Appl Biochem Biotechnol (2013) 169:624–635
DOI 10.1007/s12010-012-0017-x

Effects of Phytohormones and Jasmonic Acid on Glucosinolate Content in Hairy Root Cultures of *Sinapis alba* and *Brassica rapa*

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Received: 23 October 2012 / Accepted: 4 December 2012 /
Published online: 27 December 2012

Abstract Although some study have established hairy root cultures from brassicaceous plants with glucosinolates (GS) as characteristic secondary metabolite, studies are missing which compare hairy roots with the corresponding mother plants. Therefore, two different plant species—*Sinapis alba* and *Brassica rapa* subsp. *rapa pygmeae teltoviensis*—were transformed with the *Agrobacterium rhizogenes* strain A4. Aliphatic and indolyl GS were present in *B. rapa*, exhibiting larger quantities in leaves than in roots. Aromatic *p*-hydroxybenzyl GS were found particularly in the leaves of *S. alba*. However, the proportion of indolyl GS increased suddenly in transformed hairy roots of *S. alba* and *B. rapa*. Cultivation with the phytohormone kinetin (0.5 mgL^{-1}) enhanced GS accumulation in *B. rapa* hairy roots, however not in *S. alba*, but 2,4-D (0.4 mgL^{-1}) induced de-differentiation of roots in both species and reduced GS levels. GS levels especially of 1-methoxyindol-3ylmethyl GS increased in hairy roots in response to JA, but root growth was inhibited. While 2 weeks of cultivation in 100 to 200 μM JA were determined at optimum for maximum GS yield in *S. alba* hairy root cultures, 4 weeks of cultivation in 50 to 100 μM JA was the optimum for *B. rapa*.

Keywords Hairy root culture · Glucosinolates · *Brassica rapa* · *Sinapis alba* · Jasmonic acid · Kinetin · 2,4-D

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Introduction

Agrobacterium rhizogenes naturally causes the development of "hairy root diseases" at infection sites in several dicotyledones and some monocotyledonous plants [1]. Transformed hairy roots as an in vitro culture are considered a valuable source of root-derived secondary metabolites. Hairy roots have a number of characteristics that make them extremely advantageous for use in the production of secondary metabolites compared to cell cultures [2]. In culture, they exhibit high growth rates and stable differentiated phenotypes and consequently, biochemical stability [3]. Hairy root cultures can generally be cultivated in phytohormone-free media due to their ability to produce their own phytohormones [4]. They can also be used as model systems to investigate the biosynthesis of secondary metabolites and ways to influence, such as by apply precursors and signaling molecules [5–7]. The rol genes of *A. rhizogenes* cause the excessive root hair phenotype, due to the intensified synthesis of growth plant hormones [8]. However, they can also induce secondary metabolite biosynthesis by turning on the transcription of defense gene [9–11]. Interestingly, secondary metabolite accumulation depends on plant growth and the developmental stage; consequently, the metabolite composition of plants and hairy root cultures can differ considerably. For example, the meroterpene bakuchiol is abundant in aerial parts of *Psoralea drupacea* Bunge, but is absent in cell suspension or hairy root cultures [12]. However, hairy root cultures of *Salvia miltiorrhiza* Bunge exhibited a greater biosynthetic capacity for the production of diterpene diketones compared to the herbal mother plant [13].

Hairy roots grown under controlled cultivation conditions provide promising prospects for studies of cultures' metabolism and secondary metabolite production. Elicitation is a process to enhance the secondary metabolite production of plants using their ability to respond to different stress factors. Elicitors are defined either as biotic, e.g., polysaccharides derived from plant cell walls (pectin or cellulose), microorganisms (chitin or glucans), and phytohormones like jasmonic or salicylic acid or abiotic, e.g., inorganic salts, and physical factors like high pH or UV-B [14]. We therefore, need to understand the induced signaling pathways induced by elicitors and to gain knowledge of elicitor dosage and timing of harvest after elicitation in order to optimize secondary metabolite production. Jasmonic acid is used as an elicitor in several studies and has been found to increase metabolites such as glucosinolates, anthocyanins, and phenolic acids [15, 16]. The production of secondary metabolites in the plant tissue generally depends on cell differentiation and proliferation as well as growth regulators [17]. High levels of external auxins may promote increase hairy root growth, but increase or decrease secondary metabolite production depending on the plant species [18, 19]. Since there is no universal effect of a particular elicitor on different plants or cell culture systems and since effects are dosage dependent [14], studies on different plant species are required to determine the optimal system for producing certain secondary metabolites.

