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Riboflavin Excretion from the Excised Roots of *Hyoscyamus niger*

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Abstract

Excised roots of *Hyoscyamus niger* growing in darkness excrete yellow pigment into the liquid culture medium. The pigment has been identified as riboflavin by paper chromatography, thin-layer chromatography, photolysis, and spectrophotometry. The increase of biomass of *H. niger* roots in the flasks is uniform during the growth period (ca. 30 days), having no exponential phase. A significant decrease in pH was observed in Murashige-Skoog (MS) and Knop-M media during the first 4 days following inoculation. The pH of media then increased slowly until the cultures reached their stationary phase (ca. 35 days) and then increased rapidly. The excretion of riboflavin into the media (expressed per volume of media) was low during the first 4 days of incubation and increased rapidly thereafter during the 6th to 14th days of growth. The maximum content of riboflavin in the medium, expressed per dry weight of the roots, was observed during the 14th to 15th days after inoculation. A decrease in the iron content of the MS medium caused an increase in the excretion of riboflavin; the additional iron in the MS medium resulted in a decrease in the excretion of riboflavin.

Keywords: Excised roots, *Hyoscyamus niger*, iron, pH, riboflain

Introduction

Hyoscyamus niger L. has been regarded as a rich source of pharmaceutically important tropane alkaloids, such as hyoscyamine and scopolamine. These alkaloids are synthesized in the roots of the plants, and accordingly, *in vitro* root cultures are the best sources of these alkaloids (Hashimoto et al., 1986). Most of the reported excised root cultures required auxins for induction and maintenance. The root cultures of *H. niger* are not typical, as these are readily established and grow without the presence of exogenous phytohormones and produce large amounts of tropane alkaloids (Shimomura et al., 1991; Robbins & Dräger, 1995; Pudersell et al., 1999). In contrast to other root cultures previously investigated by our group (*Armoracia rusticana*, *Solanum tuberosum*, *Prunus avium*, etc.) the liquid culture medium of *H. niger* turned yellow during the growth period of the roots in the dark on a rotatory shaker. If roots were cultured under light, the yellow color of the media dissipated, but it did not dissipate in the dark after inactivation of the enzymes by heating.

These observations led to the supposition that the compound with yellow luminescence, released by *H. niger* root culture into the growth medium, might be a flavin pigment. The excretion of riboflavin by the roots of intact tobacco plant has already been observed in 1958 (Pound & Welkie, 1958). Later, this was observed for several dicotyledonous species (sugar beet, lettuce, pepper, etc.) under iron stress. Additional iron (Fe^{3+}) in the liquid culture medium caused a decrease in the excretion of riboflavin (Welkie & Miller, 1989). Under iron and temperature stress, a decrease in riboflavin excretion from roots was observed, but riboflavin excretion did not have a one-to-one ratio with the root mass (Welkie, 1995). The amount of riboflavin excreted into the culture medium increased due to the excessive administration of manganese. It has been suggested that excessive manganese leads to iron deficiency (Shimizu et al., 1998). Salt stress did not cause an increase in riboflavin excretion from the intact plant roots (Welkie & Miller, 1992).

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To our knowledge, such drastic color changes of sterile media with excised roots has not been examined previously. The aims of this study are to demonstrate that the yellow pigment excreted by the roots of *H. niger* is riboflavin and to explain the dynamics of its formation and the relationship of its formation with the pH and iron content of the medium.

