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TYROSOL GLUCOSYLTRANSFERASE ACTIVITY AND SALIDROSIDE PRODUCTION IN NATURAL AND TRANSFORMED ROOT CULTURES OF *RHODIOLA KIRILOWII* (REGEL) REGEL ET MAXIMOWICZ

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The study examined tyrosol glucosyltransferase activity and the efficiency of salidroside production in natural and transformed root cultures of *Rhodiola kirilowii* (Regel) Regel et Maximowicz. Neither enzyme activity nor salidroside accumulation were detected in natural and transformed root cultures maintained in media without tyrosol. To induce TGase activity in biotransformation reactions, tyrosol was added to natural and transformed root cultures on the day of inoculation. The first peak of TGase activity (0.23 U/μg) was detected on day 9 in natural root culture, accompanied by the highest salidroside content (15.79 mg/g d.w.), but TGase activity was highest (0.27 U/μg) on day 15. In transformed root culture, day 18 showed the highest TGase activity (0.15 U/μg), which coincided with the highest salidroside content (2.4 mg/g d.w.). Based on these results, tyrosol was added to the medium on the days of highest previously detected activity of TGase: day 15 for natural root cultures and day 18 for transformed root cultures. This strategy gave significantly higher yields of salidroside than in the cultures supplemented with tyrosol on the day of inoculation. In natural root culture, salidroside production reached 21.89 mg/g d.w., while precursor feeding in transformed root cultures caused a significant increase in salidroside accumulation to 7.55 mg/g d.w. In all treatments, salidroside production was lower in transformed than in natural root cultures.

Key words: *Rhodiola kirilowii*, TGase, salidroside, transformed root culture, biotransformation.

INTRODUCTION

Plants are a source of important drugs and drug candidates, as they are able to produce bioactive molecules derived mainly from primary metabolites through chemical modifications including hydroxylation, methylation, oxidation and glycosylation reactions (Shilpa et al., 2010). Many novel natural products from plant cell culture have been created, including new metabolites formed by biotransformation (Stöckigt et al., 1995).

Glycosylation is an important modification process and often is described as the final step in biosynthesis of natural compounds. Enzymes that catalyze glycoside formation are called glucosyltransferases. Glucosyltransferase have been classified in 90 families (Coutinho et al., 2003) on the basis of their structure and stereochemistry. Glucosyltransferase facilitates the transfer of nucleotide diphosphate-activated sugars to low molecular weight molecules (Vogt and Jones, 2000; Bourne and Henrissat, 2001). In plants these transfer reactions generally use UDP-glucose with acceptors that include hormones such as auxins and cytokinins, and secondary metabolites such as flavonoids and phenolic compounds (Lim and Bowles, 2004).

Rhodiola kirilowii (Regel) Regel et Maximowicz (Crassulaceae) is a perennial plant from the Asian region, used in herbal medicine. Root extracts of *Rhodiola* spp. in preparations used throughout Europe, Asia and the United States have been described as having biological activity (Krajewska-Patan et al., 2008; Yu et al., 2011).

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Salidroside (phenylpropanoid glycoside) is the main bioactive component of *Rhodiola* species such as *Rhodiola* rosea, *R. sacra*, *R. crenulata* and *R. sexifolia*, and is found mainly in plant roots. It is known for its adaptogenic, antioxidant, cardiovascular-protective and antianoxic properties. Due to its high pharmacological value, salidroside is a candidate drug for the treatment of cardiovascular and cerebrovascular diseases (Liu et al., 2006; Wiedenfeld et al., 2007; Peng et al., 2009; Guo et al., 2010). The low yield of salidroside in *R. kirilowii* calls for the development of biotechnology methods to improve production of this compound in vitro. One approach is conversion of exogenous *p*-tyrosol to salidroside in plant cultures in vitro.

The salidroside precursor *p*-tyrosol is a small phenolic molecule whose biosynthetic pathway and regulation are not completely understood. There are two different views on salidroside production in the literature. The first holds that tyrosol is produced from a *p*-coumaric acid precursor by a decarboxylase derived mainly from phenylalanine. The second states that the precursor of tyrosol may be tyramine synthesized from tyrosine (Zhang et al., 2011).

