cell lines.^{4–8} The myrosinase–GL system can represent also a mechanism of recognition for specialised pathogens, as for *Phyllotreta cruciferae*, an insect belonging to *Chrysomelidae* that attacks several *Brassicaceae*.⁹

In particular, GBS hydrolysis gives an unstable isothiocyanate, which spontaneously forms indole-3-carbynyl (Fig. 1). Considerable evidence indicates antitumoural activity related to this compound, as well as to some of its by-products (e.g. 3,3'-diindolylmethane), especially against human mammary cancer.^{10–12} These findings have suggested a possible protective effect against breast cancer associated with the consumption of GBS-containing vegetables, such as broccoli or Brussels sprouts, due to a shift in oestrogen metabolism.¹³ Moreover, a recent study has pointed out a significant correlation between increased consumption of dietary GBS and decreased

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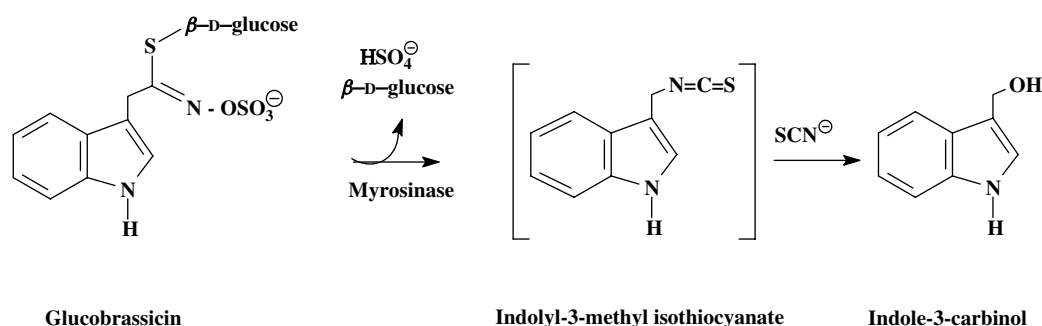


Figure 1. Myrosinase-catalysed hydrolysis reaction of glucobrassicin.

levels of urinary metabolites of tobacco-specific lung carcinogen in smokers.¹⁴ The consumption of vegetables rich in GBS is thought to yield significant amount of indole-3-carbinol or related substituted compounds, but *in vivo* studies on GBS degradation pathway were not yet carried out, due to the difficulties to obtain sufficient amounts of pure compound to perform experiments. GBS is in fact usually present at quite low concentration in most vegetable sources, like broccoli or cauliflower, and always mixed together with other indole GLs, therefore complicating its extraction in pure form. GBS organic synthesis is otherwise difficult and expensive.^{15,16} Recently, screening among different accessions of Italian woad highlighted the sample that came from 'Casolavalsenio' as having a relatively abundant leaf content of GBS and for a lack of other types of GLs.¹⁷ Moreover, treating the leaves with jasmonic acid (JA) provoked a five-fold increment of GBS, due to a selective induction of indole GLs, as already described for other *Brassica* species.^{18–21} JA is a well known endogenous bioregulator, acting at low concentration, that can exert inhibitory effects or promote the synthesis of storage proteins as well as compounds involved in protection mechanisms.²² Considering the high cost of JA, it would be interesting to find alternative ways to enhance the content of GBS in the leaves of 'Casolavalsenio', prior to GBS extraction, in order to set up a model of large scale production at low cost for this compound.

There are other factors that can positively affect the biosynthesis of indolic GLs such as foliar wounds provoked by insect attack, fungal infection, and artificial punctures.^{23,24} More generally, agronomic practices can also affect GL content, as a balanced sulfur and nitrogen nutrition.²⁵ In particular, since sulfur is part of the molecule, a lack of this element could represent a limiting factor, as shown for *Brassica juncea* L. and *Brassica napus* L.²⁶ The effect of leaf wounding and combined S–N fertilisation was thus assayed under greenhouse and field conditions to verify the possibility of producing GBS from woad leaves. As a result of a new purification method that has been published recently,²⁷ the availability of GBS in good amounts and at low cost could finally permit *in vivo* studies to be performed in order to clarify the anti-cancer role of GBS-rich vegetables, like broccoli, in the human diet.²⁸

MATERIALS AND METHODS

Materials

The woad seed utilised belongs to the Italian accession of 'Casolavalsenio', which is grown at a nursery managed by Bologna province (Italy) together with a mountain community.