Materials and Methods

Plant material

Sterile plants of henbane (*Hyoscyamus niger* L.) were established from seeds, obtained from the Biocenter of Viikki of the University of Helsinki. Seeds were sterilized with 9% chlorinated lime with some drops of Tween 80 as a detergent. After the washing of the seeds three times with distilled and sterilized water, these were planted onto the sterile 1/5 diluted Murashige-Skoog (Murashige & Skoog, 1962) medium with 8% agar and 1% sucrose. Seed germination and plant growth was carried out in a plant growth room at 22 ± 2 °C with a 16/8 h day/night cycle and under cool white fluorescent lamps (20 µmol·m^{-2}·s^{-1}). From 3-week-old seedlings, the sprouts were excised and transferred onto the 1/2 macronutrients of Murashige-Skoog (MS) solid medium with 2% sucrose. The shoots formed 3–4 cm plants with 1.5–2 cm roots within 4 weeks. From these plants, the roots were cut to obtain excised root culture.

Roots were grown in a plant growth room in the dark at 25 ± 2 °C on a rotatory shaker (90 rpm) in 250-ml Erlenmeyer flasks containing 50 ml growth medium. A root inoculum of ca. 100 mg fresh weight was transferred into the fresh medium after every 28 days. MS complete medium was used for the multiplication of roots, and the medium, containing Knop's (1865) macronutrients and MS miconutrients and organics (Knop-M), was used for the experiments. Sucrose was added at 20 g/l.

Isolation of flavins

The growth medium (40 ml) was filtered through filter paper (Filtrak, VEB Spezialpapierfabrik, Niederschlag, Germany) and passed through a C_{10} column (5 × 2.3 mm). The yellow substance similar to riboflavin was retained at the top of the C_{10} column during application and washing with water. The column was washed with 40 ml of water to remove growth medium components and other substances. The yellow pigment(s) were eluted with ethanol of various concentration in the effluent volume, which depended on the ethanol concentration. The effluent obtained with 30% ethanol was routinely used for paper chromatography (PC), thin-layer chromatography (TLC), and spectrophotometric measurements.

Chromatography

Paper chromatography was performed on Whatman chromatography paper 1 Chr. (Whatman International Ltd, Maldstone, England). The mobile phases used were BAW, upper phase of *n*-butanol-acetic acid-water (4 : 1 : 5, v/v); PAW, pyridine-amyl alcohol-water (1 : 1 : 2); and WI, water saturated with isoamyl alcohol. For thin-layer chromatography, Silufol plates 150 × 150 mm (Kavalier, Czechoslovakia) were used. The mobile phase for TLC were BAW and AAW, and the upper phase was *n*-amyl alcohol-acetic acid-water (3 : 1 : 3, v/v). R_f values were determined from the center of the spot.

Photolysis of riboflavin

Photolysis of riboflavin and other yellow substances was carried out on a silica plate or chromatography paper under UV of high-pressure mercury lamp with a UV filter by the following method. The spot of riboflavin solution or the yellow fraction of ethanol effluent from C_{10} column was chromatographed in one direction. The chromatogram was dried, illuminated in UV light for 5–30 min, and thereafter chromatographed once more with the same solvent in the other direction. The products of photolysis were developed on the second run on the paper or silica plate.

Riboflavin assay

Concentration of riboflavin was determined using a fluorimeter Analiz (Geologorazvedka, Saint Petersburg, Russia) modified by Dr. A. Tohver. The maximum of exitation wavelength was 436 nm, and emitted light was passed through a 500-nm cutoff filter. Concentration was determined using standard curves established with authenic riboflavin (Chemapol, Praha, Czechoslovakia). Spectra were recorded using a Specord UV-Vis spectrophotometer (Carl Zeiss, Jena, Germany). All laboratory procedures were carried out in darkness or with dim red safe light.

Results

Identification of riboflavin

PC and TLC examination of the yellow fraction revealed only one yellow luminescent spot on chromatograms, which had R_f values close to that of a riboflavin spot (Table 1). Examination of the products of photolysis on paper and thin silica layer could be regarded as an alternative method for the identification of photolabile substances. The chromatographic method used in the current study revealed three distinct products of photodegradation for both riboflavin and yellow substances on the paper (Table 2); six photolabile substances were discovered on silica gel (Table 3). Comparison of the mobility of the products of photolysis of yellow substance from *H. niger* root culture media resulted in the same results.

