Root tip -dependent, active riboflavin secretion by *Hyoscyamus albus* hairy roots under iron deficiency

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Summary

Hyoscyamus albus hairy roots with/without an exogenous gene (11 clones) were established by inoculation of *Agrobacterium rhizogenes*. All clones cultured under iron deficient condition secreted riboflavin from root tips into the culture medium and the productivity depended on the number and size of root tips among the clones, although the addition of sucrose was essential for riboflavin production. A decline of pH was observed before riboflavin production and root development using either a root tip or propagated roots: propagated roots were employed for further work due to their lesser variation. Additions of proton-pump inhibitors, N,N'-dicyclohexylcarbodiimide (DCCD) at 100 and 10 μM and erythrosine B at 100 μM, suppressed the pH decline at 100 and 10 μM accompanied by inhibition of riboflavin secretion and root growth; at 10 μM of erythrosine B, pH decline occurred with a moderate delay, but both growth and riboflavin efflux were inhibited. Neither inhibition of the pH decline nor riboflavin production was observed at 1 μM. To examine the necessity of acidification and riboflavin secretion by the roots themselves, artificial pH reduction of culture medium with organic acids and the addition of exogenous riboflavin with/without pH reduction were performed. When hairy roots were cultured in iron-deficient medium acidified with citric acid (pH 4.0) or malic acid (pH 3.7), pH increased rapidly to around 5 overnight, following which riboflavin production and root growth occurred. Addition of riboflavin did not affect riboflavin secretion by the roots, but acidification with citric acid (pH 4.0) helped achiever greater riboflavin production and earlier pH elevation. These results indicate that riboflavin efflux does not directly connected to active pH reduction, and more significantly active riboflavin secretion occurs by internal requirement in *H. albus* hairy roots under iron deficiency.

1. Introduction

Iron is an essential metal for all living organisms and involved in redox reactions such as the electron transport chain in respiration and photosynthesis for the maintenance of homeostasis [9]. Iron deficiency is quite common for plants that stand on calcareous soil. Plants utilize water-soluble ferric iron from soil, but its uptake by roots is often limited due to its extremely low solubility especially at neutral pH. To address this problem, plants have evolved two general strategies: one is that roots of so-called strategy II plants (graminaceous plants) synthesize and produce iron chelators, called phytosiderophores, which form highly stable complexes with ferric iron and uptake these complex inside the root cells. Another strategy I plants (non- graminaceous plants) are known to release protons from the roots to the rhizosphere by activation of a proton-pump H^+-ATPase so as to reduce soil pH, by which the solubility of ferric irons increases [9, 19, 30, 39]. In addition, the release of organic acids such as citric and malic acid occurs, which work as reagents for iron-chelation [4, 11, 13, 14]. Ferric iron-chelators are modified to the ferrous iron by membraneous reductase and then the ferrous iron is transported into cells by an iron regulated transporter.

It was also found that not all but some strategy I plants from Aizoceae to Umbelliferae, including tobacco, beet, sunflower and cucumber, excrete riboflavin [39], and also unique riboflavin sulfates in the case of sugar beet [33, 34], in the rhizosphere under iron-deficient conditions. Recently, cultured roots of *Hyoscyamus niger* were also found to be able to secrete riboflavin without their aerial parts [36]. Even though there is abundant knowledge on the phenomena mentioned above, there is little data to date on the biochemical mechanism of riboflavin production and on the reason for riboflavin secretion in rhizosphere. Riboflavin is an aqueous and neutral compound not considered to be an iron-chelator. Riboflavin contains

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nitrogen atoms, and hence is a very valuable compound for all plants, so that riboflavin excretion in certain

species seems to exact a high energy cost for such iron deficiecy.

Flavins, including riboflavin and its derivatives flavinmononucleotide (FMN) and

flavinadenindinucleotide (FAD), are very important compounds for all organisms, the same as iron, and

work as co-enzymes in various redox reactions, especially on the plasma membrane [1].   In higher plants,

flavoproteins, flavin-conjugated proteins, include nitrate reductase, which affects nitrate assimilation [1],

polyamine oxidase, regulates the cellular polyamine levels [17], the blue light photoreceptors phototropin

and cryptochrome, which transduce blue light energy into a physiological signal [28, 38], and so on.   In

contrast, the functions of protein-free flavins are not well understood, although higher plants contain

considerable amounts of free riboflavin, 10 μM, in the cytoplasm [18].   Humans cannot biosynthesize

riboflavin and therefore need to take it in as Vitamin B_2 from foods.   Bacteria and yeast are able to

produce riboflavin and improved strains are used for biotechnological production in large scale industrial

applications [6, 7, 25, 42].   Interestingly, iron is known to be a potent inhibitor of riboflavin production in

these cases [2, 6, 32].   Plant-based riboflavin production is also of interest as a biotechnological source of

riboflavin.

We found that *Hyoscyamus albus* hairy roots cultured in iron-deficient medium were able to secrete

riboflavin into the medium during experiment using transgenic roots mentioned in the following section,

independent of the previous results reported for *H. niger* cultured roots [36].   To understand the

mechanism of riboflavin secretion by iron-deficient plant roots, we employed thi*s H. albus* hairy root

system to exclude complex factors involved in an intact plant system, because intact plants develop iron

sensors in both root and shoots, transmit signals between them and communicate with each other [30].

Here we examine the effects of proton-pomp inhibitors, medium acidification with external organic acid,

and riboflavin addition on pH change and riboflavin productivity.   We describe that riboflavin secretion

occurs from the root tips and the amounts are dependent on number and size of root tips among various root

clones.   In addition, external supply of riboflavin does not affect the riboflavin secretion by the roots

themselves, but the addition of organic acid in order to acidify the medium helps to elicit increased

riboflavin production.

2. Results

2.1. Riboflavin secretion by various hairy root clones under iron-deficient condition

Hyoscyamus albus is a Solanaceae tropane alkaloid-producing plant.   We have established 11 transgenic hairy root clones of *H. albus* with/without the gene *hyoscyamine 6β-hydroxylase* (*H6H*), which enzyme converts hyoscyamine to scopolamine [20], for scopolamine production as part of a series of transgenic investigations [26, 27, unpublished data].   All clones (encoding Ha 1~16) were analyzed for insertion of the *H6H* gene as well as the *rolB^{TL}* and *rolB^{TR}* genes [3] derived from *Agrobacterium rhizogenesis* ATCC15834 by genomic PCR.   The established hairy root clones bearing exogenous genes are listed in Table 1.   Wild type root cultures of *H. albus* were obtained from the root tip of the seedling germinated under sterile conditions, too.   Ha 1 to 7 with both *rolB^{TL}* and *rolB^{TR}* grew thick swelling roots with less branching, whereas Ha 11 to 16 with solely *rolB^{TL}* developed thin hairy roots with a lot of branching, regardless of being with/without the *H6H* gene (Table 1).   Interestingly, non-transformed root cultures grew in a very similar manner to Ha 11~16 without growth hormone, as reported for *H. niger* excised root cultures [31, 36].