Greenhouse experiment

In 1998, 'Casolavalsenio' seeds were sown, without separating them from the siliqua, in plastic 70 × 80 cm trays filled with natural loamy soil, according to a completely randomised block design with two replicates (trays), obtaining 96 plants per trays. Trays had been regularly watered at field capacity throughout the experiment. Plants were grown at 14/10 h day/night cycles with temperature ranging from 15 to 25 °C and relative humidity (RH) from 70 to 90%.

Treatments were represented by:

- superficial abrasions of the leaves by carborundum powder
- puncturing of the leaves by a brass brush, obtaining about 25 holes cm⁻²
- spraying a 5 mmol L⁻¹ solution of JA (Sigma-Aldrich S.r.l. Milan, Italy) in 0.1% Triton X-100 (Sigma-Aldrich).
- control (untreated)

and were performed on 1-month-old plants, grown at two nitrogen and sulfur levels: 0 and 100 kg ha⁻¹ for both elements, applied as ammonium nitrate and potassium sulfate. Each treatment was repeated after 2 days.

Leaves were collected 48 h after the second treatment, cutting the plants at the crown level, then immediately stored at –20 °C and freeze-dried prior to GBS analyses.

Field experiment

In the spring of 1999 'Casolavalsenio' seeds were sown at the experimental farm of the University of Bologna, at Cadriano (Italy). The experimental design was a split–split plot with three replicates and three factors:

- nitrogen fertilisation, supplied as ammonium nitrate at sowing on whole plots at two levels: 0 and 100 kg ha⁻¹

- sulfur fertilisation, supplied as potassium sulfate at sowing on sub-plots at four levels: 0, 100, 200 and 300 kg ha⁻¹
- treatments, i.e. leaf puncturing and control (not treated) on sub-sub-plots, each measuring 1 m × 1 m.

Seeds were manually sown in rows at 2 cm depth and 20 cm between rows. The field was watered three times a week and weeds were removed manually. An emergence of 65% was observed with a final investment of about 120 plants m².

Leaves were punctured by a brass brush 40 days after sowing, repeating the treatment after 2 days as in the greenhouse experiment. All the leaves from the three central rows of each plot were collected 48 h after the second treatment, immediately stored at -20 °C, then freeze-dried prior to analyses.

Phyllotreta atra experiment

About 20 exemplars of *Phyllotreta atra* F. coming from the experimental plots were collected and confined in an isolator together with 12 woad plants, in pots, at the two-leaf stage, in a greenhouse. Outside the isolator, 12 plants in pots were used as the control. Insects were allowed to feed on the leaves for 1 day, then leaves from the two groups were collected and freeze-dried prior to analyses of GBS.

Glucosinolate analyses

After collection, woad leaves were freeze-dried with an Edwards Pirani 1001 Minifast Do.1 lyophiliser (Milan, Italy), then ground to powder and stored in a desiccator. Each sample was analysed twice according to the ISO 9167-1 official method.²⁹ About 400 mg aliquots of freeze-dried leaves were extracted by adding 5 mL of boiling 70% ethanol plus 200 µL of standard solution of sinigrin as internal standard. The mixture was homogenised for 5 min at 75 °C using a U-Turrax T25 (IKA-Werke, Staufen, Germany) homogeniser and the extracts were centrifuged. Residue was re-extracted with further 5 mL of boiling 70% ethanol and re-centrifuged. Supernatants were combined to give a final volume of 10 mL. Each extract (1 mL) was loaded into a minicolumn filled with 0.6 mL of DEAE Sephadex A-25 anion exchange resin (Amersham Biosciences, Milan, Italy) conditioned with 25 mmol L⁻¹ acetate buffer pH 5.6. After washing with 3 mL of buffer, 150 µL of purified sulfatase³⁰ were loaded into the minicolumn and were left overnight at room temperature. The desulfo-GLs were then eluted with 3 mL of distilled water and finally used for high-performance liquid chromatography (HPLC). Desulfo-GLs were analysed using an Agilent Model 1100 HPLC system (Agilent Technology Italia S.p.A, Milan, Italy) with an Inertsil ODS3 column (250 × 3 mm, 5 µm). Chromatography was performed at a flow rate of 1 mL min⁻¹, at 30 °C, by eluting with a gradient of water (A) and acetonitrile (B) as follows: isocratically 1% B for 1 min, linear gradient to

