

Tropane alkaloid production and riboflavin excretion by *Hyoscyamus niger* L. hairy root cultures

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Abstract. The production of tropane alkaloids and excretion of riboflavin was comparatively investigated in hairy root clones of *Hyoscyamus niger* transformed with *Agrobacterium rhizogenes* strain LBA 9402 and wild-type (WT) root culture of *H. niger*. From six selected hairy root clones, one clone (C-1) was relatively similar to WT roots in morphological features and growth intensity. The other, fast-growing (FG) root clones grew twice better than WT roots and had very variable morphological features. Clone C-1 produced, depending on the medium (MS, B5, Knop-M), 5 to 15 times more scopolamine and 1.5 to 2 times more hyoscyamine than WT roots. Most of the FG root clones produced less tropane alkaloids than WT roots, but there was a strong dependence on the composition of media. The excretion of riboflavin from C-1 clone was somewhat lower than that from the WT roots. The excretion of riboflavin from the FG root clones occurred at a detection level, but increased under iron deficiency of media.

Key words: *Hyoscyamus niger*, wild-type roots, hairy roots, tropane alkaloids, riboflavin.

Hyoscyamus niger L. (Solanaceae) has been regarded a rich source of tropane alkaloids such as hyoscyamine and scopolamine. Wild-type (WT) root culture of *H. niger* has been studied for the production of tropane alkaloids *in vitro* (Hasimoto et al., 1986; Jung et al., 2001; Pudersell et al., 2003). Most of the reported WT roots, also other Solanaceae WT roots, require auxins for vigorous growth; however, *H. niger* WT root cultures have been shown not to be typical. They proliferate vigorously in liquid medium without auxins and produce a large amount of alkaloids (Shimomura et al., 1991; Robins & Dräger, 1995; Pudersell et al., 2003).

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Infection of plant cells by *Agrobacterium rhizogenes* usually results in hairy root formation at the sites of infection. The integration of the transferred DNA of Ri plasmids causes metabolic changes in auxin and cytokinin syntheses, which result in morphogenetic alterations (plagiotrophic growth, high branching, etc.) of the formed hairy roots (Zambryski et al., 1989). The *A. rhizogenes* strain used for the transformation of these root clones (Vanhala et al., 1995) has a significant influence on the phenotype of the hairy root clone, on its growth rate, and tropane alkaloid production.

A. rhizogenes-mediated hairy root cultures are fast growing (Hamill et al., 1986; Jung & Tepfer, 1987) and genetically stable (Aird et al., 1988), and the Ri-transformed state is maintained during long periods of culture (João & Brown, 1994). Hairy roots produce secondary metabolites even in higher amounts than the parent plants (Kamada et al., 1986; Christen et al., 1989; Oksman-Caldentey et al., 1989).

The results of our preceding work showed that the WT root culture of *H. niger* produces increased levels of the alkaloid scopolamine of higher economic value and decreased levels of hyoscyamine compared with the field culture of *H. niger* (Pudersell et al., 2003). Pudersell and co-workers (Pudersell et al., 2004) established that excised WT roots of *H. niger* excrete a large amount of riboflavin into the growth medium.

The aim of this study was to investigate the capability of clones of hairy roots of *H. niger* to produce tropane alkaloids and the excretion of riboflavine into the culture medium.

MATERIAL AND METHODS

Bacterium strain

Agropine strain *A. rhizogenes* LBA 9402 (gift from Norwich laboratory, England) carrying the kanamycin resistance (*NPT II*) and β -glucuronidase (*gus*) genes was used for transformation. *A. rhizogenes* was grown in YMB (0.5 g L⁻¹ K₂HPO₄, 0.2 g L⁻¹ MgSO₄, 0.1 g L⁻¹ NaCl, 10 g L⁻¹ mannitol, 0.4 g L⁻¹ yeast extract, pH 7.0) medium. The bacterium was agitated to intensive growth by subculturing in YMB medium at 25°C in the dark on a rotatory shaker (90 rpm) for 24 h before its use in transformation.