The enzyme H6H is a 2-oxoglutarate dependent dioxygenase, requiring presence of Fe^{2+} ion together with molecular oxygen and ascorbate for the reaction.   To know the effect of iron concentration on tropane alkaloid productivity, we added ferrous iron in liquid Murashige-Skoog (MS) [22] medium at various concentrations (0, x1, and x10).   Increased iron concentration effectively produced both scopolamine and hyoscyamine, but productivity of both compounds decreased without iron (data not shown).   On the other aspect,   it was noticed that the medium colour turned yellow when hairy roots were cultured without the iron.   Chemical analysis indicated the yellow compound had absorption maxima at 267, 372 and 444nm, and greenish fluorescence under UV-illumination, which were coincident with authentic riboflavin.

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Behaviours of riboflavin and the yellow compound by HPLC chromatography and acid/alkali treatment were also identical. Therefore, it was confirmed that *H. albus* hairy roots expressed iron deficiency-dependent riboflavin production. Search of the literature regarding riboflavin secretion under iron deficiency hit a paper which mentioned a very similar phenomenon using non-transformed root cultures of *H. niger* [36].

HPLC analysis revealed that riboflavin content in the free-form was negligible in root tissues cultured in the iron-deficient liquid medium, as given later (Table 2). Then we speculated that riboflavin once appeared was actively excreted in the liquid medium from the hairy roots. Riboflavin excretion was further checked using a solid medium without iron, showing that riboflavin was actively secreted from root tips into the solid medium (Fig. 1). In the case that root tips were allowed to grow in the air, yellow drops appeared at the tips and then dropped down to the solid medium. The solid medium turned visible yellow colour with greenish fluorescence under UV-illumination.

H. albus roots were able to grow without iron, but the growth was inferior to and root morphology different from the roots supplied with sufficient iron. Roots without iron grew shorter, but more branching was formed; root tips, especially the subapical zones, became swollen with a great many dense hairs, as reported previously [16, 29].

Using all hairy root clones as well as non-transformed root cultures, we determined the riboflavin production and root growth under iron-deficient condition, resulting in the finding all of the clones, including the wild type (WT) roots, produced riboflavin (Fig. 2). Since root tip seemed to be the main site of riboflavin excretion (Fig. 1), the number and size of root tips were also counted in addition to riboflavin production (Fig. 2). Under iron deficiency, the number of root tip varied extremely from 3 to 96/culture among clones, in which Ha 16 was most (81±13), followed by Ha 7 (43±5). The size of root tip measured at the most wide part varied between 0.4 and 1.6 mm in diameter/tip, as well. There was a tendency that hairy root clones with both $rolB^{TL}$ and $rolB^{TR}$ (Ha 1~7) had bigger size of root tip than clones with solely $rolB^{TL}$ (Ha 11~16): the tip size more than 0.8 mm in diameter was only found in clones Ha 1~7, although Ha 1 was thinner than others. Hairy root clones without external *H6H* gene (Ha 7 and 16) produced

greater riboflavin, but also higher production was found in some *H6H* positive clones (Ha 2, 4 and 5).

Among them, clone Ha 16 showed the highest riboflavin productivity (0.263±0.087). As a basal medium,

B5 medium [8] was better than MS medium (data not shown). Suitable sucrose concentration for

riboflavin production was examined, revealing that 1~2% was better than 3%, while without sucrose, no

riboflavin was produced (data not shown). Therefore, clone Ha 16 cultured in B5 basal medium

supplemented with 1% sucrose was used throughout the experiment thereafter.

2.2. Effect of with/without pre-culture of hairy roots on riboflavin production and pH

It is well-known fact that roots of the so-called strategy I plants acidify the rhizosphere during iron

deficiency to enhance the solubility of ferric iron [39, 40, 41]. Since *H. albus* belongs to the strategy I

plants, the relationship between riboflavin secretion and pH changes under iron deficient conditions was

determined using hairy roots. When a root tip (ca. 2 cm in length) was directly inoculated into iron-free

medium as the starting material, pH was gradually reduced down to around 4.0 until 10 days, and then

maintained a nearly steady state. Riboflavin production as well as root growth started once the pH value

had reached a minimum (Fig. 3), although the root weight cultured in iron-free medium was less than one

third of that in normal medium. Due to the large variations in riboflavin production and sometimes even

no growth in the iron-free medium of a root tip, we also examined the effect of iron-deficiency using

propagated roots. A root tip was pre-cultured for 2 weeks in the normal medium with iron and then the

medium was changed to iron-free medium for a further 2-week culture. In this case, much earlier pH

reduction together with riboflavin production was found after 3 days in iron-free medium, and then the pH

was slightly restored. The minimum pH with propagated roots was higher (ca. 4.5) than that without

pre-culture (ca. 4.0) (shown as control in Figs. 4 and 5). Since hairy roots with pre-culture grew in a

relatively constant manner with less variation, we used propagated roots for the following experiments.

Riboflavin contents were analyzed for the medium and tissue cultured with/without iron, showing nearly

95% of riboflavin was present in the culture medium under iron starvation (Table 2). Therefore,

riboflavin contents only in culture medium was determined hereafter.

2.3. Effects of proton-pump inhibitors on pH and riboflavin production

The medium acidification was observed together with riboflavin secretion by *H. albus* hairy roots (Fig. 3). The proton-pump (H^+-ATPase) mainly contributes to rhizosphere acidification under metal deficiency [9, 11, 30]. To determine the involvement of the proton-pump in medium acidification and riboflavin secretion, we used proton-pump inhibitors. From various inhibitors, the plasma membrane specific inhibitors, DCCD and erythrosine B [21], were chosen and independently applied to propagated roots at different concentrations (0, 1, 10, and 100 μM), and changes of pH and riboflavin production were pursued for a further 2 weeks. DCCD strongly inhibited H^+ efflux, resulting in almost no pH decline at 10 and 100 μM for 2 weeks (data not shown), whereas erythrosin B acted more moderately; at 10 μM, pH decline was delayed moderately and reached the minimum after 10days in culture, although at 100 μM, pH was maintained at around 5.5 throughout the experiment (Fig. 4). Beside these differences, riboflavin secretion was not detected, except in a 10 and 100 μM erythrosine B-treated 10-day old samples, and root growth was potently inhibited with DCCD and erythrosine B at 10 and 100 μM. The treatment with 10 μM erythrosine B induced pH reduction, but severely inhibited riboflavin secretion. Nearly no effect appeared by either of DCCD and erythrosine B at 1 μM.