Glycosylation of tyrosol is thought to be the final step in salidroside biosynthesis (Xu et al., 1998a) (Fig. 1). However, the efficiency of this reaction depends on tyrosol glucosyltransferase (TGase) activity. There are a few literature reports (Xu et al., 1998b; Yu et al., 2011) discussing the activity of TGase in the biotransformation process in *Rhodiola* spp. These investigations were concerned mainly with *R. sachalinensis*. Xu et al. (1998b) suggested that low yield of salidroside in *R. sachalinensis* cell culture was due to the low efficiency of glycosylation and a lack of synchronization between TGase activity and tyrosol accumulation in the cells. Ma et al. (2007) showed that conversion of tyrosol aglycone to salidroside in *R. sachalinensis* could be regulated by UGT73B6. Overexpression of the UGT73B gene resulted in increased salidroside content in transformed *R. sachalinensis* plants (2-fold increase over untransformed control) and transformed calli (2.4-fold increase).

Yu et al. (2011) achieved isolation of two other uridine diphosphate (UDP)-glucosyltransferase cDNAs: *UGT72B14* and *UGT74R1*. The first transcript was more abundant in roots and the second was highly expressed in calli of *R. sachalinensis*. UGT72B14 gave the highest salidroside production in vitro and in vivo.

Supplementation with tyrosol as a precursor for the biotransformation reaction at the proper time was found to markedly increase salidroside content in *R. rosea* callus cultures (György et al., 2005). In cell suspension of *R. rosea* cultured in modified Murashige and Skoog (MS) medium, Furmanowa et al. (2002) demonstrated biotransformation of *p*-tyrosol to salidroside. Adding exogenous tyrosol to the culture enhanced salidroside yields by 1.8% in green and 2.3% in yellow cell lines.

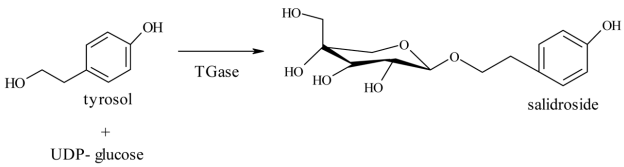

Fig. 1. Salidroside biosynthesis catalyzed by tyrosol glucosyltransferase (TGase).

Krajewska-Patan et al. (2007) studied enhancement of salidroside biosynthesis by biotransformation of *p*-tyrosol in callus culture of *R. rosea*. They supplemented tyrosol in callus tissue cultured on solid medium and in compact callus aggregate (CCA) in liquid medium, achieving significantly higher salidroside content in CCA (4.3%) and in callus culture on solid medium (3.1%) than in plant roots in vivo (2%).

Biotransformation reactions can also be performed in transformed root culture. Transformed root cultures of *R. sachalinensis* have been studied as a potential source of salidroside (Zhou et al., 2007). Transformed root cultures were established by transformation with *Agrobacterium rhizogenes* and cultivated on liquid MS medium. Medium supplementation with tyrosol resulted in increased biomass and salidroside accumulation.

The studies discussed above demonstrate the great potential of biotransformation for efficient production of salidroside in various kinds of *Rhodiola* plant cultures. Analysis of TGase activity is part of the effort to improve the biotransformation process in these cultures. In this study we examined the effect of TGase activity on the efficiency of conversion of exogenous tyrosol to salidroside in natural and transformed *R. kirilowii* root cultures. We also determined tyrosol and salidroside content. This is the first report of a study of TGase activity in natural and transformed lines of *R. kirilowii* root cultures.