The absorption spectrum of the yellow substance had maxima at 224, 269, 370, and 450 nm, and the solution of riboflavin in 30% ethanol had maxima at 224, 270, 373, and 454 nm. The difference in absolute heights of the spectra was

Table 1. Mobility of riboflavin and yellow substance (Ys) on paper and silica layer.

	R_f				
	Paper chromatography			Thin-layer chromatography	
Spot due to	BAW	PAW	WI	BAW	AAW
Riboflavin	0.24	0.30	0.43	0.44	0.22
Ys	0.26	0.32	0.40	0.44	0.22

BAW, upper phase of *n*-butanol : acetic acid : water (4 : 1 : 5, v/v); PAW, pyridine : amylalcohol : water (1 : 1 : 2, v/v); WI, water saturated with isoamyl alcohol; AAW, upper phase of *n*-amyl alcohol : acetic acid : water (3 : 1 : 3, v/v).

Table 2. R_f values of the products of photolysis (PP) of riboflavin (Rbf) and the yellow substance (Ys) from growth medium (paper chromatography).

BAW			PAW			WI		
Spot of PP	Ys	Rbf	Spot of PP	Ys	Rbf	Spot of PP	Ys	Rbf
Yellow	0.23	0.22	Blue	0.15	0.16	Blue	0.12	0.11
Yellow	0.36	0.34	Yellow	0.31	0.31	Yellow	0.25	0.25
Blue	0.52	0.50	Blue	0.63	0.64	Yellow	0.41	0.39

BAW, upper phase of *n*-butanol : acetic acid : water (4 : 1 : 5, v/v); PAW, pyridine : amyl alcohol : water (1 : 1 : 2, v/v); WI, water saturated with isoamyl alcohol.

Table 3. R_f values of products of photolysis (PP) of riboflavin (Rbf) and the yellow substance (Ys) from growth medium (thin-layer chromatography).

AAW			BAW		
Spot of PP	Ys	Rbf	Spot of PP	Ys	Rbf
Yellow	0.35	0.35	Yellow	0.41	0.41
Yellow	0.47	0.47	Yellow	0.48	0.48
Yellow	0.54	0.54	Yellow	0.63	0.63
Yellow	0.63	0.63	Yellow	0.68	0.68
Yellow	0.71	0.71	Yellow	0.74	0.74
Blue	0.77	0.77	Blue	0.85	0.85

AAW, upper phase of *n*-amyl alcohol : acetic acid : water (3 : 1 : 3, v/v). BAW, upper phase of *n*-butanol : acetic acid : water (4 : 1 : 5, v/v).

probably due to some nonluminescent substance(s) from the effluent.

The growth of roots and riboflavin excretion

The growth of the excised roots depended on the weight of inoculated root material. If the inoculum was very small (1–1.5 mg of dry weight), the weight of roots increased 20-fold during 28 days. If ca. 60 mg of root material was inoculated, the gain in biomass was only three-fold (Table 4). These data are in agreement with the experiments of Kanokwaree and Doran (1997) with *Atropa belladonna* hairy roots. An increase in the number of inoculated root tips from three to nine reduced the growth up to 40%. The excretion of riboflavin into the medium depended on the weight of inoculum as well. The excretion of riboflavin (expressed on the volume unit of the medium) increased during the growth cycle (exept the cases of very high biomass: 60–65 mg of dry weight). However, the results of the expression of the amounts of excreted riboflavin for the 1 g final dry weight of roots are the converse of this. In flasks with very small inoculum (1–2 mg), the production of riboflavin was 1.9 mg/g dry weight of roots. In flasks with large inocula, the production of riboflavin was twofold lower (Table 4).