2.4. Effects of artificial acidification and riboflavin addition

The results obtained with the proton-pump inhibitors did not indicate whether the pH decreased was essential for the initiation of riboflavin production because root growth was inhibited too extensively to conclude the necessity of proton-pump activation. Even riboflavin production was almost diminished by treatment with 10 μM erythrosine B, where pH decline was delayed moderately but occurred. To determine the need for medium acidification by the root itself under iron-deficient conditions, artificial

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acidification of the culture medium was performed.   Since effluxes of organic acids into rhizosphere were observed under nutrient deficiency [4, 11, 13], we prepared the iron-free medium adjusted its pH at 4.0 and 3.7 using citric and malic acid, respectively, to be 10 μmol/flask (400μM) and supplied the medium to hairy roots.   The effect of culture in the acidic medium was pursued for 2 weeks, and showed the pH increased rapidly to around 5.0, followed by a steady pH (ca. 5.2), as a result of either the citric or malic acid (Fig. 5).   External acidification using these organic acids did not inhibit riboflavin production at all, indicating that acidification by the root itself is not necessary for riboflavin exudation.   These treatments even helped with riboflavin production and root growth.

The next question was whether roots are eager to produce riboflavin by themselves.   There is a possibility that roots cease secreting riboflavin when they sense a sufficient amount of riboflavin in the rhizosphere.   To investigate, exogenous addition of riboflavin with/without artificial acidification in the culture medium was performed.   Pre-propagated roots were cultured for 2 weeks in the iron-free media supplemented with various concentrations of riboflavin together with/without acidification.   Since the maximal riboflavin production was ~0.4 μmol/flask (16 μM) in our experimental system (Table 2, Fig. 3), riboflavin was added at 0.1~1.6 μmol/flask (4~64 μM).   Citric acid was employed for medium acidification (pH 4.0) to be 10 μmol/flask (400 μM) in this study, and without citric acid, the pH was adjusted to 5.8.

Riboflavin content and pH were analysed after 2 weeks of culture, where the riboflavin added at the initial stage was subtracted from the total amounts (Fig. 6).   Regardless of the exogenous riboflavin, hairy roots continued secreting riboflavin in the rhizosphere.   The results indicate that artificial acidification enhanced riboflavin productivity near two-fold, and the riboflavin amount in the medium seemed not to affect the riboflavin production by roots themselves.   The medium pH after 2 weeks exhibited a tendency to elevate, depending on a combination of the riboflavin concentration and acidification; pH varied between 4.8~5.0 without artificial acidification, and between 5.1~5.4 with acidification.

3. Discussion

Here we confirmed that root systems including hairy roots and non-transformed roots of *Hyoscyamus albus* actively excrete riboflavin under iron-deficient condition without their aerial parts, the same as has been reported for *H. niger* non-transformed roots [36]. Using a *H. albus* hairy root system under iron deficiency, riboflavin was shown to be actively secreted, apparently from root tips (Fig. 1). This dose not demonstrate directly that the root tip *per se* is the site of riboflavin biosynthesis, but the difference of the riboflavin amounts among the 11 hairy root clones and a wild type root seemed to correlate with the number and size of root tips (Fig. 2). Then riboflavin amount was plotted against root tip size (diameter) multiplied by root tip number, showing a significant correlation (n = 12, r = 0.832) is present (Fig. 7). Although there is no other report describing a correlation of root tip number/size and riboflavin secretion, this correlation supports the root tip to be the likely site of riboflavin secretion and biosynthesis. This observation can be used as a general selection marker for biotechnological use of high riboflavin-producing root clone.

A functional change is usually accompanied by histological/morphological changes. As reported here and previously [16], subapical swollen root tips were formed under iron deficiency, especially double (iron and copper) deficiency accelerated the more pronounced development of the swollen root tip [29]. Very recently, Vorwieger et al. succeeded to isolate *Arabidopsis bHLH* transcription factor genes, which are induced under iron deficiency, and ectopically to express them in transformed tobacco plants, resulting in iron deficiency independent riboflavin excretion [37]. There transgenic plants showed a significantly increased root diameter due to a higher diameter of cortex cells and a higher number of rhizodermal cells. In the stress response against iron shortage, Landsberg observed differentiation of rhizodermal transfer cells in the subapical zone of *C. annuum* roots, where visible starch grains disappeared almost completely, along with an increase in the number of mitochodria [16]. He suggests that most sugars in the roots are immediately used for energy delivery under iron deficiency, because starch deposits were retained only in iron-sufficient roots. The requirement for sugar by roots under iron-deficient conditions is supported by

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our experiment: no riboflavin was secreted from *H. albus* hairy roots without a supply of sucrose in the iron-deficient medium. Such high turnover of energy must result in organic acid production and a supply of reductants, such as $NADH_2/NADPH_2$, at which point riboflavin secretion may be involved. Since root tips at the subapical zone containing rhizodermal transfer cells seems to be the place of H^+ efflux and organic acid production [16], it is possible that riboflavin production occurs also in the same or adjacent area.

Iron deficiency-dependent decrease in pH in root culture medium together with riboflavin secretion was also observed here in this study (Fig. 3). In the case of *H. niger* root cultures, the minimum pH was between 4.4~4.6 [36]. In sugar beet [34], pH reached approximately 3.7 after 15days, and pH was maintained at 3.5~4.0 in muskmelon [10]. In *H. albus* hairy roots, the minimum pH was around 4.0 when a single root tip was subjected to iron deficiency and around 4.5 when propagated roots did. In the case of *Beta vulgaris* plants, no riboflavin production was observed under iron deficient conditions when the nutrient medium was buffered at pH 7.7 [34]. These results seem to indicate that acidification is essential to trigger riboflavin production. Since proton efflux by proton-pump H^+-ATPase must contribute to the pH decline, as indicated by Jones [11] and Scmidt [30], we determined the effect of proton-pump inhibitors, DCCD and erythrosine B, on pH decline and riboflavin production in *H. albus* hairy roots under iron deficiency.. In our experiments system, all of pH decline, riboflavin secretion and root growth were severely inhibited with 10 and 100 μM DCCD and 100 μM erythrosine B. With application of erythrosine B at 10 μM, pH was down to around 4.5 by which riboflavin release as well as root growth was potently inhibited (Fig. 4). This case shows that pH decline does not always induce riboflavin secretion.

Is it necessary to reduce pH by active proton efflux from the root itself to trigger of riboflavin production? *H. albus* hairy roots were cultured in the iron deficient medium acidified with citric acid (pH 4.0) or malic acid (pH 3.7), because these acids contribute to pH decline. In addition, neither citric acid nor malic acid harm root tissues, since these chemicals are actually produced in response to iron deficiency [4, 11, 16]. Surprisingly, the medium pH increased rapidly (overnight is sufficient) and riboflavin production was initiated (Fig. 5), indicating that roots need not make the rhizosphere acidic by themselves

to elicit riboflavin.   This and previous results indicate that active pH decrease is not directly correlates with riboflavin release, although both pH decline and riboflavin secretion were commonly observed under iron deficient condition in riboflavin releasing plants.