MATERIAL AND METHODS

NATURAL ROOT CULTURE

Seeds of *Rhodiola kirilowii* were collected from the Medicinal Plant Garden of the Institute of Natural Fibres and Medicinal Plants in Poznan. The seeds were germinated in vitro on Linsmaier and Skoog (LS) medium (Linsmaier and Skoog, 1965) without growth regulators, consisting of 3% sucrose and 0.78% agar, in darkness at 24°C. Then shoot tips

were excised from the in vitro-germinated seedlings and rooted on LS medium supplemented with 10 mg/l adenine sulfate (AS), 1 ml/l kinetin (Kin), 5 ml/l indole-3-butyric acid (IBA) and 3% sucrose, solidified with 0.78% agar. Natural root culture was established from roots of 1-month-old plantlets. Roots were cultured in 20 ml LS liquid medium supplemented with 10 mg/l AS, 1 ml/l Kin and 5 ml/l IBA in Erlenmeyer flasks on an INFORS rotary shaker (105 rpm) in darkness at 24°C. Every 4 weeks, roots (0.415 ± 0.07 g) were subcultured on fresh LS medium.

TRANSFORMED ROOT CULTURE

Transformed roots were successfully established by transformation with *Agrobacterium rhizogenes* LBA 9402. Integration of the bacterial DNA into the *R. kirilowii* genome was confirmed by polymerase chain reaction (PCR) (Zych and Pietrosiuk, 2008). For our study the best-growing transformed root clone (RKTR1) was used. Transformed root cultures were grown in Erlenmeyer flasks containing 20 ml modified Gupta and Durzan (DCR) medium (Gupta and Durzan 1985) without phytohormones on an INFORS rotary shaker (105 rpm) in darkness at 24°C. Every 4 weeks, roots (0.4 ± 0.06 g) were subcultured on fresh DCR medium.

The growth of the two root lines, natural and transformed, was studied simultaneously. From day 3 through day 27, samples were collected every 3 days from three flasks of each culture. The roots were washed with deionized water, blotted dry and weighed (fresh weight, f.w.). Then the roots were lyophilized and dry weight (d.w.) was recorded. Each experiment was performed in duplicate.

TYROSOL SUPPLEMENTATION

Salidroside production in natural and transformed root cultures was examined according to two experimental protocols. In the first, 2.5 mM tyrosol was added to 20 ml liquid medium in sterile conditions on the day of inoculation. From day 3 through day 27, samples from three flasks of each culture were harvested every 3 days. In the second protocol, 2.5 mM tyrosol was added to the medium on the day of the highest TGase activity. Samples were collected every 24 h for five consecutive days. Samples were also taken from reference cultures that were not supplemented. Each experiment was performed in duplicate.

CHEMICAL ANALYSIS

Chemical analysis was performed as described by Wiedenfeld and Pietrosiuk (unpublished data 2007). Salidroside and tyrosol were extracted from lyophilized roots and postculture media. To obtain salidroside and tyrosol fractions, root samples collected during the study were sonicated in methanol. Extraction was performed three times for 30 min at 40°C. The extracts were evaporated and the dry residue was partitioned in carbon tetrachloride/methanol/water (5:4:1, v/v/v). The methanol/water extract was evaporated to dryness and the residue was dissolved in n-butanol/water, separated into two fractions and evaporated. The majority of aglicons were expected to be in the butanolic fractions, and the glycosides in the water fractions. Lyophilized postculture media were dissolved in methanol, filtered and evaporated. The steps that followed were the same as those used for root analysis. The dry residue from each fraction was dissolved in methanol and analyzed with a Dionex high-performance liquid chromatography (HPLC) system equipped with an automated sample injector (ASI-100) and UVD 340S detector, and gradient elution with acetonitrile/phosphoric acid/methanol (7:85:8-20:60:20, v/v/v) at 1 ml/min for 30 min. Separation was done on an EC 250/4.6 Nucleosil 120-7 C18 column (Macherey-Negal, Düren, Germany) and the eluent was monitored at 205, 220, 250 and 275 nm. Tyrosol and salidroside (ChromaDex, Inc. California, USA) were used as standards and were analyzed under the same conditions. Peaks were assigned by spiking the samples with standards and comparing the retention times and UV spectra.