22% B for 21 min, linear gradient to 1% B for 3 min. Elution of desulfo-GLs was detected by a diode array, monitoring the absorbance at 229 nm, and the amount of GBS was determined using sinigrin as the internal standard and the relative response factor.³¹ GBS was identified by comparison with a synthetic reference sample and characterised by ¹H- and ¹³C-NMR spectroscopy, in agreement with previous reports.^{15,27}

Statistical analyses

Data were submitted to analysis of variance by the MSTATC 2.10 program and the means were separated using Duncan's test at a significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Greenhouse experiment

In accord with the method given by Bodnarik and Palaniswamy,²⁴ woad leaves were repeatedly treated (physically or chemically) in order to increase the effect on GBS induction, allowing plants to react between the treatments and produce GBS. Moreover, we chose to treat young leaves because (1) they are biosynthetically more active than mature leaves, and (2) some authors have reported a dilution effect, i.e. a decrease of GL concentration correlated with age, due to leaf expansion.^{26,32,33}

In order to obtain samples containing only GBS, to facilitate a subsequent purification, at leaf collection the cutting was carefully maintained above the crown, because woad roots contain GLs (both aliphatic and indolic) other than GBS.¹⁷ It was ensured that the leaves were not damaged, in order to avoid myrosinase action, and therefore GBS breakdown.

Freeze-dried leaves of 'Casolavalsenio' woad contained a strongly predominant individual GLs such as GBS, as pointed out in a previous study¹⁷ (Fig. 2).

Analysis of variance revealed a significant enhancement of GBS content due to both fertilisation and treatments, with a significant interaction between the factors studied. Fertilisation alone gave a three-fold increment of GBS, if compared to the unfertilised and untreated control (Table 1). Moreover, the highest levels of GBS reached in the fertilised trays underlines

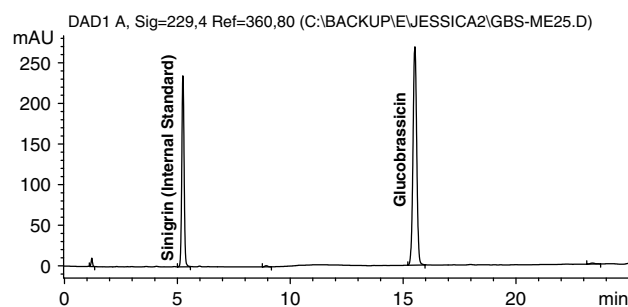


Figure 2. HPLC chromatogram of woad leaves of 'Casolavalsenio' accession. Injection volume: 20 µL; internal standard concentration in the injected solution: 0.23 mmol L⁻¹.

Table 1. Glucobrassicin content ($\mu\text{mol g}^{-1}$ d.w.) in freeze-dried leaves of *Isatis tinctoria*, 'Casolavalsenio' accession, in relation to the different treatments and to S–N fertilisation levels (0 and 100 kg ha⁻¹), in the greenhouse experiment

Treatment	Glucobrassicin	
	N ₀ + S ₀	N ₁₀₀ + S ₁₀₀
Control	8.1 ± 0.4 ^c	23.9 ± 1.0 ^c
Abrasions	17.8 ± 0.4 ^b	28.7 ± 0.4 ^b
Puncturing	19.7 ± 0.6 ^b	30.2 ± 0.6 ^b
Jasmonic acid	58.9 ± 3.6 ^a	63.4 ± 3.3 ^a

N₀, N₁₀₀, S₀ and S₁₀₀ refer to nitrogen and sulfur levels of 0 and 100 kg ha⁻¹, respectively.