As shown with tobacco [35] and red pepper [16], H^+ efflux occurs only in root tips at the subapical zone near 1 cm in length: even proton influx happens at root portions 2 cm apart from the root tips [35]. When we cultured a root tip directly in acidic medium (pH 4.0), the roots neither grew nor produced riboflavin (data not shown).   In contrast, when we cultured propagated roots with the same acidic medium, pH was rapidly increased, followed by riboflavin secretion (Fig. 5).   This difference could be caused by morphological differences between a root tip (about 2 cm in length, with a few branches) and propagated roots (more than 10 cm of elongated roots with a great many branches).   It is possible in the case of propagated roots the mature part of the roots can help increase the pH, as indicated with tobacco plants [35], whereas proton influx was not sufficiently occurred in the case of a root tip only.   Interestingly, a single root tip was able to grow in iron deficient medium when it made the medium acidic by itself, where the pH was gradually decreased (Fig. 3).   Therefore, a root tip may acquire resistance to low pH conditions.

The addition of citric acid (pH 4.0) increased riboflavin productivity by nearly two-fold and helped root growth (Fig. 6).   Under metal deficient conditions, the increased secretion of citric and malic acids was found, and these acids contributed to form complexes with metals [4, 12, 13].   From *H. albus* hairy root cultures under iron deficient condition, the secretion of citrate and malate was also detectable since 3 days and their amounts increased linearly and after 2 weeks, reached to be around 0.2 and 2.1 mM, respectively.   The addition of citric and malic acid in the culture medium did not seem to affect the net secretion of citric and malic acid (data not shown).   Jones and Darrah investigated on influx and efflux of organic acids in intact, sterile maize roots using ^{14}C-labelled citrate, concluding the influx of organic acids can be expected to be limited importance in rhizosphere [13].   Since uptake of organic acids is presumably unlike, it is possible that the external supply of these organic acids saves energy for *H. albus* hairy roots to descent pH by themselves and thus accelerate further riboflavin production (Figs.5 and 6).   However, more focused work will be required to know the process of organic acid secretion by root culture system

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and the actual action of external organic acids under iron deficiency.

Why and how do *H. albus* hairy roots release riboflavin under iron starvation? Lorenz et al. described that higher plants contain 10 μM of free riboflavin in the cytoplasm [18]. In *H. albus* hairy roots, free riboflavin was detected around 15~30 μM from tissues cultured for 2 weeks with and without iron conditions (Table 2). Since riboflavin concentration in the medium is lower than that in the tissues, riboflavin efflux seems to occur via passive diffusion. However, external application of riboflavin in the medium to be up to 64 μM did not disturb internal riboflavin secretion (Fig. 6), indicating riboflavin is actively secreted from the root cells. Consequently, riboflavin content in the iron deficient medium became extremely high: near 95 % of free riboflavin was found from the medium. Since the riboflavin produced by the roots themselves did not evidently interact with the riboflavin presented in the rhizosphere, riboflavin secretion by the roots must be caused by internal necessity, not by pressure from environmental requirements in the rhizosphere   It is not yet determined whether free-form riboflavin secretion occurs via *de novo* synthesis or via dissociation from binding-form riboflavin such as flavoproteins.

As already mentioned, proton and organic acid effluxes generally occur from root tips of strategy I plants under iron starvation, but not all these plants produce riboflavin in the rhizosphere. Among 57 species in 12 families determined, riboflavin production was confirmed in 25 species [39]. However, still question is remained why some species excrete riboflavin under iron starvation and others do not. Riboflavin is a sufficiently valuable compound, containing carbon and nitrogen, that it must not be wasted in the rhizosphere. Since root exudates serves not only as nutrients for microbial growth, but also contains chemical molecules that promote chemotaxis of soil microbes to the rhizosphere   [4], riboflavin may be somehow used for association between plants and microbs to overcome iron deficiency. It is not possible at this point to describe the actual function of riboflavin in the rhizosphere, but detailed histological, biochemical and ecological research on a possible mechanism of riboflavin secretion by the roots under iron-deficient stress will provide the required information from the basic to the biotechnological aspects to allow this determination.

4. Material and Methods

4.1. Chemicals

Riboflavin, FMN, FDA, DCCD, erythrosine B, DMSO, and citric acid were purchased from Wako Chemicals and malic acid from Nacalai Tesque. Other chemicals were used the highest grade available in commercial.

4.2. Establishment of transformed and non-transformed roots

Hyoscyamus albus L. seeds were germinated aseptically. The seeds were treated with 0.1% $HgCl_2$ for 3 min, then washed three times with sterile distilled water, and cultured on Murashige and Skoog (MS)[22] medium solidified with 0.2 % gellan gum under continuous light (ca. 2000 mmol m^{-2} s^{-1}). For transformation, *Agrobacterium rhizogenes* strain 15834 harboring the *hyoscyamine 6β-hydroxylase* (*H6H*) gene was used the same as in the previous report [27]. Methods of transformation, establishment of transformants, and detection of gene insertion were also described in the same report. As a non-transformed roots, a root tip was isolated from the sterile seedling and cultured on the hormone-free MS medium.

4.3. Culture methods

Hairy roots as well as untransformed roots were kept at 25ºC in the dark on a solid MS medium and subcultured on the same medium every month. For experimental purposes, two-week-old roots were always supplied and two kinds of culture methods were used: 1) a primary root tip with a few lateral roots (ca. 2 cm in length) was directly transferred to Gamborg B5 medium [8] modified as follows below and cultured for 3 weeks; 2) a primary root tip was pre-propagated in the normal B5 medium for 2 weeks and

then the medium was exchanged to a modified B5 medium described below, followed by further culture for 2 weeks.   All cultures were performed in a 100 ml conical flask containing 25 ml of liquid medium or a 50 ml conical flask containing 15 ml of medium and incubated at 25 ºC in the dark on a rotary shaker (80 rpm).

Iron deficient B5 medium was prepared by elimination of Fe-EDTA from B5 basal medium supplemented with sucrose (0, 1, 2, and 3%) before autoclaving at 121˚C for 15 min.   All chemicals used for the following work were filter-sterilized before addition to the culture medium.   DCCD was solved in DMSO at 100 mM and erythrosine B in distilled water at 5 mM; these stocks were further diluted 10 and 100 times, respectively.   DCCD solutions (25 μl) and erythrosine B solutions (500 μl) were added to be 100, 10, 1 and 0 μM in the final solution.   Citric and malic acid were prepared as 100 mM aqueous solutions, respectively, and a 100 μl aliquots of each was added to 25 ml of iron-free B5 medium, resulting in a pH of 4.0 and 3.7, respectively.   Riboflavin aqueous solution (0, 1, 4, 8, and 16 ml) prepared at 100 μM (no further concentration was obtained) was mixed with iron-free sterile B5 basal medium (25, 24, 21, 17 and 9 ml, respectively) to be 25 ml in total volume (to be 0, 0.1, 0.4, 0.8 and 1.6 μM in the final concentration):   according to the required volume of the iron-free basal medium, each strength basal medium was prepared to become the same concentration eventually.   Medium pH was always adjusted to pH 5.8 at the initial stages, except for the acidification experiments mentioned above.