Brassicaceae comprise plants of wide economic importance and are used, for example as spices, oils, vegetables, and pharmaceuticals. Glucosinolates (GS) are the characteristic group of secondary plant metabolites found in this plant family and plants of the order Brassicales. GS consist of a sulfur-linked β-D-glucopyranose moiety and an amino acid-derived side chain [19]. According to their side chain, they are classified as aliphatic, aromatic, or indolyl glucosinolates. There is a lot of controversial data on the impact of GS on human health [20–22]. Many GS have an anti-carcinogenic effect, such as the aliphatic 4-methylsulfinybutyl GS, the indolic 3-indolylmethyl, and the aromatic 2-phenylethyl [23]. Other GS, however, have shown to have adverse effects, as it is shown for the aliphatic (2*R*)-2-hydroxy-3-butenyl GS and the indole 1-methoxyl-3-indoylmethyl GS [24].

Although hairy roots have been produced in *Brassica napus*, *Brassica nigra*, and *Brassica juncea* [25, 26], only a few studies have investigated the GS content in hairy root cultures [27–29] and none have compared the hairy root GS profiles with the corresponding mother plants. The aim of this study was therefore, to compare GS profiles of different plant parts with those of hairy root cultures and to use elicitor treatments to enhance the production of GS in root cultures. This is an addition for the development of technology to produce specific bioactive ingredients using hairy roots. Two different plant species—*Sinapis alba* (white mustard) *Brassica rapa* subsp. *rapa pygmeae teltoviensis* (teltover turnip)—with different GS profiles were chosen to this purpose and transformed using the *A. rhizogenes* strain A4.

Materials and Methods

Induction of Hairy Root Culture

Two different species of the Brassicaceae family, *S. alba* (white mustard, *S. alba*) and *B. rapa* subsp. *rapa pygmeae teltoviensis* (teltover turnip, *B. rapa*), were used for the transformation. Seeds were surface-sterilized by immersion in 70 % ethanol followed by 10 % sodium hypochlorite each for 1 min. The seeds were then rinsed three times in sterile deionized water and placed on petri dishes with hormone-free MS medium for germination. The seeds were incubated at 22 °C in 24 h light. Seedlings were used for inoculation with the *A. rhizogenes*.

The *A. rhizogenes* strain A4 was a gift from David Tepfer (INRA, France) and a continuous culture was established on MYA medium (pH=6.6). An adequate bacteria density above (OD_{600}>1.6) in the inoculation medium must be achieved for plant transformation to succeed. For this purpose, 2 ml MYA supplemented with 50 $\mu g ml^{-1}$ was inoculated with the *A. rhizogenes* strain and kept overnight on a shaker at 150 rpm and at 28 °C. The *A. rhizogenes* culture was transferred to 50 ml MYA and kept for another day on a shaker at 28 °C. After 2 days of bacterial culturing, roots from 4-day-old aseptic seedlings were placed in 2-ml tubes filled with this culture for 1 min. The roots were then placed in 250-ml flasks containing 60 ml hormone-free MS medium [30] supplemented with 3 % sucrose. One day later, cefotaxime with final concentration of 200 $mg L^{-1}$ was added to eliminate the bacterium. Only root inoculates that developed the hairy root phenotype were sub-cultivated every 2 to 4 weeks. Hairy roots (also in the experiments) were cultivated using 60 ml hormone-free MS medium (30 $g L^{-1}$ sucrose) in Erlenmeyer flasks on a shaker (110 rpm) at 26 °C in the dark. The pH of the solution was adjusted to 5.8 by adding potassium hydroxide. To conduct the experiments, 0.5 to 1.0 g sterile, fresh hairy roots was used for each replication.

Experiment to Compare Glucosinolate Profile of Plants with Hairy Root

In order to compare GS profiles of hairy root cultures with plants, 4-week-old hairy root cultures of *S. alba* and *B. rapa* were harvested in liquid nitrogen. Six replications were taken per plant species and freeze-dried for GS analysis. Six-week-old plants were used for comparison purposes. The plant were cultivated in separate pots (9 cm diameter, 8 cm height) filled with soil containing peat (Werkband e.V., Type T) in a greenhouse at about 24 °C during the day and 20 °C at night. Supplemental PAR irradiation (150 $\mu mol m^{-2} s^{-1}$) was provided to give a 16-h photoperiod. After 6 weeks, whole plants of species, including roots, were harvested. The soil was washed off the roots; the plant was cut at the base and upper plant

parts and roots were harvested separately in liquid nitrogen and freeze-dried for GS analysis. The chemical analysis was conducted in six replications.

Elicitor Treatment with Phytohormones

2,4-Dichlor-phenoxy acetic acid (2,4-D) and 6-furfuryladenine (kinetin) from the phytohormone group auxins and cytokinins respectively, were selected as elicitor treatments for the hairy root cultures. A stock solution of 0.4 g 2,4-D L^{-1} and 0.5 g kinetin L^{-1} was prepared and 1 mL was filter sterilized and added to 1 L MS medium with 200 mgL^{-1} cefatoxin each. 60 ml of the MS medium with 2,4-D, kinetin or without phytormones (control) was filled in a 250 mL Erlenmeyer flask and about 500 mg hairy root culture of *B. rapa* and *S. alba* was added. The experiment was conducted in four replications. Roots were kept on a shaker (110 rpm) at 26 °C in the dark for duration of the experiment. The fresh weight increase of hairy root cultures was determined after 14 and 28 days. Upon completion of the experiments, the hairy root culture samples were flash-frozen in liquid nitrogen, freeze-dried and used for GS analysis.