TYROSOL GLUCOSYLTRANSFERASE ASSAYS

TGase activity was measured in natural and transformed roots without supplementation (control) and in roots supplemented with 2.5 mM tyrosol, by the HPLC method described by Yu et al. (2011), slightly modified. The roots were homogenized in an ice-cold mortar in 0.1 M potassium buffer (pH 7.5, 5 ml) containing 0.8 mM β-mercaptoethanol and 1% (w/v) polyvinylpyrrolidone. After centrifugation (15,000 g for 15 min at 4°C) the supernatant was used as the crude enzyme preparation. The mixture for determination of enzyme activity consisted of a sample of crude enzyme, 250 μM tyrosol, 2 mM UDP-glucose and 50 mM Tris-HCl buffer (pH 7.5). The mixture was incubated for 20 min at 30°C in an Eppendorf thermomixer. After 0, 10 and 20 min the reaction was terminated by adding methanol (200 μl). After centrifugation (12,000 g for 10 min) the samples were subjected to HPLC analysis using the same conditions described above for phenolic glycosides. Enzyme activity is expressed in units U/μg protein. Roots were taken from both lines starting from the first day of the growth cycle and thereafter at 72 h intervals. TGase activity was measured in triplicate for both lines in each trial. All the data are means

±SE from each experiment. The results were analyzed using STATISTICA PL 10 (StatSoft, Krakow, Poland).

PROTEIN CONTENT DETERMINATION

Protein content was determined according to Bradford's (1976) method with a standard curve prepared using bovine serum albumin. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

RESULTS AND DISCUSSION

ROOT GROWTH

We examined the increase of fresh and dry biomass of natural and transformed roots of *Rhodiola* kirlowii cultivated in media supplemented with tyrosol (precursor) and in media without tyrosol added (control culture). Preliminary experiments (data not shown) had shown that natural and transformed roots differed in their requirements for minerals in the media. LS medium proved most suitable for culture of natural roots; no biomass increase was observed on DCR medium. For transformed roots DCR medium proved best for growth promotion. The growth curves of *R. kirilowii* natural and transformed roots in control culture are compared in Figure 2. The transformed roots had higher growth rates (final weight/inoculum weight) than natural roots (2.2-fold higher f.w., 1.4-fold higher d.w.). Media supplementation with tyrosol had a detrimental effect on transformed root growth, which was half that of control cultures (Fig. 3).

Xu et al. (1998b) discussed the connection between biomass increase and the biotransformation reaction in *Rhodiola sachalinensis* cell suspension cultures. They fed suspension cultures with 3 mM tyrosol at 24 h intervals from day 8 to day 10. As a consequence of this strategy, growth of cells was severely inhibited and biomass rapidly decreased. Similar data were reported by Krajewska-Patan et al. (2007) in *Rhodiola rosea* callus culture and by Wu et al. (2003) in *R. sachalinensis* suspension culture of compact callus aggregate. The decrease of biomass growth was proportional to the tyrosol concentration and ranged from 10% to 20%. In transformed root cultures of *R. sachalinensis*, on the other hand, the growth of transformed roots was greatly improved by addition of tyrosol (Zhou et al., 2007). The general inference from most studies of cultures of *Rhodiola* species in vitro is that tyrosol feeding inhibits biomass increase.

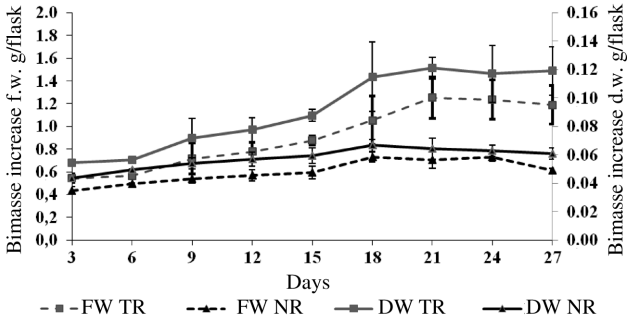

Fig. 2. Fresh and dry biomass growth curves of natural and transformed *R. kirilowii* root cultures over the growth cycle.