Transformation procedure

Leaves from sterile plants were cut into pieces of ca 0.5 × 0.5 cm and co-cultivated with a 24-h-old *A. rhizogenes* suspension culture diluted four times in 1/2 MS (Murashige & Skoog, 1962) liquid medium (ca 10⁵ cells mL⁻¹) for 30 min. After that the leaf segments were dried with sterile filter paper, placed onto the MS solid medium (0.7% agar) containing 500 mg L⁻¹ cefotaxime

(Claforan®, Rousell, France) and 100 mg L⁻¹ kanamycin (to select kanamycin-resistant primary hairy roots), and incubated in illuminated phytotrone (light intensity 20 μmol m⁻² s⁻¹) at 22±2 °C. About a fortnight after the inoculation the formation of 2–4 roots on the segments of leaves was observed.

One root was excised from each of the leaf pieces. These roots were called hairy root clones. Roots were transferred to 300 mL conical flasks containing 50 mL MS liquid medium supplemented with 2% sucrose and 200 mg L⁻¹ cephotaxime for purification of roots from bacteria. The root cultures were subcultured 6 times after every 3 weeks. The root cultures were purified and multiplied on a shaker at 90 rpm in a dark phytotron at 25±2 °C. After the third subculture in the purification medium, 6 well-growing hairy root clones that were morphologically different from the WT root clone were selected from 30 hairy root clones.

Growing conditions of root culture

Root segments of 3 cm were transferred into the fresh medium after every 28 days. For the multiplication of roots the MS complete medium, B5 medium (Gamborg et al., 1968), and Knop (Knop, 1865) macronutrients medium supplemented with MS micronutrients and organics (Knop-M) plus 2% sucrose were used. The growth of the roots was followed by transferring 100 mg of fresh roots or four 3-cm root tips (depending on the experiment) to 50 mL of liquid media in 300 mL conical flasks.

β-glucuronidase (GUS)-assay

The presence of GUS activity in the root clones was detected histochemically (Jefferson, 1987) using 5-bromo-4-chloro-3-indolyl-β-D-glucouronide (x-gluc) as substrate.

Determination of the content of tropane alkaloids

For HPLC determination of tropane alkaloids in the root material of *H. niger* alkaloids were extracted with ether according to a previously elaborated methodology, and the content of both tropane alkaloids, hyoscyamine and scopolamine, was determined (Pudersell et al., 2003). The authentic preparations of the respective pure alkaloids (Sigma) were used as reference substances.

Determination of riboflavin

The concentration of riboflavin was determined fluorimetrically using a fluorimeter Analiz (Geologorazvedka, St. Petersburg, Russia). The maximum excitation wavelength was 436 nm and the emitted light was passed through a 500 nm cut-

off filter. The riboflavine content in the medium was calculated using comparison with standard curves established with authentic riboflavin (Chemapol, Prague, Czechoslovakia).

The data were subjected to analysis of variance. The means were compared using Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Clones of hairy roots selected for the present study had various morphological features. Clone 1 was morphologically similar to WT roots. There was no branching characteristic of typical hairy roots, but roots were thickened compared with the WT root culture. Roots of clone 6 were very thick, and lateral roots were very short. Other hairy root clones were highly branched semblance, with shorter (C-2) or longer (C-9) lateral roots and various density of lateral roots (Fig. 1).

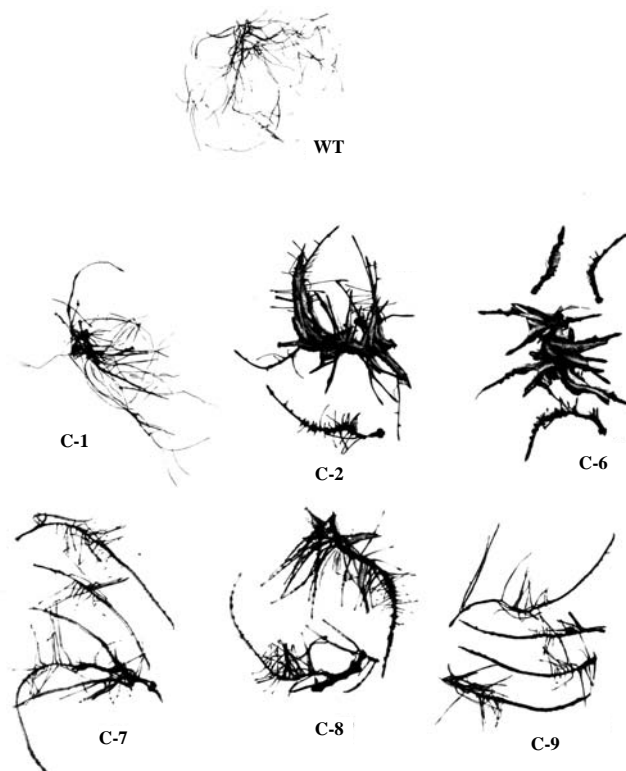


Fig. 1. Part of a bundle (contact copies) of wild-type (WT) and hairy root clones (C).