Elicitor Treatment with Jasmonic Acid

Jasmonic acid (JA, Sigma, USA) was dissolved in 5 % ethanol solution, filter sterilized (0.22 μm), and used as a stock solution for preparing 50, 100, 200, and 280 μM solutions in a hormone-free MS medium with 200 mgL^{-1} cefatoxin. The fresh weight of hairy root cultures aliquot of *S. alba* and *B. rapa* was determined. Approximately 500 g was transferred to each Erlenmeyer flask, which contained 60 ml MS medium. The experiment was conducted in three replications for each JA concentration and the control. Erlenmeyer flasks with roots were kept on a shaker (110 rpm) at 26 °C in the dark. The fresh weight was determined after 7, 14, and 28 days of treatment, by removing the root from the medium and draining for one min. The root samples were then flash-frozen in liquid nitrogen and subsequently freeze-dried for GS analysis.

Glucosinolate Analysis

GS were extracted from 20 mg lyophilized tissue in 70 % boiling MeOH and re-extracted twice as described in detail in Mewis et al. [31] before loading onto DEAE Sephadex A-25 ion exchanger (acetic acid-activated; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) mini columns. Allyl GS for *S. alba* and 4-hydroxybenzyl GS for *B. rapa* were used as an internal standard to quantify GS in extracts. After several column wash steps [20], GS in extracts were desulfated with 75 μl aryl sulfatase solution (Sigma-Aldrich Corp., H-1 from *Helix pomatia*). Desulfated extracts were analyzed by HPLC (Dionex Summit P680A APLC-System) equipped with an LPG-3400SD pump, WPS-3000SL an automated sample injector and fitted with a C-18 reverse-phase column (Acclaim TM 120, 250 mm–2.1 mm, Dionex) with solvents (A) ultrapure H_2O and (B) 40 % acetonitrile (HPLC grade in ultrapure H_2O). The multi-step gradient was run at a flow rate of 0.4 ml/min. The 43 min run consisted of 0.5 % (*v*/*v*) B (1 min), to 20 % B in 7 min, 2 min hold, to 50 % B in 9 min, 3 min hold, to 99 % (*v*/*v*) B in 6 min, 5 min hold, in 5 min back to 0.5 % B and a 7 min final hold at 1 % (*v*/*v*) B. The eluent was monitored by photodiode array detection (DAD-3000 diode array detector, Dionex) between 190 and 360 nm. GS peaks at 229 nm were identified using retention time and UV spectra. Statistical differences in GS content among treatments were determined by ANOVA followed by Tukey's HSD test (SYSTAT 9, SPSS Inc., Chicago, USA).

Results

Glucosinolate Profile of Brassicaceous Plants and Corresponding Hairy Root Cultures

The GS profile of plant organs and hairy root cultures differed considerably. Aliphatic or aromatic GS were found in *B. rapa* and *S. alba* plants, respectively, whereby indolyl GS dominated in hairy root cultures (Fig. 1). The aliphatic compounds (*R*)-2-hydroxy-3-butenyl GS, 2-hydroxy-4-pentenyl GS, and 4-pentenyl GS were found in quantitative amounts only in leaves of *B. rapa*. The 4-hydroxyindol-3ylmethyl GS was the dominant indolyl GS in leaves; 1-methoxyindol-3ylmethyl GS was the most abundant compound in roots. Roots of *B. rapa* exhibited significant higher GS levels than leaves. However, total GS levels in hairy roots did not differ to that in leaves (Fig. 1). Interestingly, only four indolyl GS were found in hairy roots: 4-hydroxyindol-3ylmethyl GS, indol-3ylmethyl GS, 1-methoxyindol-3ylmethyl GS, and 1-methoxyindol-3ylmethyl GS. In contrast, high levels of aromatic GS were detected in leaves and roots of *S. alba* with *p*-hydroxybenzyl GS as the main compound. Leaves of *S. alba* had the highest overall GS content, at about 60 $\mu mol\,g^{-1}$ dry weight. Significantly lower GS levels were determined for hairy roots and roots.