The excretion of riboflavin from roots to Knop-M medium was lower than in MS medium, although the increase of root biomass did not differ greatly (Table 5). The growth of biomass of excised roots of *H. niger* was relatively linear during the whole growth period without any exponential phase and reached a stationary phase at days 33–35 of growth. Excretion of riboflavin (expressed per volume unit of the medium) increased rapidly on days 6–14. Following this, a very small increase in the content of riboflavin in the medium was observed until the end of experiment (40th day). The expression of riboflavin content per dry biomass of roots

Table 4. Growth and riboflavin excretion to MS medium from excised roots of *H. niger* depending on inoculum dry weight (28 days).

Inoculum (mg)	Final weight of roots (mg)	Increase of weight (fold)	Content of riboflavin	
			In medium (μg/ml)	To mg/g dry weight of roots
1–1.5	30 ± 4	20	1.14 ± 0.18	1.90 ± 0.26
4–5	65 ± 6	16	1.73 ± 0.17	1.47 ± 0.12
14–16	119 ± 8	8	3.21 ± 0.12	1.45 ± 0.05
30–35	140 ± 15	5	3.72 ± 0.18	1.36 ± 0.06
60–65	170 ± 21	3	2.31 ± 0.22	0.92 ± 0.06

Table 5. Excretion of riboflavin from *H. niger* excised roots into MS and Knop-M media (25 days; inoculum 25–30 mg dry weight; n = 15).

Medium	Dry weight of roots (mg)	Riboflavin content	
		In medium (μg/ml)	To mg/g dry weight of roots
MS	149 ± 12	3.62 ± 0.20	1.21 ± 0.09
Knop-M	132 ± 14	2.16 ± 0.16	0.82 ± 0.10

demonstrated that the maximum content of riboflavin in the medium was observed on days 14–15. This shows that the formation of riboflavin can be impeded, while the root growth continues with its former intensity (Fig. 1).

Changes of media pH

The pH of medium decreased rapidly during the first days of growth and then increased slowly until days 31–35. An abrupt increase in the pH (<8) was observed only then, if the growth of the roots was stopped (Fig. 1).

Fundamental changes in the pH value of the medium were observed during the first 10 days of growth for the roots. Small differences were observed, if the roots of *H. niger* were grown in the different media or with different amounts of inoculum. In the MS medium with small inoculum (ca. 4 mg), the minimum pH was observed on the fourth day; with a greater inoculum (ca. 30 mg), this became the third day. At the same time, the decrease in pH was greater (5.2 → 4.4) in the case of small inoculum, when compared with the culture with greater inoculum (5.2 → 4.6) (Fig. 2). The pH of Knop-M medium reached its minimum on the fourth day of growth, but in contrast to the MS medium, a decrease in pH was greater in the case of greater inoculum (Fig. 3).

The excretion of riboflavin into the medium was slow during the period of rapid decrease of the pH of media and increased rapidly as the minimum of pH was reached (Figs. 2 and 3).

Iron content and riboflavin excretion

Increase or decrease of the iron content in MS medium resulted in changes to the excretion of riboflavin into the media. Excretion of riboflavin was highest if the iron was depleted in the MS medium. Addition of $Fe^{III}EDTA$ two times greater than normal into the MS medium (normal 100.6 μM) caused sufficient decrease in riboflavin excretion. At the same time, the changes of iron content in medium did not cause any specific changes in the growth of the root cultures (Table 6).

The pH of all kinds of media was adjusted to 5.8 before autoclaving. Depending on the content of iron in the media, the pH of media decreased during autoclaving – the increase in pH was higher if the medium contained more iron. After 15 days of incubation for the roots, the pH of the media were almost equal (4.6–4.7). The pH of MS medium without the iron decreased by 0.9 units, and the pH of MS medium with double the normal iron level increased by 0.1 units (Table 6).

Discussion

In all reports, the excretion of riboflavin from roots into the nutrient solution has been observed using liquid culture of intact plants. The Hogland and Arnon (1938) nutrient solution has been used that does not contain iron. Grown in this solution, numerous intact plants excrete riboflavin into the nutrient solution.