In the time-course experiments, 3 flasks were harvested by vacuum filtration, and separated into roots and media.   Fresh weight of roots and the number/size of root tips, in the case of various hairy root clones (Fig. 2), were measured and stored at–20 ^{o}C until analysis.   Tip size in diameter (mm) at the most large part was measured with a digital micrometer calliper (Mitutoyo DC-150P)   In the case of media, the pH and riboflavin content were assayed immediately before storage.

4.4. Analysis of riboflavin, organic acid and pH

Culture media were centrifuged (10,000 rpm, 15 min) before analysis to eliminate small particles

coming from root tissues.   Flavins, including riboflavin, were extracted from the root tissues as follows [5].

Roots (ca.100 mg fresh weight) were homogenized with distilled water (1 ml) in a mortar, and the

homogenates were transferred to Eppendorff tubes and heated at 80 $^{\circ}$C for 15 min.   After cooling and

centrifugation (10,000 rpm, 15 min), the supernatants were taken up and applied for analysis.

Yellow compound found in the medium cultured without iron was identified as riboflavin in

comparison to authentic riboflavin, FMN and FAD, using a spectrophotometer, UV-illumination, and HPLC.

Riboflavin contents in the media were usually determined by a UV-VIS spectrophotometer (Shimazu

UV-1600) at 444 nm.   Root extracts and medium samples treated with erythrosine B, which has absorption

at 444 nm, were separated by HPLC (Jasco Intelligent HPLC system) on an Inertsil ODS-3 column (GL

science Inc, Japan, 4.6×150 mm).   The HPLC conditions were employed according to the reported method

[5, 24] with certain modifications, as follows: flow rate, 0.8 ml/min; column temp, 40ºC; detection, 371

nm;   solvent A, MeOH; solvent B, 10mM NaH_2PO_4 (pH5.5, adjusted with 1M NaOH); isocratic analysis

of A: B= 35:65.   Concentration was determined using standard curves established with authentic

riboflavin in both methods.

Organic acid contents in the medium were measured by HPLC according to the known method [23].

An apparatus and column were same as riboflavin analysis.   Other conditions were as follows: flow rate,

0.5 ml/min; column temp, 25ºC; detection, 220 nm;   solvent A, MeOH; solvent B, 2% NaH_2PO_4 (pH2.3,

adjusted with phosphoric acid); isocratic analysis of A: B= 2:98.   Concentration was determined using

standard curves established with authentic compounds. Medium pH was measured using a pH meter (TOA

Electronics, HM-5ES).

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Figure Legends

Fig. 1. Riboflavin secretion from root tip into solid medium without iron.

Photos: **a**), Roots with yellow tips under visible light (early stage); **b**), Roots the same **a** under UV-illumination. Fluorescence remained mainly in the root tips;　**c**), Fluorescence which spread to the medium (later stage) was detected under UV-illumination.

Fig. 2. Root tip mumber, its size, growth and riboflavin production by various hairy root clones and wild type roots of *H. albus* cultured in iron-deficient medium.　Starting material, a root tip (ca.2 cm in length);　culture periods, 3 weeks;　N (= 3) ± standard deviation.

Fig. 3. Time courses of root growth, medium pH and riboflavin secretion. A root tip was cultured directly in the iron-deficient medium.　N (= 3) ± standard deviation.

Fig. 4. Effects of the proton-pump inhibitor erythrosine B, on root growth, medium pH and riboflavin secretion. A root tip was cultured with iron for 2 weeks, then the medium was exchanged to iron-deficient medium supplemented with the inhibitor at various concentrations (0, 1, 10, and 100 μM), and culture was continued for 2 weeks. N (= 3) ± standard deviation.

Fig. 5. Effects of acidification of the culture medium using citric acid (pH 4.0) or malic acid (pH 3.7) on root growth, medium pH and riboflavin secretion.　The culture method is referenced as a legend in Fig. 4.　N (= 3) ± standard deviation.

Fig. 6. Effects of the presence of riboflavin in the medium on fresh weight, medium pH and net riboflavin secretion.　The culture method is referenced as a legend in Fig. 4, except for the additions of riboflavin (0, 0.1, 0.4, 0.8, and 1.6 μM) together with citric acid (initial pH 4.0) or without (initial pH 5.8).　N (= 3) ± standard deviation.　Riboflavin added at the initial stage was subtracted from the total amounts by calculation, so the net amount of secreted riboflavin is shown.

Fig. 7. Correlation between riboflavin production and root tip number multiplied by size by various hairy root clones and wild type roots of *H. albus* cultured in iron-deficient medium.　Calculation was performed using data in Fig. 2.

Table 1. Transformed and non-transformed *H. albus* roots with(+) /without (-) exogenous genes.

clone No.	H6H	rolB^{TL}	rolB^{TR}
Ha 1	+	+	+
Ha 2	+	+	+
Ha 4	+	+	+
Ha 5	+	+	+
Ha 6	+	+	+
Ha 7	-	+	+
Ha 11	+	+	-
Ha 12	+	+	-
Ha 14	+	+	-
Ha 15	+	+	-
Ha 16	-	+	-
WT	-	-	-

Table 2. Riboflavin distribution in the root tissues and medium cultured for 14 days under iron sufficient (+) and deficent (-) conditions. N(=3) ± sd.