Effects of Phytohormones on Growth and Glucosinolate Content of Hairy Root Cultures

To test the possibility that phytohormones have an enhancing effect on GS accumulation and the growth of brassicaceous hairy root cultures, the MS medium was supplemented with 2,4-D and kinetin. The auxin derivative 2,4-D, at a concentration of 0.4 $mg\,L^{-1}$, had a strong effect on hairy root culture growth in both species, and the percentage fresh weight increase in 4 weeks was significantly lower in those treatments than in cultures in a hormone-free medium (Fig. 2). *B. rapa* hairy root culture was more sensitive to 2,4-D compared to *S. alba* hairy root culture, and in fact stopped growing. The cytokinin kinetin had a different effect on growth of the two different hairy root cultures. The biomass increase of *S. alba* hairy root cultures was significantly lower with kinetin at a concentration of 0.5 mL^{-1} in the medium compared to root

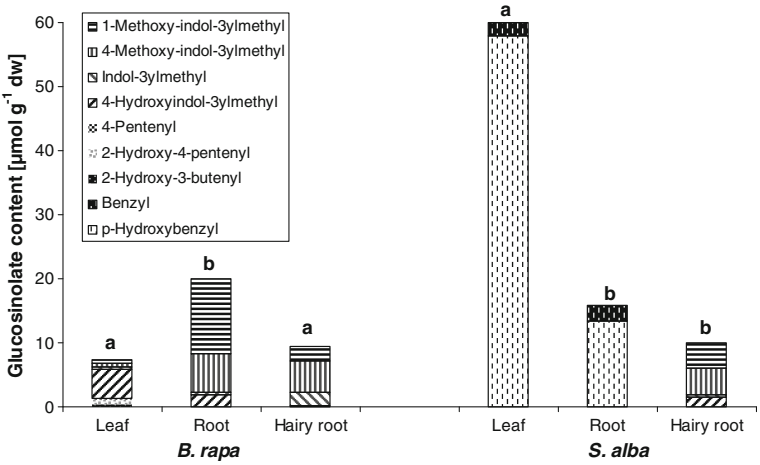

Fig. 1 Glucosinolate content in different organs of *B. rapa* and *S. alba* compared to levels in hairy root cultures (*different letters* indicate significant differences between treatments within one plant species, Tukey's test $p \leq 0.05$)

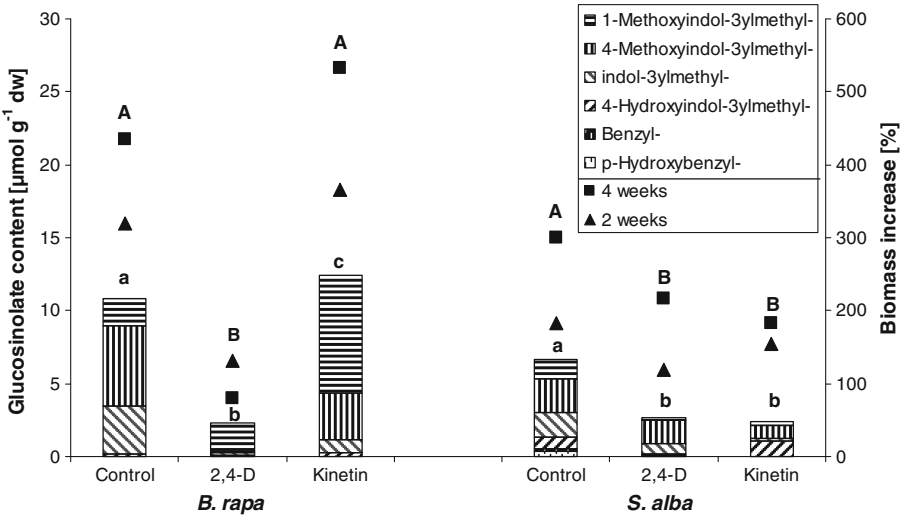

Fig. 2 *Bars* represent GS contents in hairy root cultures of *B. rapa* and *S. alba* supplemented with 2,4-D or kinetin compared to the control after 4 weeks; items indicate an increase in fresh weight after 2 and 4 weeks of cultivation; (*different small* (GS) and *capital letters* (biomass increase in 4 weeks) indicate significant differences between treatments within one plant species, Tukey's test $p \leq 0.05$)

cultures in the hormone-free medium. However, a slight, insignificant increase in percentage fresh weight was observed in *B. rapa* (Fig. 2). Supplementation with 2,4-D reduced the GS content in *B. rapa* hairy root cultures about fivefold compared to the controls, although only amounts of indol-3ylmethyl GS and 4-methoxyindol-3ylmethyl GS were significantly reduced after 4 weeks of cultivation (Tukey's test $p \leq 0.05$). 2,4-D also decreased the indolyl GS content in *S. alba* hairy root cultures (Fig. 2). Kinetin had the opposite effect on GS accumulation in the two hairy root cultures. GS levels in *S. alba* hairy root cultures were significantly lower after 4 weeks of cultivation in MS with kinetin compared to the hormone-free medium. In contrast, total GS levels were significantly higher in *B. rapa* hairy root cultures after cultivation using kinetin due to a fivefold increase of 1-methoxyindol-3ylmethyl GS compared to levels in the control (Fig. 2). However, levels of indol-3ylmethyl and 4-methoxyindol-3ylmethyl GS were lower in hairy root cultures with kinetin supplementation.