A decrease in pH of the nutrient solution and the chlorosis of the plants was simultaneously observed (e.g., Welkie & Miller, 1989; Welkie et al., 1990). The pH of the nutrient solution started to decrease after an initial 6 days of increase (fifth to eighth days). During this period of pH increase, the content of riboflavin of the nutrient solution increased very slowly. If the plants grown in the Hogland and Arnon solution had 2 mg/l (35.7 μM) $Fe^{III}EDTA$ added, the excretion of riboflavin was very low or practically absent. The pH of solution increased rapidly on the sixth to seventh days of growth and stabilized at 8.0–8.2 until the end of the experiment (14th day) (Welkie, 1993).

In our experiments, the MS and Knop-M media were used, in which the content of iron is 100 μM; it is threefold more

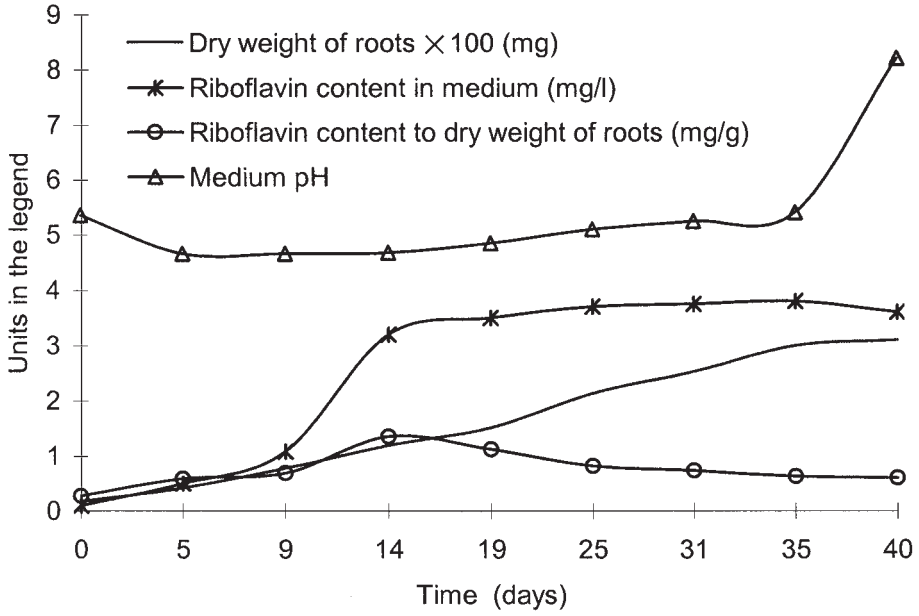

Figure 1. Growth of *H. niger* roots in MS medium, excretion of riboflavin to medium, and the change of medium pH.

