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Galloylglucoses and riccionidin A in *Rhus javanica* adventitious root cultures

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Abstract

Adventitious root cultures of *Rhus javanica* L. produced large amounts of galloylglucoses (gallotannins) and an anthocyanidin, riccionidin A, formerly found only in liverworts. Production of both galloylglucoses and riccionidin A in the adventitious root culture system was suppressed by light. The *Rhus* root culture showed the highest productivity for those secondary metabolites in a modified Linsmaier–Skoog (LS) liquid medium containing 30 mM NH_4^+ and 30 mM NO_3^- as nitrogen sources in the presence of 10^{-6} M 3-indoleacetic acid (IAA). © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Rhus javanica*; Anacardiaceae; Adventitious root; Galloylglucose; Anthocyanidin; Riccionidin A

1. Introduction

We previously reported the production of gallotannins by callus tissues induced from leaves of *Rhus javanica* (Taniguchi, Takeda, Yabu-uchi, Yoshida & Yazaki, 1997). In a continuing study of the tissue culture of *R. javanica*, we established an adventitious root culture capable of producing large amounts of gallotannins and an anthocyanidin, riccionidin A, which was however undetectable in its callus tissue. This paper describes the characterization of the polyphenolics produced in the cultured roots, and the effects of light, plant hormones, and nitrogen sources.

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2. Results and discussion

Adventitious roots were generated from axenic leaves of *R. javanica* on Linsmaier–Skoog (LS) agar medium (Linsmaier & Skoog, 1965) supplemented with 3-indoleacetic acid (IAA, 10^{-5} M) and kinetin (10^{-5} M). The roots were then subcultured in the liquid LS medium containing 10^{-6} M IAA and 10^{-5} M kinetin at 25° in the dark.

2.1. Characterization of metabolites produced in the cultured roots

HPLC analysis showed that the extract of the cultured roots contained a large amount of galloylglucoses (GGs, 30 mg/g dry weight). The observation comparable to that in callus cultures induced from the same plant source (Taniguchi et al., 1997), although the chromatograms differed from each other. The relative amount of each GG based on the total GGs content was; penta-GG 6.6%, hexa-GG 12.6%, hepta-GG

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26.4%, octa-GG 25.5%, nona-GG 17.2%, deca-GG 8.2% and undeca-GG 3.5%, respectively. The contents of nona- to undeca-GGs in the dried root tissues were greater than those detected in the callus tissues, while the contents of penta- to hepta-GGs were less in the root culture (Table 1).

Formation of reddish pigments in the adventitious root tissues was observed, and these seem to be only localized in the walls of epidermal tissue (Fig. 1). The HPLC analysis of 1% HCl–MeOH extract of the root tissue with a photo-diode array detector revealed the presence of a main pigment having an absorption maximum at 497 nm. This spectrum was different from that of chrysanthemin which had been reported as an anthocyanin in the leaves of *Rhus* species (Karimdzhanov, Islambekov, Mavlyanov & Ismailov, 1986). Thus, the pigment was isolated from the cultured roots as described in Section 3, and was identified as riccionidin A (**1**), which was previously found as a cell wall pigment in the liverwort, *Ricciocarpos natans* and other related species. It was identified by comparison of its ^{1}H- and ^{13}C-NMR spectral data with those of the reported values (Kunz, Burkhardt & Becker, 1994), as well as by its UV–Vis maximum at 486 nm in acidic solution (pH 2), and the bathochromic shift to 510–550 nm in weak acidic or neutral solution. This UV–Vis spectral feature might arise from a retroflavonoid structure (Saito & Shibata, 1975) with an ether linkage between the B and C rings. Although this anthocyanidin was not detectable in the calli or in the intact leaves of *R. javanica* at all, its occurrence in the intact roots has been confirmed by reversed-phase HPLC, thereby revealing that this compound is not unique to the adventitious root cultures. The amount of riccionidin A (**1**) in the adventitious root culture (0.32 mg/g fresh weight) was, however, higher than that within the intact roots of *R. javanica* (0.18 mg/g fresh weight) or the liverwort *Ricciocarpos natans* (up to 0.11 mg/g dry weight) (Kunz & Becker, 1995). This is the first example of this type of anthocyanidin in higher plant tissues.