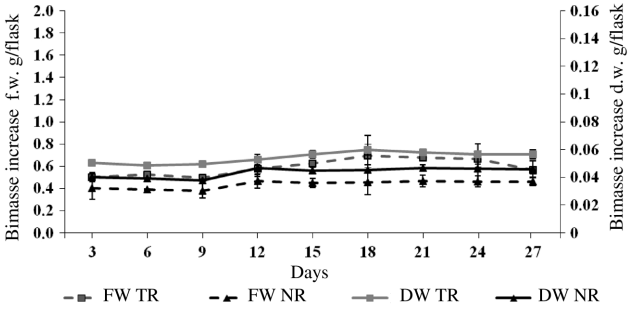

Fig. 3. Fresh and dry biomass growth curves of natural and transformed *R. kirilowii* root cultures after supplementation with *p*-tyrosol on the first day of the cycle.

EFFECT OF UDP-GLUCOSYLTRANSFERASE ACTIVITY AND TYROSOL SUPPLEMENTATION ON SALIDROSIDE PRODUCTION

In preliminary investigations we examined tyrosol glucosyltransferase activity in untreated control root cultures and in roots after medium supplementation with tyrosol on the day of inoculation. No enzyme activity was detected in untreated transformed and natural root cultures maintained on media without tyrosol. Neither tyrosol, the precursor in salidroside biosynthesis, nor salidroside were detected in roots.

As tyrosol can rapidly and markedly induce salidroside production (Zhou et al., 2007), we decided to induce TGase activity by biotransformation. In natural root cultures after tyrosol supplementation on the day of inoculation we detected TGase activity as early as day 3 (Fig. 4). The first considerable increase in enzyme activity occurred on day 9 (0.23 U/μg) and it reached its highest value (0.26 U/μg) on day 15. Chemical analysis of roots showed an increase in total salidroside content (water and butanol fractions) from day 3 to day 18 (Fig. 5) The salidroside yield was highest on day 9 (15.78 mg/g d.w.), suggesting synchronization with the first peak of TGase activity. The level of salidroside was also

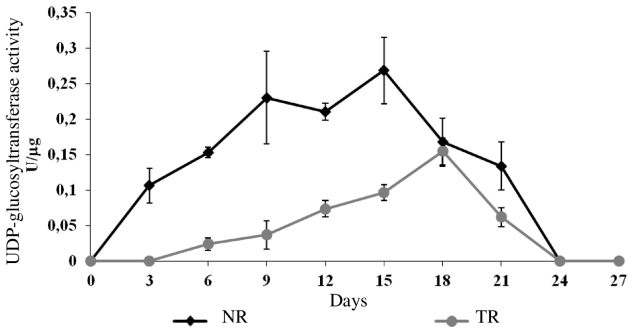

Fig. 4. Tyrosol GTase activity of natural and transformed *R. kirilowii* root cultures over the growth cycle.

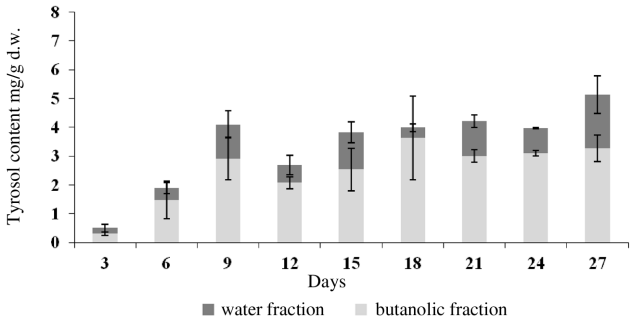

Fig. 6. Tyrosol content in natural *R. kirilowii* root culture during the growth cycle after supplementation with *p*-tyrosol on the first day of the cycle.