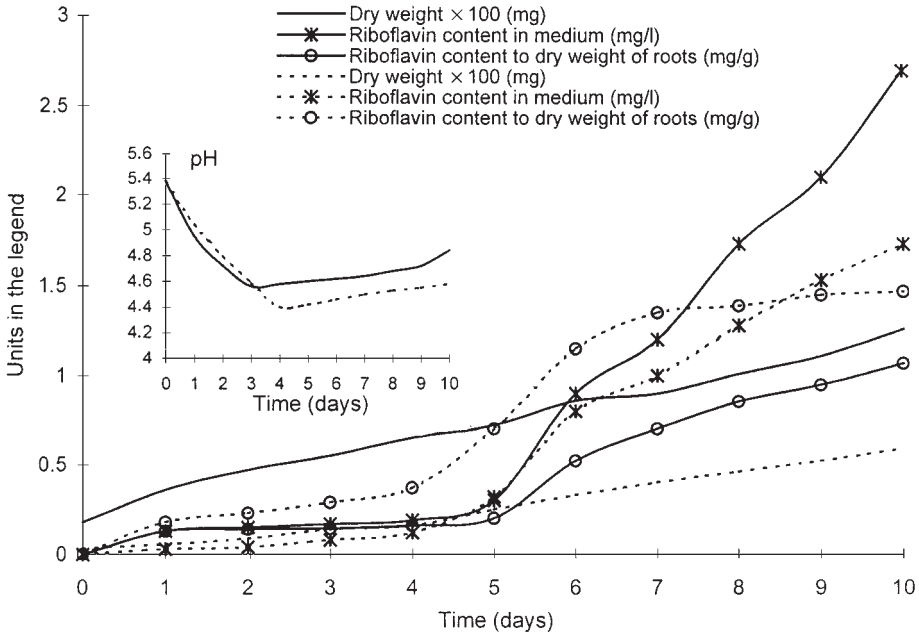

Figure 2. First 10 days of growth of *H. niger* roots in MS medium, excretion of riboflavin to medium, and the change of medium pH (inset: inoculum ca. 30 mg ——; inoculum ca. 4 mg - - -).

than in experiments that use the intact plants of pepper with a Hogland and Arnon solution of high iron content, as reported by Welkie (1993). In both media, the excised roots of *H. niger* excreted high amounts of riboflavin into the solution. Our results suggest that a correlation exists between the iron content in medium and release of riboflavin by the roots as described by Welkie (1993). If iron was depleted from the MS medium, the content of riboflavin in the medium increased ca. twofold. If the iron concentration was doubled in the MS medium, the riboflavin excretion decreased by twofold when compared with roots grown in MS medium with normal iron concentration.

The pH of liquid medium of *H. niger* excised roots decreased rapidly for the first 3–4 days in MS media (NH_4^+ – 20.6 mM; NO_3^- – 39.4 mM; PO_4^{3-} – 1.24 mM) and Knop-M (NH_4^+ – 0 mM; NO_3^- – 10.9 mM; PO_4^{3-} – 1.8 mM). Thereafter, the pH of the media increased slowly for ca. 35 days of culture and then increased rapidly. A rapid increase in pH

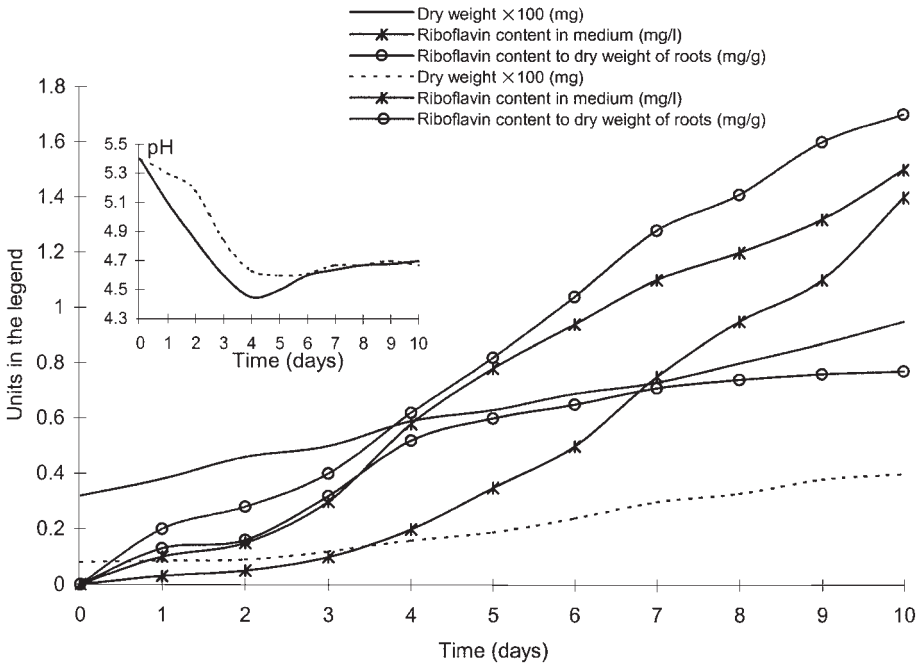

Figure 3. First 10 days growth of *H. niger* roots in Knop-M medium, excretion of riboflavin to medium, and the change of medium pH (inset: inoculum ca. 30 mg ——; inoculum ca. 4 mg - - -).