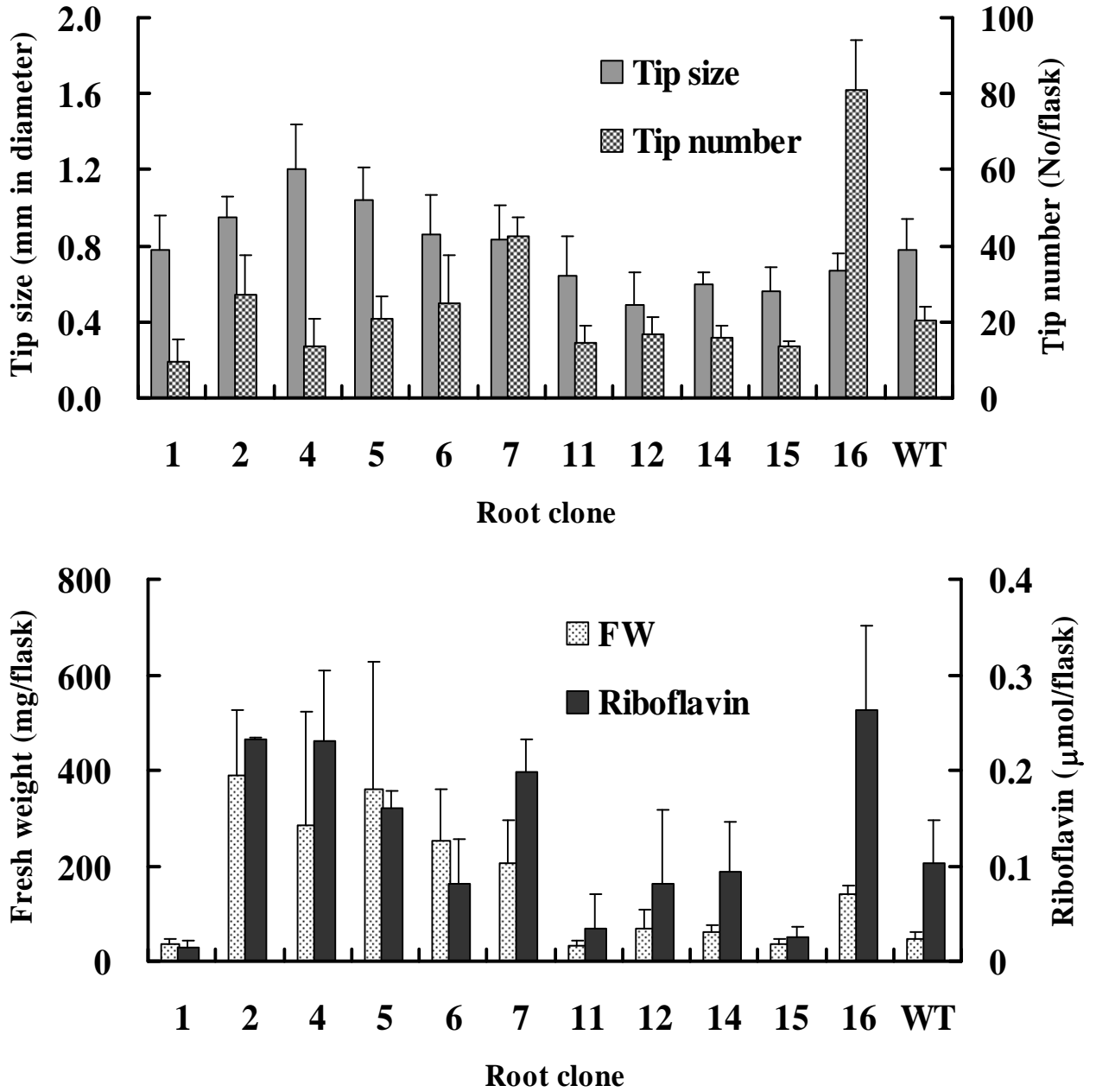| Culture condition	Fe (+)		Fe (-)	
Riboflavin contents	Root tissue	Medium	Root tissue	Medium
(µmol/flask)	0.013 ± 0.001	0.062 ±0.010	0.021 ± 0.006	0.348 ± 0.073
(µM)	14.9 ± 2.5	2.5 ± 0.4	29.4 ± 10.2	13.9 ± 2.9

Fig. 1 Higa et al.
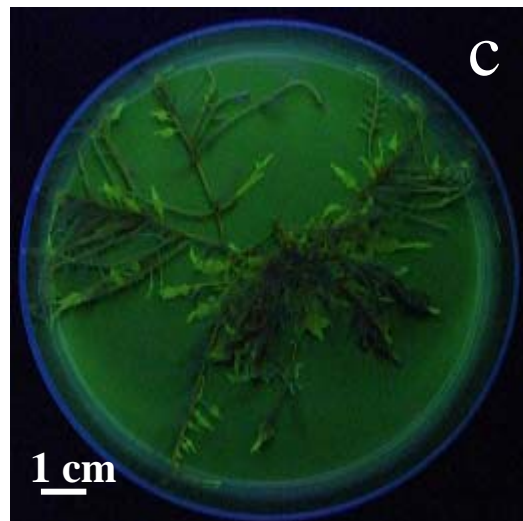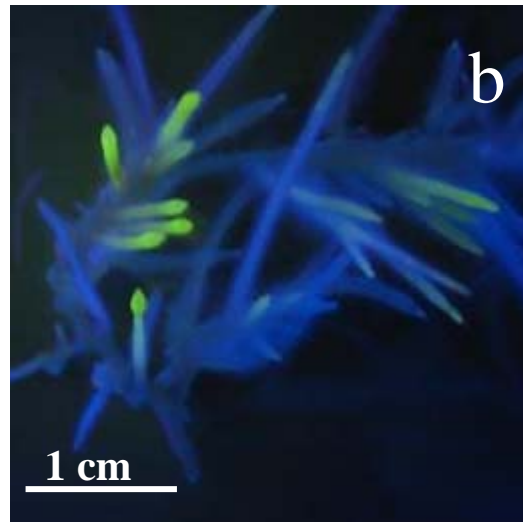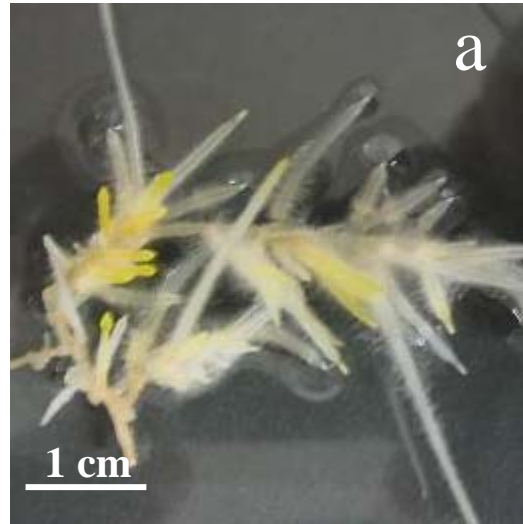
Fig. 2. Higa et al.

Fig. 3. Higa et al.