The activity of GUS was detected in most hairy root clones except clone 7 (Table 1). As hairy root clones were selected by kanamycin resistance, all they obviously contain the *NPT II* gene. This is not surprising as hairy root clones have often shown the absence of one or more genetic markers (Vries-Uijtewaal et al., 1989; Dobigny et al., 1995).

As root clones growing vigorously in the liquid medium without auxins were selected for the experiments, we cannot assert that our data are valid for the whole spectrum of the potential hairy roots of *H. niger*. The growth of some Ri-transgenic root clones may be even less than that of WT roots (Dobigny et al., 1995). In our selection the growth of most fast-growing (FG) hairy root clones (C-2, C-6, C-7, C-8, C-9) exceeded that of WT roots more than twice. The excretion of riboflavin into the B5 medium from FG roots was very low, remaining practically at the detection level. The growth of hairy root clone 1 was slightly better than that of WT roots, but its capacity of excreting riboflavin into the medium was lower (Table 1).

The content of hyoscyamine in FG roots was up to six times less than in WT roots. In hairy root clone 1 the content of hyoscyamine was twice less than in WT roots (Table 1).

The content of scopolamine in hairy root clones varied greatly. In hairy root clone 8 the content of scopolamine was five times less than in the WT roots, in clone 6 (morphologically most different from the WT roots) the content of scopolamine was slightly (1.4 times) higher than in the WT roots, but in clone 1 the content of scopolamine was even 15 times higher than in the WT roots (Table 1).

Table 1. Growth, excretion of riboflavin, and the content of tropane alkaloids in the wild-type and hairy root cultures of *Hyoscyamus niger* grown in B5 medium (28-day culture; inoculum 100 ± 10 mg fresh roots)¹

Root clone	GUS activity	Growth, dry weight mg flask ⁻¹	Riboflavin content in the medium, mg L ⁻¹	Hyoscyamine content in the roots, mg g ⁻¹	Scopolamine content in the roots, mg g ⁻¹
Wild type	–	147 ± 12 ^d	3.12 ± 0.18 ^a	2.12 ± 0.22 ^a	5.86 ± 0.24 ^c
Hairy root					
C-1	+	189 ± 18 ^c	2.18 ± 0.11 ^b	1.14 ± 0.02 ^b	88.87 ± 1.77 ^d
C-2	+	383 ± 23 ^a	0.07 ± 0.01 ^c	ND	ND
C-6	+	341 ± 19 ^{ab}	0.09 ± 0.01 ^c	0.351 ± 0.007 ^c	8.55 ± 0.17 ^b
C-7	–	346 ± 28 ^{ab}	0.06 ± 0.02 ^c	0.373 ± 0.007 ^c	2.03 ± 0.41 ^d
C-8	+	366 ± 20 ^{ab}	0.08 ± 0.01 ^c	0.285 ± 0.006 ^d	1.04 ± 0.21 ^d
C-9	+	329 ± 16 ^b	0.02 ± 0.02 ^d	ND	ND

ND – not detected.

¹ Values are means of 10 replicates ± standard error; means within columns having different letters are significantly different according to DMRT at $P > 0.05$.

Excised WT roots of most tropane alkaloid-synthesising plants (*Hyoscyamus muticus*, *H. albus*, *Atropa belladonna*, *Datura* sp. etc.) do not grow well in media without exogenous growth regulators. Therefore WT root cultures grown in media with auxins were used for the comparative examination of alkaloid production. Zehra et al. (1998) found that the hairy root cultures of *H. albus* produce less scopolamine than WT roots (grown in a medium containing 0.5 mg L⁻¹ IBA). Our experiments show that in the case of hairy roots of *H. niger* a hairy root clone (C-1) may produce large amounts of scopolamine compared with WT roots.