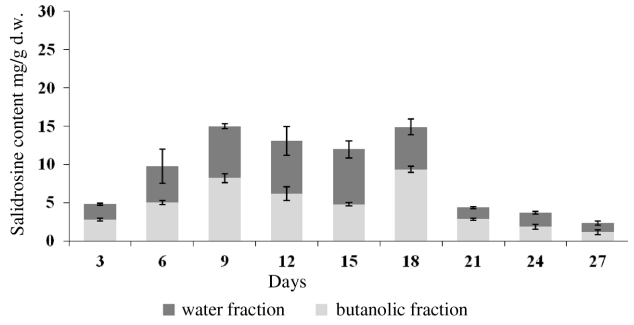

Fig. 5. Salidroside content in natural *R. kirilowii* root culture during the growth cycle after supplementation with *p*-tyrosol on the first day of the cycle.

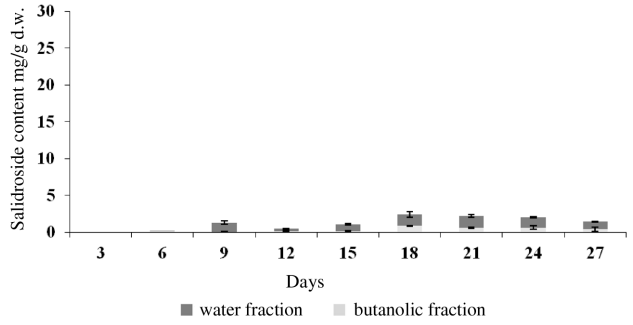

Fig. 7. Salidroside content in transformed *R. kirilowii* root culture during the growth cycle after supplementation with *p*-tyrosol on the first day of the cycle.

high on day 18 of the growth cycle (15.41 mg/g d.w.), demonstrating a lag between salidroside production and the highest detected maximum of TGase activity on day 15. Total salidroside content on day 15 reached 11.96 mg/g d.w., significantly ($P < 0.01$) lower than on day 18 (15.41 mg/g d.w.).

Culture of natural roots with tyrosol added to the medium initiated salidroside production and also tyrosol accumulation in root tissues (Fig. 6). The amount of tyrosol was highest on day 27, when total salidroside content was at its lowest and no TGase activity had been detected in the previous 6 days. In natural *R. kirilowii* root cultures, tyrosol accumulation was not closely synchronized with TGase activity. Tyrosol content was low in the exponential growth phase up to day 9. This was also the day of the first peak of TGase activity (Fig. 4). The highest TGase activity was noted on day 15, which did not coincide with the highest tyrosol content. From day 18 to the end of the culture period, tyrosol content remained high even though TGase activity was no longer detected (Fig. 6).

In transformed root cultures with tyrosol added on the day of inoculation, tyrosol content was rela-

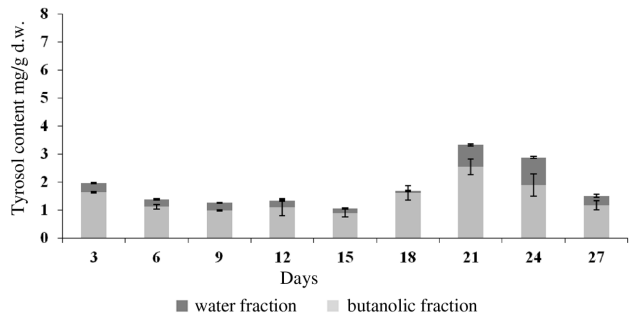

Fig. 8. Tyrosol content in transformed *R. kirilowii* root culture during the growth cycle after supplementation with *p*-tyrosol on the first day of the cycle.

tively high on day 3 but TGase activity was low and had just begun to increase (Fig. 8). The first sign of TGase activity was noted on day 6 of the growth cycle. It increased to its highest value (0.154 U/µg) on day 18, thereafter falling sharply until the end of culture; TGase activity was not detected after day 21 (Fig. 4). The increase in total salidroside content was correlated with increased TGase activity: salidroside yield was highest (2.4 mg/g d.w.) on day