The morphological structure of hairy root cultures was influenced differently by the two phytohormones. Kinetin did not change the root morphology of cultures from both plant species as displayed in Fig. 3b and e, compared to hormone-free cultures shown in Fig. 3a and d. Contrary to this, 2,4-D supplementation changed the typical hairy root phenotype. Fine hairy roots were not formed in the MS medium with 2,4-D at a concentration of 0.4 mgL^{-1}. Adding 2,4-D resulted in the partial disorganization of the hairy root morphology and calli were formed in *S. alba* (Fig. 3c) and *B. rapa* (Fig. 3f). *S. alba* hairy roots also produced nodules in medium with 2,4-D.

Impact of Jasmonic Acid on Growth and Glucosinolate Content of Hairy Root Cultures

Hairy root cultures of *S. alba* and *B. rapa* were treated with different concentrations of JA to study glucosinolate elicitation and the effect on growth. The fresh weights of *S. alba* hairy root cultures were significantly lower in all treatments with JA compared to the control without JA after 7, 14, and 28 days (Fig. 4). The highest fresh weight decrease compared to the control sample was observed in the highest concentration of JA, namely 280 µM, on all

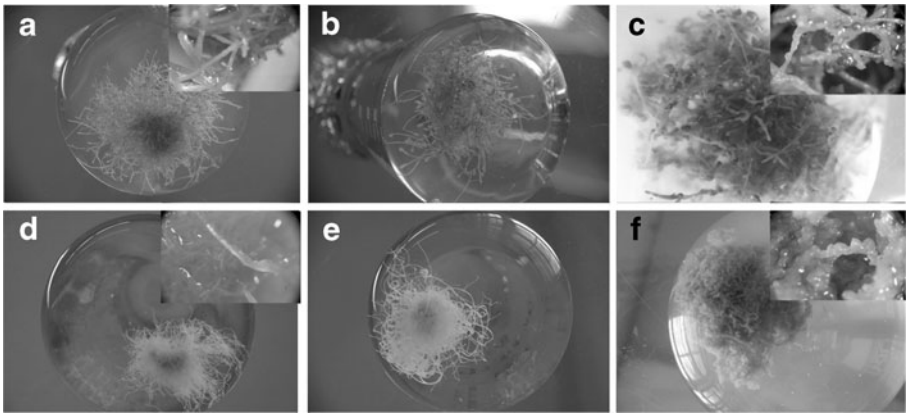

Fig. 3 Morphological structure of hairy root cultures of *S. alba* (*upper row*) and *B. rapa* (*lower row*) in MS medium after 4 weeks with or without supplemented phytohormones: control (**a**, **d**) kinetin (**b**, **e**), and 2,4-D (**c**, **f**) (top-right pictures magnified 16 times)

sampling days. The impact of JA on the GS content of *S. alba* hairy root cultures was also analyzed on days 7, 14, and 28 after adding the elicitor JA. Seven days after cultivation in the MS medium with different JA concentrations, GS levels were only significantly higher in hairy root cultures with 50 μM JA and significantly lower in the treatment with 280 μM. On day 14, the levels of GS in hairy roots were also the lowest with the highest concentration of JA. The highest level of GS, with mean 18.2 $\mu mol\,g^{-1}$ dry weight was observed after 14 days of the cultivation of *S. alba* hairy root with 100 μM jasmonic acid. The content was significantly higher than in the control (Fig. 4). On day 28, the GS contents of hairy roots were not significantly different, apart from the lower JA concentration (Fig. 4). Two weeks of cultivation with the supplementation of 100 to 200 μM were determined to be the optimum harvest time for the highest GS yield in *S. alba*.

Interestingly, single GS abundance in *S. alba* hairy root cultures was different at the three harvest times, and depended on the treatment. While 4-hydroxy-3-indolylmethylglucosinolate is the most produced GS in all treatments, after 7 days of cultivation, the dominating GS on days 14 and 28 was 1-methoxyindol-3ylmethyl GS in cultures treated with 50, 100, and 200 μM, with about 50 % of the total GS content. The highest concentration of 1-methoxyindol-3ylmethyl GS was found in the treatment with 100 μmol JA. In contrast, 4-hydroxyindol-3ylmethyl GS remains the major compound in hairy roots grown under control conditions and at a concentration of 280 μM.

The inhibitory effect of JA on biomass was also observed in *B. rapa* hairy root cultures, although it was not as intense as in *S. alba* hairy root cultures. Generally, the growth rate—about 200 % increase of *B. rapa*—is not as intense as that of *S. alba*, amounting to 600 % biomass increase within 28 days. For this reason, data are only presented for days 14 and 28. The growth rate in *B. rapa* hairy root cultures was faster in the first 14 days of cultivation in the controls (Fig. 5). In contrast, JA treated root cultures with 50 and 100 μM JA grew faster in the second cultivation period. Similarly to the effects of JA on *S. alba* hairy root cultures, JA supplementation significantly reduced biomass production for all concentrations (Fig. 5). A reduction in fresh weight increase of hairy root cultures was observed at concentrations above 100 μM. The total GS contents of root cultures treated with 50 and 100 μM JA were significantly higher than the control on days 14 and 28 (Fig. 5), with about 6.5- and 4-fold increase, respectively. At the highest JA concentration of 280 μM, the GS contents of hairy