The maintenance of *H. niger* root cultures in media with various formulations (MS, Knop-M, B5) demonstrated that the morphology of roots remained unchanged (data not given). The composition of the media had relatively little effect on the growth of all types of roots. In the B5 medium (ratio NH₄⁺/NO₃⁻ = 2/25) the growth of roots was slightly more than in the MS medium (20/39), and in the Knop-M medium (0/10) the growth of roots was slightly lower than in the MS medium (Table 2). Our results are consistent with those of other authors, reporting that ammonia salts or a high ratio of NH₄⁺/NO₃⁻ in media inhibit the growth of roots (Oksman-Caldentey et al., 1994; Yamamoto & Kamura, 1997). Ammonium is very diffusive and it easily accumulates into the tissues, becoming very toxic if not immediately metabolized (Richter, 1993). Excessive ammonium has direct or

Table 2. Growth, excretion of riboflavin, and tropane alkaloid production in wild-type and hairy root clones of *Hyoscyamus niger* grown in different media (28-day culture; inoculum 100 ± 10 mg fresh roots)¹

Root clone and medium	Dry weight, mg flask ⁻¹	Riboflavin in the medium		Alkaloid content in the roots, mg g ⁻¹ of root dry weight	
		mg L ⁻¹ of the medium	mg g ⁻¹ of root dry weight	Hyoscyamine	Scopolamine
Wild type					
MS	143 ± 13 ^e	3.84 ± 0.18 ^a	1.34 ± 0.02 ^a	2.32 ± 0.22 ^z	5.87 ± 0.42 ^d
Knop-M	132 ± 8 ^f	2.95 ± 0.16 ^b	1.12 ± 0.03 ^b	2.31 ± 0.19 ^c	0.93 ± 0.08 ^f
B5	177 ± 9 ^c	3.01 ± 0.08 ^b	0.85 ± 0.02 ^c	ND	ND
Clone 1					
MS	160 ± 7 ^d	2.43 ± 0.09 ^c	0.84 ± 0.01 ^c	2.18 ± 0.11 ^c	7.89 ± 0.39 ^c
Knop-M	145 ± 10 ^e	1.86 ± 0.10 ^e	0.64 ± 0.02 ^d	4.47 ± 0.22 ^a	65.60 ± 3.28 ^a
B5	188 ± 12 ^c	2.49 ± 0.07 ^c	0.66 ± 0.02 ^d	3.75 ± 0.19 ^b	28.48 ± 1.42 ^b
Clone 8					
MS	298 ± 16 ^b	0.07 ± 0.01 ^f	0.012 ± 0.002 ^e	–	4.73 ± 0.24 ^e
Knop-M	285 ± 19 ^b	0.08 ± 0.01 ^f	0.014 ± 0.001 ^e	0.47 ± 0.02 ^e	6.12 ± 0.31 ^d
B5	360 ± 14 ^a	0.06 ± 0.02 ^f	0.008 ± 0.003 ^e	1.16 ± 0.06 ^d	3.92 ± 0.21 ^e

ND – not detected.

¹ Values are means of 10 replicates ± standard error; means within columns having different letters are significantly different according to DMRT at $P > 0.05$.

indirect repressive effect on nitrate accumulation (Crawford, 1995). However, the effect of the ratio $\text{NH}_4^+/\text{NO}_3^-$ varies from one clone to another. In the case of a low concentration of ammonia salts or a low ratio of $\text{NH}_4^+/\text{NO}_3^-$ (B5 medium) the growth of roots increases in many plants (Oksman-Caldentey et al., 1994; Bensaddek et al., 2001). The same happened in our experiment with *H. niger* roots.

The effect of the composition of the media on the excretion of riboflavin into the media was relatively low, too. The excretion of riboflavin into the Knop-M medium was somewhat reduced (Table 2).

Differently from root growth, the content of tropane alkaloids was quite significantly affected in all types of *H. niger* roots by the composition of the medium. The production of hyoscyamine in the WT root cultures grown in the MS and Knop-M media was almost the same. However, in hairy root clone 1 the production of hyoscyamine in the Knop-M medium was more than twice that in the B5 medium and 1.5 times higher than in the MS medium.

In clone 8 the content of hyoscyamine remained below the detection limit, and in the B5 medium it was more than twice that in the Knop-M medium. The content of scopolamine was the highest in clone 8 hairy roots grown in the Knop-M medium and the lowest in the B5 medium (Table 2).