Table 6. Excretion of riboflavin from *H. niger* excised roots depending on iron content in MS medium (15 days; inoculum 15–17 mg dry weight; n = 15).

Fe content (μM)	Dry weight of roots (mg)	Starting pH	End pH	pH decrease – increase +	Riboflavin content in medium (μg/ml)	Riboflavin content to dry weight of roots (mg/g)
0	92 ± 9	5.5	4.6	–0.9	5.60 ± 0.22	2.88 ± 0.10
50.3	89 ± 12	5.2	4.7	–0.5	4.30 ± 0.16	2.42 ± 0.09
100.6	103 ± 8	5.0	4.7	–0.3	3.03 ± 0.16	1.40 ± 0.08
201.2	86 ± 11	4.5	4.6	+0.1	1.50 ± 0.10	0.87 ± 0.11

was observed at the moment when the growth of roots had almost stopped. In experiments with hairy roots from various plants, it was established that the growth and pH profile using Gamborg et al. (1968) B5 salts medium (NH_4^+ – 2.02 μM; NO_3^- – 24.72 μM; PO_4^{3+} – 1.09 μm) resembled each other closely and reflected changes in the uptake of certain ions during the different growth phases. A significant increase in pH began at 7–12 days after inoculation (at 5.0–8.0); during the same period, the drained weight of roots also increased rapidly until the stationary phase was reached. Ammonium ions were totally and rapidly removed from the medium during the first days of culture (7–10 days decrease or stationary phase of pH) preceding the exponential growth, while only some nitrate was later used gradually. Subsequently, PO_4^{3-} was then removed (10–20 days, increase in pH) (Hilton & Wilson, 1995; Bhadra & Shanks, 1997).

In our experiments, medium with NH_4^+ (MS) and medium without NH_4^+ (Knop-M) were used. There is no clear correlation between the NH_4^+, NO_3^-, and PO_4^{3-} consumption and the pH of media. The growth of roots in both media were relatively uniform without any increases during the entire growth period and stopped at 34–40 days, evidently due to the full depletion of mineral elements and sucrose.

Thus, changes in the pH of the media of excised roots of *H. niger* differed from the pH of plant nutrient solution for intact pepper (Welkie, 1993), and the pH of media for hairy root culture of various Solanaceae species (Hilton & Wilson 1995) changes. However, this is almost the same decrease in pH as in the case of *H. niger* during the first days of culture observed in nutrient solution of iron-stressed muskmelon plants (Welkie, 1996) and in the hairy root medium of *Catharanthus roseus*. Moreover, when hairy root cultures of *C. roseus* were initiated in media with differing pH (4.2–7.3), the pH of medium decreased or increased for the fourth to fifth day onto the level of 5.0–5.5; the pH curves then turned

approximately parallel to each other (Ho & Shanks, 1992). The same changes in pH values were observed in our experiments in the MS medium of different iron content, too. Initiating media with different iron content had different pH, but after the 15th day of culture, the pH levels were approximately the same. According to Welkie and Miller (1989) and Welkie (1993), the pH decreased in nutrient media without iron or with low iron content (0.02 mg/l), and at the same time the excretion of riboflavin into the medium increased. If the media contained 0.2–2.0 mg/l of iron, the pH increased, and the excretion of riboflavin into the medium was very low. This implies that the decrease in the pH of the medium is correlated with the excretion of riboflavin. In the case of *H. niger* excised roots, the excretion of riboflavin was observed during the period of slow increase in pH of the medium.

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