Table 1
Content of each galloylglucose (GG) (mg/g dry weight) in the calli and adventitious roots of *Rhus javanica* (Figures in parentheses are percentage of GG in each plant material)

GG	Calli	Adventitious roots
5-GG	8.5 (19.3)	1.9 (6.6)
6-GG	10.6 (23.9)	3.7 (12.6)
7-GG	12.4 (28.1)	7.8 (26.4)
8-GG	7.9 (17.9)	7.5 (25.5)
9-GG	4.4 (9.8)	5.1 (17.2)
10-GG	0.4 (1.0)	2.4 (8.2)
11-GG	0.0 (0.0)	1.0 (3.5)
Total	44.2 (100)	29.4 (100)

2.2. Time-course experiments and optimization for production of GGs and riccionidin A

Time-courses of GGs and riccionidin A accumulation and growth of cultured tissues are shown in Fig. 2. The fresh weight of root tissues increased up to two times of the inoculum size in six weeks, whereas the content of GGs decreased at the early logarithmic phase, in a similar manner as callus cultures (Taniguchi et al., 1997). On the other hand, accumulation of **1** gradually increased somewhat parallel to the growth of the root tissues, but decreased during the stationary phase (Fig. 2).

Usually, production of anthocyanins is positively regulated by light in many plant species and their tissue cultures, although a few exceptions have been reported (Sakamoto et al., 1994). It should thus be noteworthy that the adventitious roots of *R. javanica* produce riccionidin A (**1**) in the dark. To clarify the effect of light on the production of this pigment as well as GGs, the root tissues cultured in darkness were subjected to light irradiation of 3000 lx (12 h/day). Thus, although root growth was not influenced by the light irradiation for three weeks, the production and/or accumulation of both GGs and **1** was apparently suppressed in light (Fig. 3). This result may imply that the biosynthesis of this anthocyanidin proceeds preferably in the dark, which is consistent with its occurrence in only the roots but not in the aerial parts of the intact plant.

The production of GGs and **1** in relation to growth of the roots cultured on LS medium supplemented with various combinations of plant hormones is shown in Fig. 4. Removal of kinetin from the medium noticeably promoted the growth and polyphenols (GGs and riccionidin A) production. Similarly, an increase in the growth as well as the polyphenol production was observed in the absence of cytokinin when 2,4-dichlorophenoxyacetic acid (2,4-D) or 1-naphthaleneacetic acid (NAA) was used as an auxin. On the other hand, the replacement of kinetin with 6-benzyladenine (BA) decreased both the growth and the pigment production, whereas the GGs production was less affected.

We previously demonstrated that the concentration of inorganic elements in the LS medium played an important role in production of an ellagitannin oligomer by *Oenothera laciniata* callus cultures in which an increase in onenothein B, an ellagitannin dimer, content was observed upon adopting the lower concentration of the LS medium (Taniguchi et al., 1998). However, in the root culture of *R. javanica*, dilution of the medium concentration gave rather negative effects on the growth and second metabolites production (data not shown).

Fig. 5 shows the effects of NH_4^+/NO_3^- ratio on

growth and GG and riccionidin A contents of the root cultures in which the total amount of nitrogen (60 mM) was kept as the standard LS medium (10^{-6} M IAA and 10^{-5} M kinetin). Although the GG contents were less affected by the change of the ratio, the production of riccionidin A increased in the medium having higher ratio in NH_4^+, i.e. 30–60 mM, whereas almost no production of riccionidin A was observed in the absence of NH_4^+. Tissue growth was maximized in the presence of 30 mM NH_4^+ and 30 mM NO_3^-.