On the basis of data by other authors (Bensaddek et al., 2001) the $\text{NH}_4^+/\text{NO}_3^-$ ratio has a significant effect on the production of tropane alkaloids by hairy roots. The reduction in the nitrate concentration (increase in the $\text{NH}_4^+/\text{NO}_3^-$ ratio) in the MS medium leads to an increased alkaloid production in the hairy roots of *A. belladonna*. The scopolamine/hyoscyamine ratio is increased when the nitrate and ammonium concentrations are raised. In *H. muticus* hairy root cultures the effect of the $\text{NH}_4^+/\text{NO}_3^-$ ratio on the hyoscyamine content follows the same pattern as on growth. The growth and tropane alkaloid production in *H. muticus* hairy root clones do not depend on the total amount of nitrogen in the medium. When the nitrogen concentration remains between 10 and 75 mM, the growth and hyoscyamine production are almost at the same level (Oksman-Caldentey et al., 1994). In *Datura quercifolia* hairy roots the growth and tropane alkaloid content are depressed by highly concentrated media. The dilution of the culture media and the reduction of nitrogen concentrations enhance growth and alkaloid content in hairy root cultures (Parr et al., 1990; Dupraz et al., 1993).

In our experiment both the reduction of the nitrogen concentration in the media and more diluted media (Knop-M < B5 < MS) exerted varying effects on tropane alkaloid production in the root cultures of *H. niger*. In the more diluted media the scopolamine content decreased in the WT roots and increased in the hairy root clone. In clone 8 the yield of total tropane alkaloids increased, but in the B5 medium, compared with the MS medium, the content of scopolamine decreased.

The excretion of riboflavin from the FG hairy root clones into the media containing 100 μM of iron was very low, practically at the detection level. For the estimation of riboflavin excretion from FG hairy roots we used the fact that

the excretion of riboflavin increases under iron deficiency in the media from intact plant roots (Welkie, 1993) as well as WT excised roots of *H. niger* (Pudersell et al., 2004). Our results (Tables 3, 4) showed increased riboflavin excretion from both WT roots and hairy root clone 8 under iron deficiency or in iron-lacking media. The greatest increase in the riboflavin (32 times, expressed on root dry weight basis) excretion was observed into the MS medium lacking iron in the case of hairy root clone 8. The riboflavin excretion into the same medium from the WT roots increased only 4.4 times. The riboflavin excretion

Table 3. Influence of Fe(III)EDTA concentration of nutrient solutions on the content of excreted riboflavin in wild-type root culture of *Hyoscyamus niger* (4 root tips; 28-day culture)¹

Medium	Dry weight of roots, mg per flask	Riboflavin content in the medium	
		mg per L of the medium	mg per g of root dry weight
MS			
Standard	18±2 ^c	0.59±0.09 ^e	1.63±0.19 ^e
1/2 Fe	28±4 ^a	1.72±0.13 ^d	3.07±0.22 ^d
0 Fe	25±2 ^a	3.83±0.12 ^a	7.20±0.18 ^b
Knop-M			
Standard	14±2 ^d	1.26±0.07 ^c	4.51±0.24 ^c
1/2 Fe	23±2 ^b	2.3±0.09 ^b	4.90±0.28 ^c
0 Fe	12±3 ^d	2.2±0.14 ^b	9.16±0.32 ^a

¹ Values are means of 10 replicates ± standard error; means within columns having different letters are significantly different according to DMRT at $P > 0.05$.

Table 4. Influence of Fe(III)EDTA concentration of nutrient solutions on the content of excreted riboflavin in fast-growing hairy root clone C-8 of *Hyoscyamus niger* (4 root tips; 28-day culture)¹

Medium	Dry weight of roots, mg per flask	Riboflavin content in the medium	
		mg per L of the medium	mg per g of root dry weight
MS			
Standard	206±20 ^a	0.12±0.03 ^e	0.029±0.008 ^e
1/2 Fe	213±16 ^a	0.17±0.02 ^e	0.039±0.007 ^e
0 Fe	116±11 ^d	2.09±0.12 ^a	0.950±0.034 ^a
Knop-M			
Standard	160±18 ^b	0.33±0.04 ^c	0.071±0.021 ^d
1/2 Fe	135±18 ^c	0.54±0.04 ^b	0.201±0.014 ^c
0 Fe	33±11 ^e	0.22±0.02 ^d	0.333±0.039 ^b

¹ Values are means of 10 replicates ± standard error; means within columns having different letters are significantly different according to DMRT at $P > 0.05$.

into the iron-deficient Knop-M media was lower than into the iron-deficient MS media. The increase (expressed on root dry weight basis) in the riboflavin excretion did not depend on the growth of roots in iron-deficient media, as in almost all media the riboflavin content increased if expressed per litre of the medium.