Without pre-culture

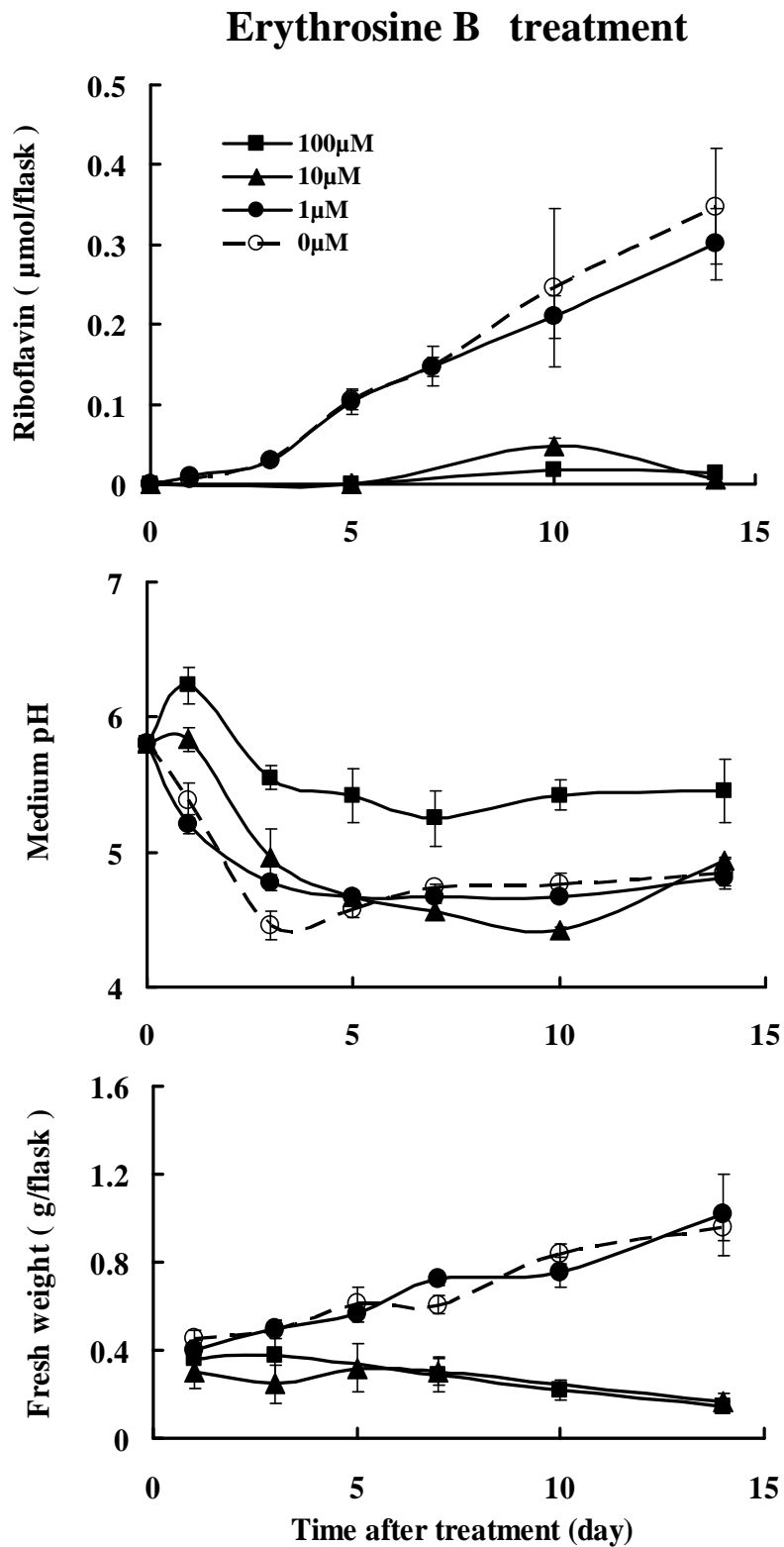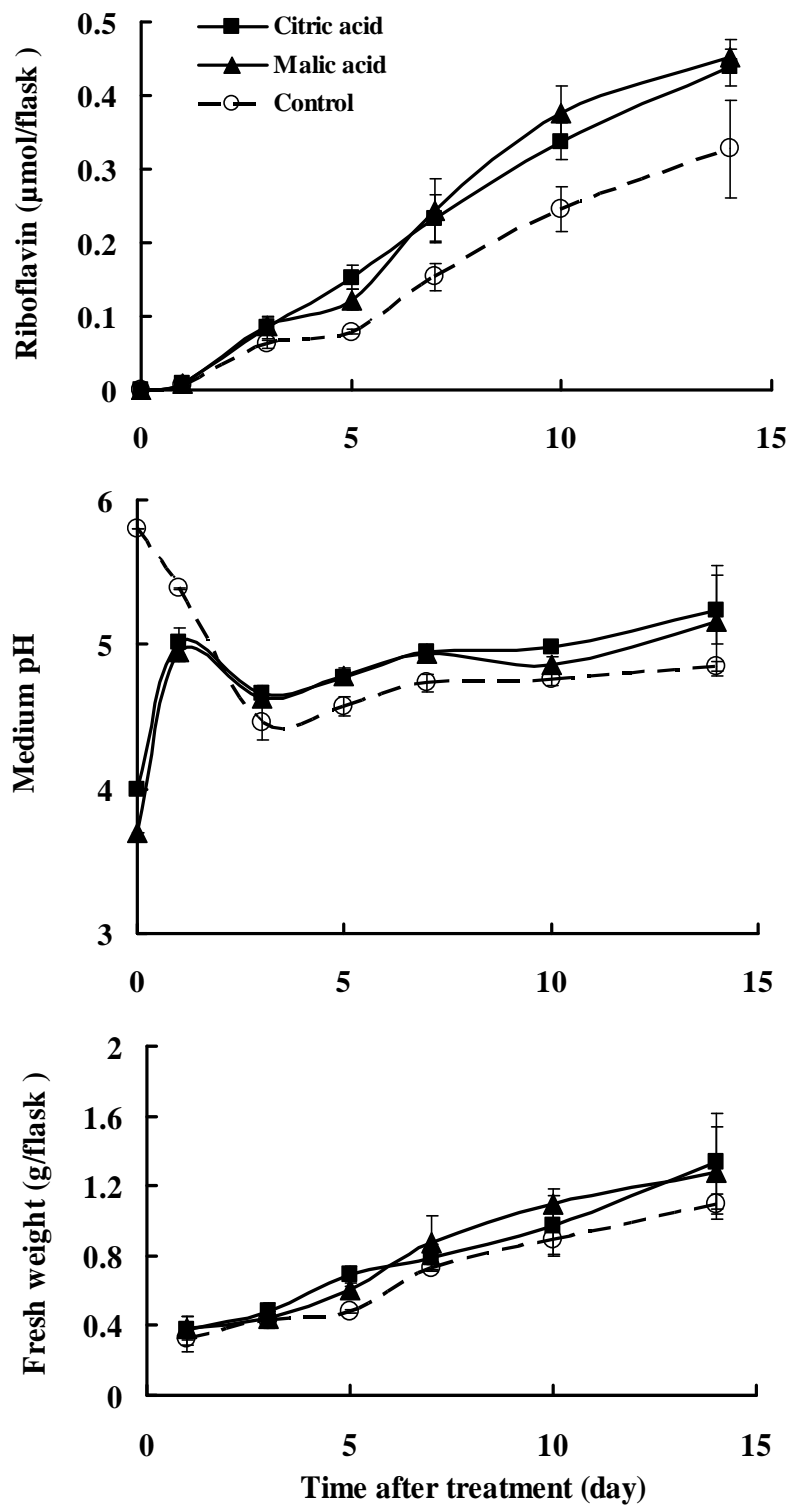Fig. 4. Higa et al.