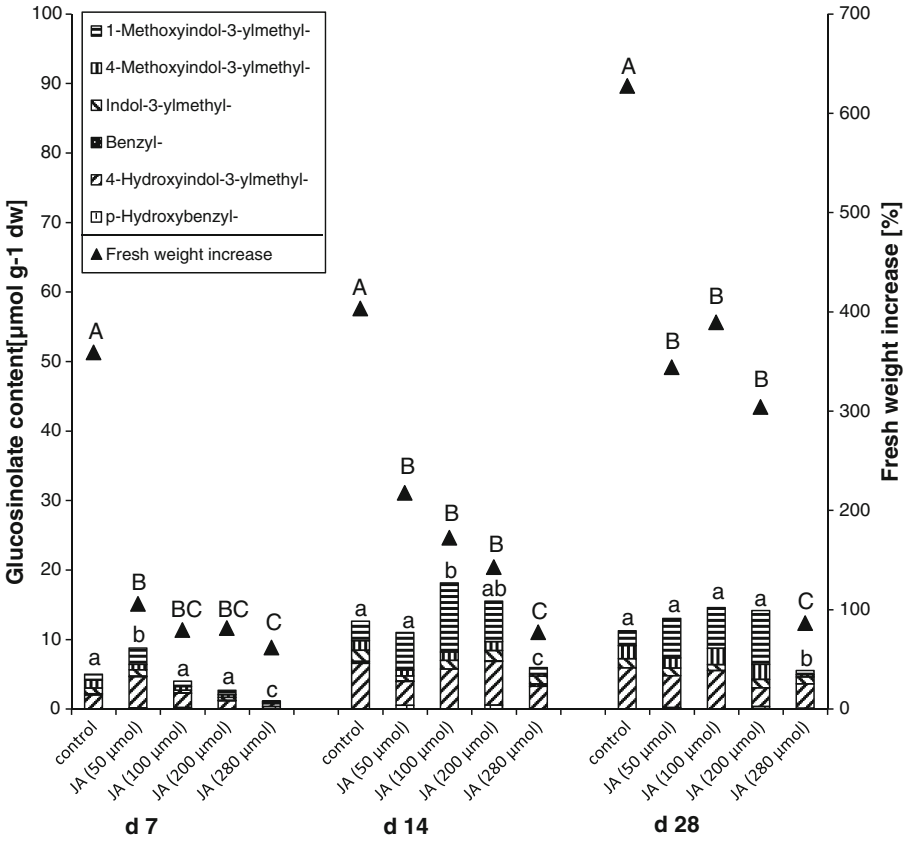

Fig. 4 Fresh weight increase and GS content of *S. alba* hairy root culture 7, 14, and 28 days after cultivation in different concentrations of jasmonic acid (values designated by different letters are significantly different based on fresh weight increase (*uppercase letters*) and GS content (*lowercase letters*) within one cultivation period, $p \leq 0.05$)

root cultures were not significantly different from the control on the two sampling days (Fig. 5). Four weeks of cultivation with a supplementation of 50 μM to 100 μM were determined as the optimum harvest time for the highest GS yield in *B. rapa*.

As determined for hairy root cultures of *S. alba*, the production of 1-methoxyindol-3ylmethyl GS was induced by JA in *B. rapa* hairy root cultures (Fig. 5). Particularly in cultures treated with 50 and 100 μM of JA, GS levels increased by about 16-fold on day 14 and about 15- and 12-fold on day 28 compared to the control. The maximum concentration of 75 μmol 1-methoxyindol-3ylmethyl GS g^{-1} dry weight in root cultures was determined for the JA concentration of 50 μmol. In general, JA supplementation reduced the amounts of 4-methoxyindol-3ylmethyl GS after 14 and 28 days of cultivation. The aliphatic allyl GS usually below the detection level, was more abundant with JA concentrations of 200 μM.

Discussion

In roots of Brassicaceae indolyl GS are the main group of GS [21]. Most of the species produce more than one special GS. GS are widely distributed in the Brassicales; in addition