These data are consistent with other observations in *Pinus tarek* (loblolly pine) tissue cultures whose increased levels of NH_4^+ notably increased amounts of monolignols being formed (summarized in Lewis,

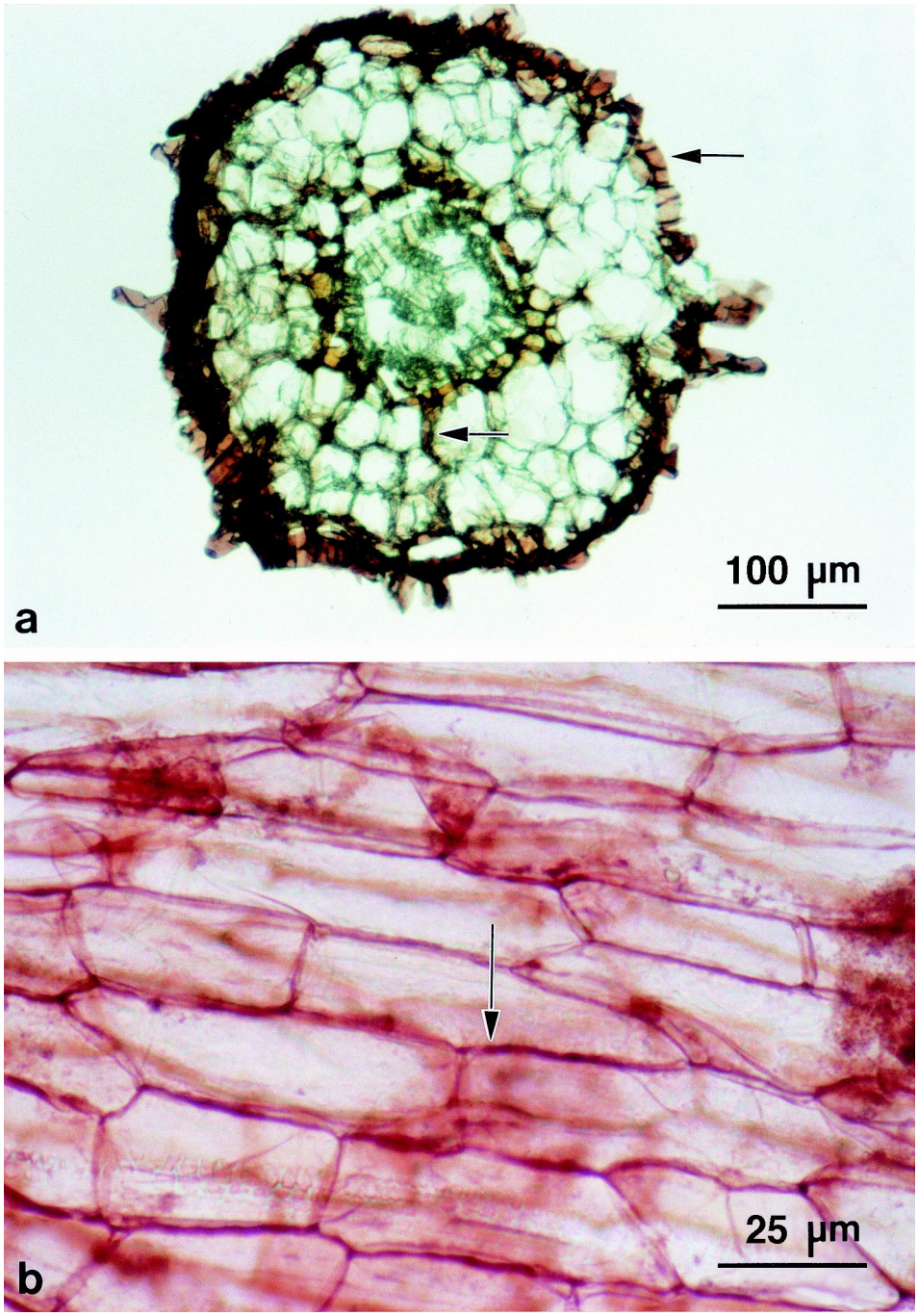


Fig. 1. Localization of pigments in adventitious roots of *Rhus javanica*. Root tissues were cultured in LS medium containing 10^{-6} M IAA, 10^{-5} M kinetin and 3% (w/v) sucrose in the dark at 25°C and harvested three weeks after inoculation. Fresh sections from adventitious roots were prepared by a Microslicer and not stained. (A): Transverse section. (B): Longitudinal section of epidermis. Accumulation of pigment is mainly observed in the walls of epidermal tissues.