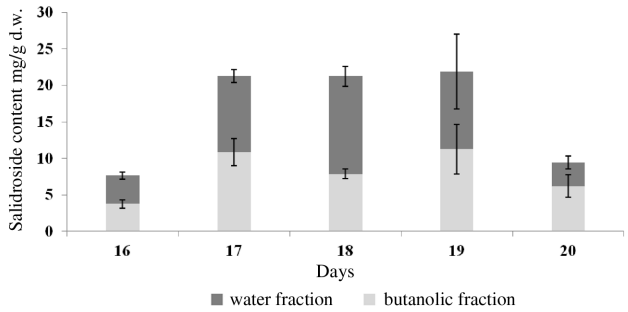

Fig. 9. Increase of salidroside content in natural *R. kirilowii* root culture after supplementation with *p*-tyrosol on the day of highest GTase activity.

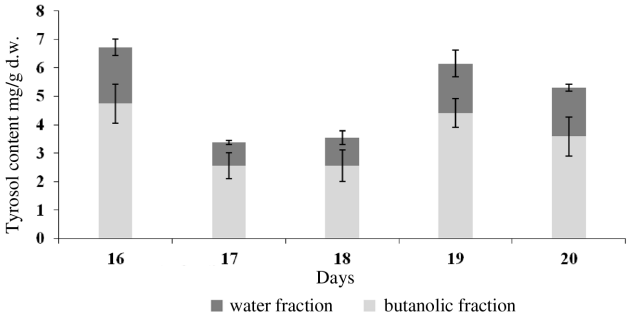

Fig. 11. Tyrosol level in natural *R. kirilowii* root culture after supplementation with *p*-tyrosol on the day of highest GTase activity.

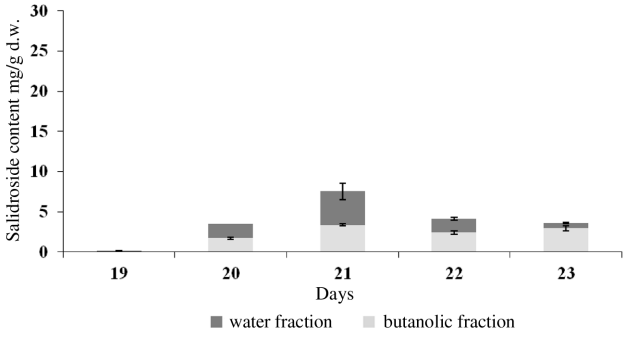

Fig. 10. Increase of salidroside content in transformed *R. kirilowii* root culture after supplementation with *p*-tyrosol on the day of highest GTase activity.

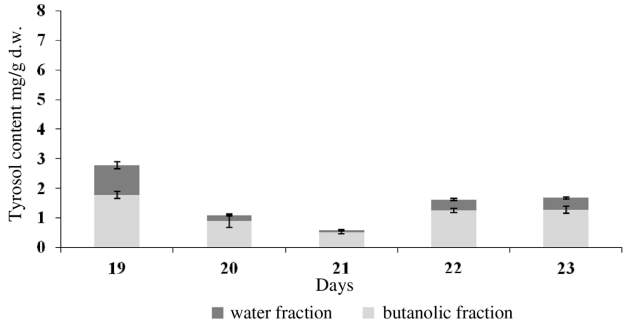

Fig. 12. Tyrosol level in transformed *R. kirilowii* root culture after supplementation with *p*-tyrosol on the day of highest GTase activity.

18 (Fig. 7). The profiles of tyrosol accumulation, enzyme activity and salidroside production in transformed roots coincided (Fig. 8). The amount of tyrosol in roots was highest (3.33 mg/g d.w.) on day 21, 3 days after the highest detected TGase activity and highest salidroside yield. After that time, both salidroside and tyrosol content decreased slowly until the end of culture.

To induce TGase activity and enhance the biotransformation reactions, tyrosol was added to the medium on day 15 to natural root cultures and on day 18 to transformed root cultures. This strategy caused a distinct rise in total salidroside production versus the control cultures. In natural root culture production of salidroside reached 21.28 mg/g d.w. 48 h after precursor feeding and remained around that level for the next 3 days; the yield was highest (21.89 mg/g d.w) 4 days after medium supplementation (Fig. 9). This maximum yield was a 1.4-fold increase in total salidroside content versus cultures supplemented with tyrosol on the first day of the cycle.