The roots of some plant cultivars that use iron efficiently (Fe-efficient plants) excrete more riboflavin than Fe-inefficient cultivars (Welkie, 1993, 1996). The significance of the excretion of riboflavin in large amounts from iron-stressed plants or excised roots is still unclear. The roots absorbed only reduced Fe^{2+} . In soil as well as in media Fe^{3+} prevails. Thus the roots have to reduce Fe^{3+} to Fe^{2+} . A specific form of riboflavin–flavin mononucleotide functions in the iron reducing process. The fact that the Fe-efficient plants excrete riboflavin into the culture media was interpreted as the ability of certain plants to increase their Fe utilization under Fe-deficiency stress by, in part, synthesizing more riboflavin that can contribute to Fe reduction at the epidermal plasmalemma (Welkie, 1996). Our results concerning the decrease in the riboflavin excretion from FG hairy roots of *H. niger* can be interpreted as the Fe-inefficiency of FG hairy roots.

CONCLUSIONS

Six hairy root clones of *Hyoscyamus niger* transformed with *Agrobacterium rhizogenes* strain LBA 9402 exhibited different patterns of growth, morphology, tropane alkaloid production, and riboflavin excretion into the medium. Clone 1, which was relatively similar to the WT root culture in morphology and growth intensity, produced more (up to 8 times) tropane alkaloids, especially scopolamine (up to 15 times) than the WT roots. The FG clone 6, which was morphologically most different from WT roots, produced 1.5 times more scopolamine than the WT roots. Other FG clones produced less alkaloids than the WT roots.

The excretion of riboflavin into the media was the highest in the case of WT roots. The excretion of riboflavin from the FG hairy roots was very low on the media of high iron level. In the absence of iron the riboflavin excretion rose up to 33 times, but the excretion level of riboflavin from the FG roots into the iron-deficient media remained up to 10 times less than that from the WT roots into the media containing 100 μM Fe.

The production of alkaloids by both WT roots and hairy root clones depended on the composition of the media. However, these dependences were not regular. The WT roots produced six times less scopolamine in the Knop-M medium compared with the MS medium. On the contrary, clone 1 produced 8 times more scopolamine in the Knop-M medium than in the MS medium. Therefore, each clone of roots needs an individual approach depending on the product we want to get with the assistance of that root culture.

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Tropaanalkaloidide süntees *Hyoscyamus niger* L. karvjuurekloonides ja riboflaviini eraldumine söötmesse

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On uuritud võrdlevalt *Hyoscyamus niger* L. hariliku (WT) juure ja *Agrobacterium rhizogenes* LBA 9402 abil saadud transgeensete karvjuurekloonide tropaanalkaloidide sünteesi juurtes ja riboflaviini eraldumist söötmesse. Välja- valitud kuuest karvjuurekloonist oli üks kloon (C-1) morfoloogiliste tunnuste ja kasvu intensiivsuse poolest sarnane WT juurtega. Ülejäänud, kiiresti kasvavad (FG) karvjuurekloonid kasvasid kaks korda kiiremini kui WT juured ja olid neist morfoloogiliselt erinevad. Karvjuurekloon C-1 sünteesis olenevalt sööttest (MS, B5, Knop-M) 5 kuni 15 korda rohkem skopolamiini ja 1,5 kuni 2 korda rohkem hüostsüamiini kui WT juured. FG karvjuurekloonid sünteesisid enamikus vähem tropaanalkaloide kui WT juured, kuid nende süntees olenes suurel määral söötme koostisest. Riboflaviini eraldumine karvjuurekloonist C-1 oli mõnevõrra väiksem kui WT juurtest. FG karvjuurekloonidest eralduva riboflaviini hulk jäi minimaalse määramispiiri lähedale, kuid selle eraldumine söötmesse suurenes tunduvalt raua defitsiidi korral.