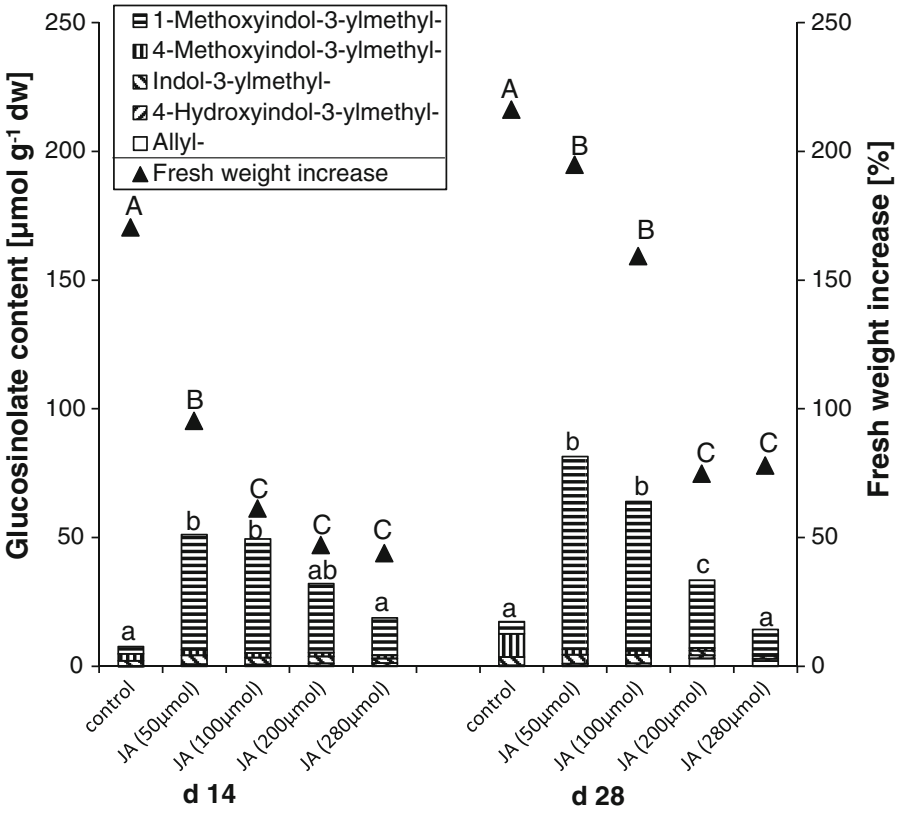

Fig. 5 Fresh weight increase and GS content of *B. rapa* hairy root culture 14, and 21 days after cultivation in different concentrations of jasmonic acid (values designated by different letters are significantly different based on fresh weight increase (*uppercase letters*) and GS content (*lowercase letters*) within one cultivation period, Tukey's test $p \leq 0.05$)

to the basic indol-3ylmethyl GS, its derivatives 1-methoxy-, 4-methoxy-, and 4-hydroxyindol-3ylmethyl GS are also present [22]. Aliphatic and aromatic GS are usually abundant in aerial plant organs, as shown for *Arabidopsis thaliana* L., *B. rapa*, and *S. alba* [16, 32, 33]. Accordingly, we found aliphatic GS in the leaves of *B. rapa* subsp. *rapa pygmeae teltoviensis* (*B. rapa*) and a high concentration of aromatic GS (e.g. p-hydroxybenzyl GS) in *S. alba.* The roots of *B. rapa* had higher GS levels and a different profile compared to leaves, as reported also by Smetanska et al. [16]. However, although roots of *S. alba* showed a similar aromatic GS profile as leaves, the total GS quantities were clearly lower. It is well documented that total GS levels and GS compositions can differ considerably between different tissues within the same plant and with a plant species [33]. After transformation of *S. alba* and *B. rapa* transformed with the *A. rhizogenes* A4, only the four indolyl GS could be detected in a quantitative amount in the present study. Genes transferred from the T-DNA of *A. rhizogenes*, which are involved in hairy root induction, include genes leading to the production of bacteria-specific metabolites, so-called opines, and genes which encode enzymes that promote the hydrolysis of conjugated forms of phytohormones [9]. The unique feature of transformed roots, e.g., altered hormone concentration and ratio, would lead us to expect a different regulation of secondary metabolism in hairy roots compared to the parent mother plant species. This observation of a changed

secondary metabolite profile of hairy roots compared to parent plant was already made in different other studies. Shi and Kintzios [34] reported for *Pueraria phaseloides* that the content of puerarin in hairy roots reached a level 1.7 times the content in the roots of untransformed plants.

The rapid accumulation of biomass and an increase in GS-specific yield are essential to enhance total yields. Overall, the elicitor treatment used in the present study, e.g., phytohormones and signaling molecules, did not enhance biomass production and tended to reduce fresh weights. Plant growth and defenses are restricted by their internal resources, and secondary metabolism often is negatively correlated with cell growth [35]. In addition, hairy root cultures must strike a balance between growth processes and the production of defensive compounds. Correspondingly, higher GS levels were found in hairy root cultures of *S. alba* and *B. rapa* supplemented with 50 up to 200 μM JA in our study. JA was also shown to modulate JA-dependent root growth inhibition in *A. thaliana* not only in hairy root cultures, whereby the P450 protein *CYP82C2* was shown to increase defense genes and the accumulation of the indolyl GS precursor tryptophan resulted in higher indolyl GS levels [36].