Each value represents the mean ± standard deviation of four data (two replicates in greenhouse, each sampled twice). Means within a column followed by the same letter are not statistically different at $P \leq 0.05$ (Duncan's test).

the importance of a balanced availability of S and N for GBS biosynthesis, as generally observed for GLs.^{25,34}

Among treatments, application of JA was the most effective, with a seven-fold enhancement of GBS content if compared to the untreated and unfertilised control and 2.5-fold if compared to the fertilised control (Table 1). This finding confirms once again the effectiveness of this compound in enhancing the indolic GL content in woad leaves, as observed in a previous study.¹⁷ Since the use of JA on a large scale is economically unfeasible, it was inserted in this experiment just as control to compare the effectiveness of alternative and cheaper treatments, such as mechanical wounding, in combination with fertilisation, with a view to obtaining suitable amounts of enriched green material for subsequent purification of the molecule.

Mechanical wounding by leaf puncturing significantly increased the GBS content, when compared to controls, even though it was to a lesser extent than JA treatment (Table 1). This was consistent with literature that reports similar effects on indole GLs in other *Brassicaceae*.^{18–20,23,24} In particular, the increment was more evident in the unfertilised trays, which showed double the control value. No significant differences were found between the two kinds of physical treatment, at both fertilisation levels, meaning that superficial abrasions are able to transmit a

wounding-like signal, obtaining the same effect as puncturing on GBS response (Table 1).

Finally, the mean GBS level of about 29.5 $\mu\text{mol g}^{-1}$ d.w. obtained in this experiment by combining fertilisation and physical treatments, even if considerably lower than those obtained with JA, was judged suitable for purification of the molecule. As GBS has a molecular weight of 486.6 (as the potassium salt), the GBS content in the treated woad leaves would theoretically correspond to about 14 g kg⁻¹ of freeze-dried material, a concentration that is considered widely acceptable when active principles are purified from vegetable material.

Field experiment

The field trial was set up to study the possibility of producing GBS on large scale by treating woad leaves with alternative means than the costly JA and to better elucidate the role of nitrogen and sulfur nutrition, which in the greenhouse trial were supplied together and at a unique dose. As an alternative to JA treatment, leaf puncturing instead of abrasions was chosen here because the first treatment showed similar effects to the second on GBS production in the greenhouse, but the intensity of damage caused by puncturing was more controllable and, theoretically, eventually reproducible by a machine used in an open field.

The split-split plot experimental design was necessary because of the low amount of woad seed available. Table 2 shows the mean values of GBS obtained in the field trial for each situation. No significant interactions were highlighted among the factors examined (N, S, wounding) by ANOVA analyses. As a mean value, the wounding treatment significantly increased GBS content by 30% (F test significant at $P \leq 0.001$). On the contrary, the different levels of N and S fertilisation did not significantly affect the leaf content of GBS, in contrast with that observed in the greenhouse, even if a trend of an increasing GBS content was observed with the first S dose (100 kg ha⁻¹) (Table 2). The absence of significant effects of fertilisation at statistical level could be due to the relatively high GBS content of the untreated and unfertilised control (16.1 $\mu\text{mol g}^{-1}$ d.w.), which was almost double compared to that

Table 2. Glucobrassicin content ($\mu\text{mol g}^{-1}$ d.w.) in freeze-dried leaves of *Isatis tinctoria*, 'Casolavalsenio' accession, untreated or treated by puncturing, in relation to different combinations of S–N fertilisation (S: 0–300 kg ha⁻¹; N: 0–100 kg ha⁻¹), in the field experiment

	Glucobrassicin					
	N ₀		N ₁₀₀		Mean	
	Control	Treated	Control	Treated	Control	Treated
S ₀	16.1 ± 4.9	27.1 ± 8.9	14.7 ± 8.6	26.6 ± 6.1	15.4 ± 1.0	26.9 ± 0.4
S ₁₀₀	21.4 ± 6.8	30.6 ± 12.5	22.5 ± 12.1	29.2 ± 14.0	22.0 ± 0.8	29.9 ± 1.0
S ₂₀₀	21.4 ± 11.2	28.1 ± 6.6	21.1 ± 9.5	27.2 ± 5.7	21.2 ± 0.2	27.7 ± 0.6
S ₃₀₀	19.7 ± 5.7	26.8 ± 6.2	20.1 ± 6.6	30.0 ± 11.7	19.9 ± 0.3	28.4 ± 2.3
Mean	19.7 ± 2.5	28.2 ± 1.7	19.6 ± 3.4	28.2 ± 1.6	19.6 ± 2.9	28.2 ± 1.3

Each value represents the mean ± standard deviation of three field replications.

recorded in the greenhouse experiment ($8.1 \mu\text{mol g}^{-1}$ d.w.) (Tables 1 and 2). Such a high value of the control could be explained both by a residual fertilisation level of the soil and, more probably, by an unexpected attack of a phytophagous insect that caused several lesions also on the control leaves, which were therefore stimulated in a similar manner to that of the mechanical treatment. This hypothesis was verified by collecting some exemplars of the insect from the experimental plots and allowing them to feed on the leaves of woad plants in pots, under greenhouse conditions.