Erythrosine B   treatment
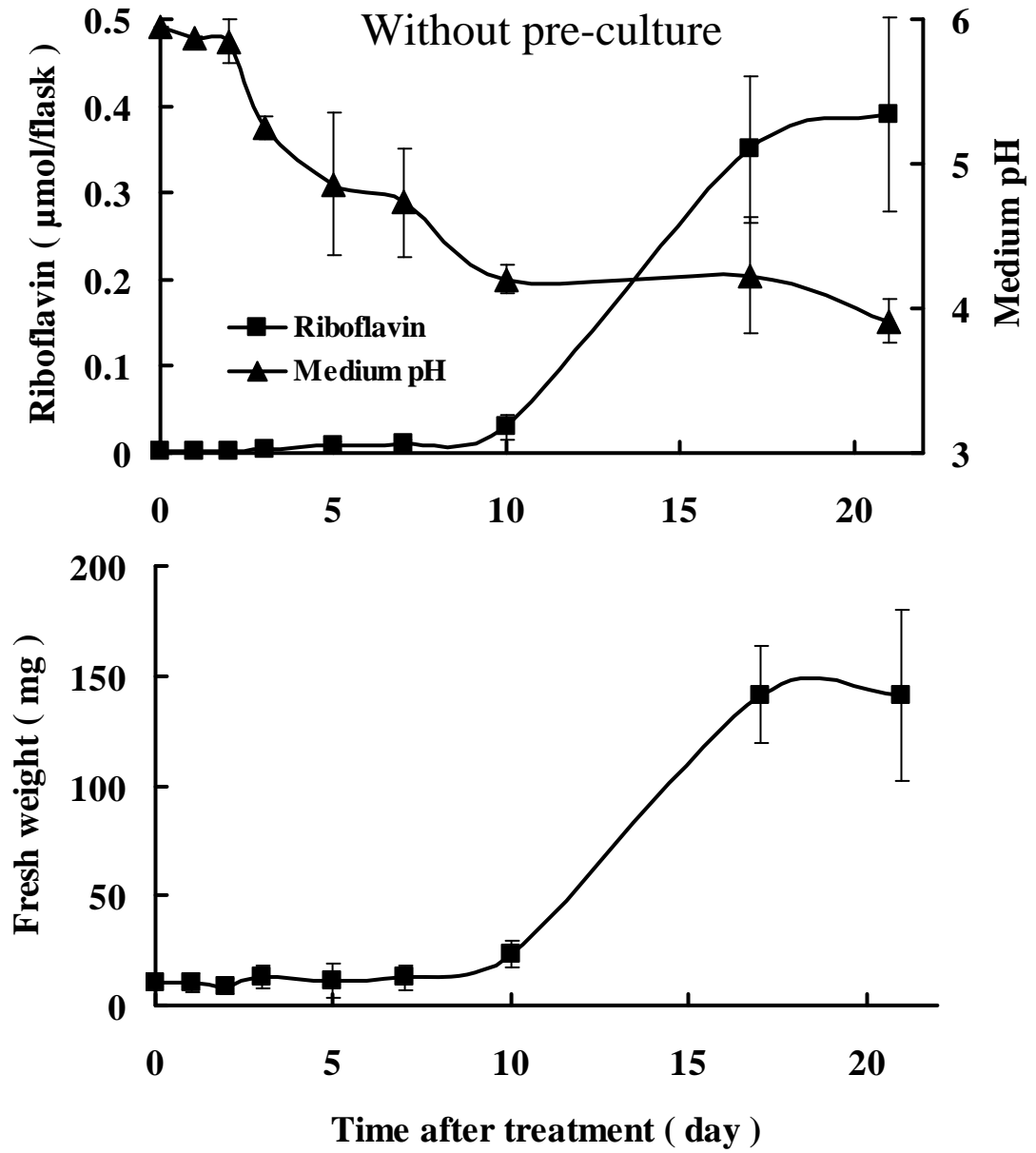
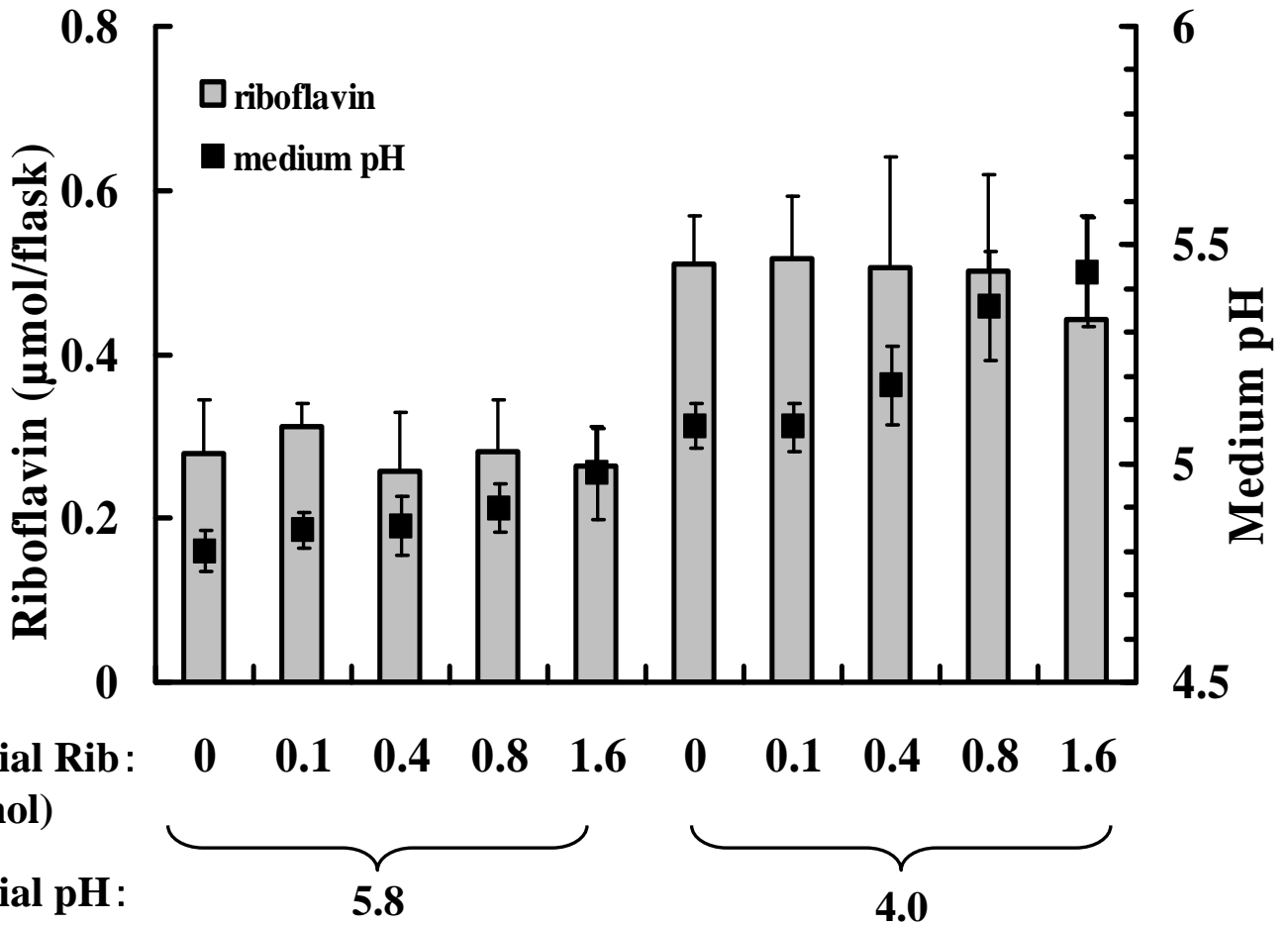Fig. 5. Higa et al.

Fig. 6. Higa et al.

Fig. 7 Higa et al.