In transformed root cultures, precursor feeding caused a significant increase in salidroside accumulation to 7.55 mg/g d.w. on day 21, 72 h after tyrosol was added to the medium (Fig. 10), 3 times higher than from transformed roots of cultures supplemented with tyrosol on the day of inoculation. Salidroside production in transformed roots was lower than in natural roots. But in transformed root culture the effect of feeding precursor on the day of the highest TGase activity was more pronounced and the increase of salidroside yield was higher as well. We also analyzed the tyrosol content of natural and transformed root cultures in which it was added on the day of highest TGase activity. In both studied root cultures, tyrosol content was highest 24 h after it was added to the medium, and this was correlated with the lowest salidroside content (Figs. 11, 12). In *R. sachalinensis* cell suspension culture, TGase activity was not synchronized with tyrosol and salidroside accumulation (Xu et al., 1998b); the TGase level was highest on day 10, while rapid tyrosol accumulation started at the beginning of the stationary growth phase on day 15. In our experiments we found a direct correlation between TGase activity and salidroside production in transformed root cultures fed tyrosol, but in the natural root cultures salidroside production was correlated with the first considerable increase of TGase activity, but there was a 72 h lag between the highest TGase

value on day 15 and salidroside accumulation.

In the investigated natural and transformed root cultures, salidroside and tyrosol was found in roots even when TGase activity was not observed after day 21. If the level of tyrosol detected in supplemented natural and transformed roots was still very high at the end of the growth cycle after the period of salidroside production, we suggest that it may have originated not only from the medium; it may have been produced by roots themselves (Figs. 6, 8, 11, 12). We speculate that induction of the salidroside biosynthetic pathway could also initiate the tyrosol biosynthetic pathway, irrespective of earlier tyrosol supplementation.

The salidroside detected in roots after day 21 may have derived from earlier activity of TGase, but its diminution is believed to be caused by the activity of β-d-glucosidase; this enzyme is reported to catalyze degradation of salidroside (Shi et al., 2007). In postculture media only traces of salidroside were detected, negligible in terms of the other results.

To date the highest intracellular salidroside content was obtained by Xiu et al. (1998b) from *Rhodiola sachalinensis* cell suspension culture (154.95 mg/g). Wu et al. (2003) also achieved a significant increase of salidroside production (57.7 mg/g), in *R. sachalinensis* suspension culture of compact callus aggregate supplemented with 4 mM tyrosol. In *Rodiola rosea* callus culture Krajewska-Patan et al. (2007) got comparable results (43.22 mg/g salidroside) after adding 2.5 mM tyrosol. In transformed root culture of *R. sachalinensis* medium supplemented with 1 mM tyrosol, salidroside accumulation increased to 6.6 mg/g (Zhou et al., 2007). In our work, feeding tyrosol on the days of highest TGase activity enhanced salidroside yield to 21.89 mg/g in natural and to 7.55 mg/g in transformed root cultures of *R. kirilowii*. As these are first results, they can only be compared with callus and suspension cultures and with other *Rhodiola* species.

Our results make it clear that natural and transformed roots of *R. kirilowii* are able to carry out biotransformation of tyrosol to salidroside and also to induce a biosynthetic pathway of tyrosol production. Regardless of the time of media supplementation with tyrosol, natural roots accumulated higher salidroside content than transformed roots did. There was a lag between the highest TGase activity and the highest salidroside yield in natural root cultures. The applied strategy of precursor feeding on days of the highest TGase activity boosted salidroside production in both the natural and transformed root cultures.

Much effort is directed at increasing the yield of salidroside from *Rhodiola* through biotransformation. Here we studied the role of a glucosyltransferase in this process, and demonstrated the dependence between tyrosol glucosyltransferase activity and salidroside production in natural and transformed root cultures of *R. kirilowii*. To our knowledge this is the first report of the major effect of TGase on salidroside production in *R. kirilowii*.

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