However, it is important to note that the mean GBS value reached by the treated leaves in the field experiment ($28.2 \mu\text{mol g}^{-1}$ d.w.) was very similar to that obtained in the greenhouse with the two physical treatments in the fertilised trays ($29.5 \mu\text{mol g}^{-1}$ d.w. as the mean value), substantially confirming the effectiveness of this method for enhancing GBS content to levels suitable for its purification.

***Phyllotreta atra* experiment**

The insect collected from the plots was classified as *Phyllotreta atra* Fabricius 1775, (*Coleoptera: Chrysomelidae: Alticinae*). *Alticinae* are highly specialised and phytophagous insects. *P. atra* is oligophagous, and host plants are Brassicas, particularly cabbage, cauliflower and rape. Insects feeding upon woad leaves under the isolator in the greenhouse caused wounds very similar to those provoked by artificial puncturing. After only 1 day of feeding, they led to a GBS increase of about 18% compared to controls (data not shown). The increase of indole GLs by insect attack is well documented: in particular *Phyllotreta cruciferae* feeding on *Brassica napus* seedlings gave an increment not significantly different from mechanical wounding.²² This could explain, in part, the lack of significant differences among fertilisation levels in the field experiment, where GBS could have been induced in the controls by insect attack.

CONCLUSIONS

In conclusion, JA treatment on young woad leaves of the 'Casolavalsenio' accession was confirmed to be highly effective in enhancing GBS content, especially in combination with N–S fertilisation, under greenhouse conditions. Since this accession contains GBS as a unique GL, to date it represents the best source of GBS for purification, together with the enhancing JA treatment described.

Mounting preclinical and clinical evidence indicates that the hydrolysis product of GBS, indole-3-carbynol, exhibits antitumour activities both *in vitro* and *in vivo*, especially towards mammary neoplasia.¹² Moreover, in a phase I clinical trial on women from a high-risk breast cancer cohort, the ingestion of indole-3-carbynol did not cause adverse effects on several hormonal variables, while a biomarker for chemoprevention increased.³⁵ Further research needs

to focus on the metabolic fate of GBS in order to clarify the role of the different GBS by-products in bringing about the anticancer properties attributed to *Brassica* vegetables and to determine which concentrations are physiologically attainable by diet. The availability of an enriched source of GBS could finally permit the purification of the compound on a gram scale, according to a high yield method published recently²⁷ and thus to perform *in vivo* experiments concerning the metabolic pathway of GBS.

The use of dietary botanicals for inhibiting cancer cell growth is increasingly being appreciated, but the question remains regarding the actual availability of these plant-derived agents once their health benefits have been unequivocally demonstrated. With this in mind a large scale production of GBS was evaluated. In the open field, where the use of JA is economically prohibitive, an alternative way of stimulation could be advantageously represented by artificial wounding that is able to provoke a remarkable increase of the compound, to more than 1% d.w., which would allow its purification. GBS, in fact, reached the same level of the corresponding treatment in a controlled environment, thus demonstrating that an enriched source of GBS is easily obtainable at low cost also in the open field. The crop cycle is very short, since the treatment is performed on young plants, but it could hypothetically be repeated, after the first collection, on the newly formed leaves, making successive harvests on the same crop, in a way similar to what is done for the dyeing crop. Otherwise, the first harvest could be dedicated to GBS production and the subsequent ones to dye extraction, not excluding obtaining an increase of the blue dye, which presents an indole structure.

In addition, the ability of *P. atra* to induce and increase in GBS could be considered in future trials: a controlled infestation of the crop could result in an effective economic and biological method to enhance GBS without any necessity of mechanical treatment.

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