However, there is also an association with the growth hormone indole-3-acetic acid and indolyl GS biosynthesis [37, 38]. In this context it is interesting to notice that indolyl GS were reduced in hairy root cultures of *S. alba* and *B. rapa* supplemented with 2,4-D medium compared to the hormone-free control. However, the concentration of 0.4 $mg L^{-1}$ 2,4-D applied may be too high for root cultures of these plant species, and hairy root de-differentiation occurred, leading to the observed lower GS contents. Considering that the transformation process with *A. rhizogenes* can result in clones that are either auxin-overproducers or hypersensitive to auxin [39, 40], it is likely that our clones belong to the second hypersensitive group. Das et al. [26] also showed that hairy root cultures of *B. nigra* and *B. juncea* cultivated in MS with 2,4-D do not produce hairy roots either singly or in combination with other phytohomones. However, kinetin supplementation was shown to profuse hairy roots, similarly to our results with kinetin and *B. rapa* root cultures. Kinetin enhanced GS accumulation in hairy root cultures of *B. rapa*, but had an opposite effect in *S. alba*. This supports the fact that there is no universal predictable effect of a particular elicitor on cell culture systems of different species. Cytokinins have been shown to have diverse effects on hairy root cultures. They direct shoot growth in many tissue cultures at high concentrations [41]; a low cytokinin (benzylaminopurine, BAP) concentration in the medium resulted in the disorganization of the root matrix of *Artemisia annua* L. with little effect on the production of secondary metabolites [42]. Cytokinin had no effect on root growth rate and secondary product accumulation in *Hyoscyamus muticus* L. root cultures [43].

The involvement of JA and its derivatives as an elicitor and signal molecule responsible for the increased production of different secondary metabolites as defense compounds has been well documented and reviewed [44–46]. In plants, JA is produced as a response to wounding and necrotrophic pathogens. Several elicitors have been studied concerning the greater production of plant secondary metabolites; JA and its derivate methyl jasmonate were proven to be very effective increasing metabolite production in plant cell cultures as well as hairy roots [47–50]. For example, JA increased betalaine in *Beta vulgaris* hairy root cultures [51], isoflavonoids in *Pueraria candollei* hairy root cultures [52], and indole alkaloids in *Catharanthus roseus* hairy root cultures [49]. JA and methyl jasmonate have also been used successfully in plants species to enhance production of secondary metabolites [53]. In brassicaceous species, indolyl GS in particular, such as 1-methoxyindoly-3ylmethyl GS, are inducible by JA [31]. For example, Doughty et al. [15] reported that the total GS concentration increased up to 20-fold in *Brassica napus* L. leaves after methyl jasmonate treatment, whereby indol-3ylmethyl GS and 1-methoxyindol-3ylmethyl GS in particular were accumulated. The same results were shown for indolyl GS in *A. thaliana* by Brader et al. [54], working with a JA-insensitive mutant. Correspondingly, we found a significant

increase (up to 16-fold) of especially 1-methoxyindol-3ylmethyl GS in hairy root cultures of *S. alba* as well as in *B. rapa*. To our knowledge, the successful enhancement of GS in hairy root cultures using elicitors has only been reported in one previous study with hairy root cultures from *Tropaeolum majus* L. [28]. Contrary to our study, in which only indolyl GS were present in abundant amounts in hairy root cultures of *S. alba* and *B. rapa*, treatment with methyl jasmonate increased the aromatic benzyl GS in hairy root cultures of *T. majus* about 1.6-fold. Another aspect to consider when obtaining secondary compounds from hairy root cultures is the amount of metabolites exuded in the nutrient medium. In further studies, the amount of glucosinolates exuded into the medium has to be taken into account. For example, the isoflavone puearin was exuded into the nutrient medium at final concentrations higher than in the hairy roots themselves [55].

Our study suggests that it is crucial to determine the optimum harvest point, efficient elicitor treatment, and dosage for the best GS yield in hairy root cultures of *S. alba* and *B. rapa*. One results of our study is that obviously, only indolyl GS can be obtained from hairy root cultures of *B. rapa* and *S. alba* in sufficient quantities. The corresponding plants may be the better choice for mining of aliphatic and aromatic GS. An interesting biotechnology approach to yield aliphatic glucosinolates in hairy root cultures of Brassicaceae would be the use of transformants overexpressing genes associated with the aliphatic glucosinolate pathway. Supplementing hairy root cultures with 0.4 $mg L^{-1}$ kinetin and not 2,4 D was proven to enhance GS in *B. rapa*, but not in *S. alba* in this study. Furthermore, the right dosage of JA (50 to 100 μM in *B. rapa* and 100 to 200 μM in *S. alba*) is an excellent inducer of 1-methoxyindol-3ylmethyl GS in *B. rapa* and *S. alba* hairy root cultures. However, JA had an inhibitory effect on root culture growth. The present study provides evidence that GS levels and profiles can be modulated by elicitor treatments. These could contribute to the development of a strategy to regulate the levels of GS in hairy roots.

Acknowledgments We would like to express our gratitude to Prof. Dietrich Knorr from Berlin University of Technology for supporting this work. We also thank Irene Hemmerich from Berlin University of Technology for the technical assistance she provided. We would like to thank Prof. David Tepfer INRA, France for providing the A4 strain.

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