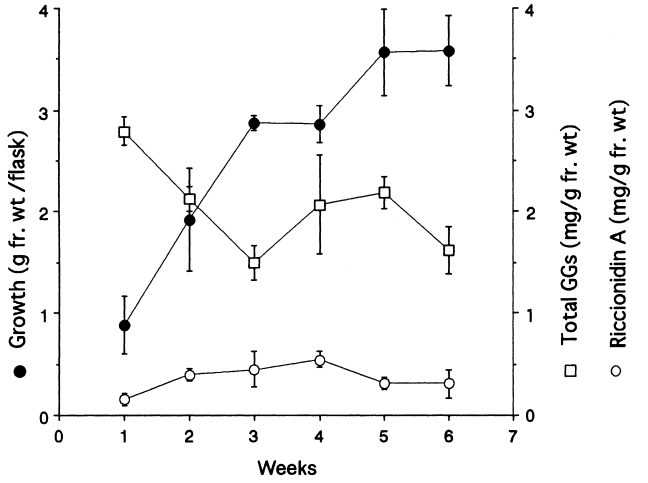


Fig. 2. Time-course of cell growth and GGs and riccionidin A production in the adventitious roots of *Rhus javanica*. Root tissues were cultured in LS liquid medium containing 10^{-6} M IAA, 10^{-5} M kinetin and 3% (w/v) sucrose at 25° in the dark. The bars indicate standard errors of the means of three replicates.

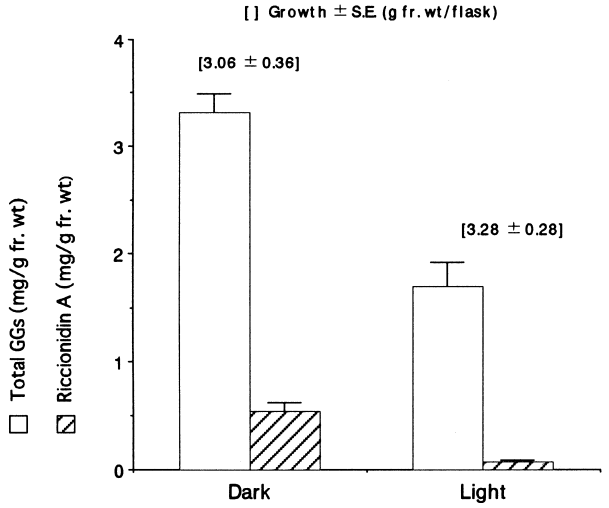


Fig. 3. Effects of light on cell growth and GGs and riccionidin A contents in the adventitious roots of *Rhus javanica*. Root tissues were cultured in LS liquid medium containing 10^{-6} M IAA, 10^{-5} M kinetin and 3% (w/v) sucrose in the dark or under illumination (3000 lx, 12 h/day) at 25°, and harvested three weeks after inoculation. The bars indicate standard errors of the means of three replicates.

Dovin & Sarkonen, 1999). That is, depending on the branch of phenylpropanoid (acetate) metabolism being affected in a particular plant species, increasing the amount of ammonium ion results in an amplification of phenylpropanoid metabolism due to its nitrogen recycling system (van Heerden, Towers & Lewis, 1996).

Interestingly, in the optimal NH_4^+/NO_3^- ratios, 60/0 mM–20/40 mM of NH_4^+/NO_3^-, removal of kinetin caused a further increase in secondary metabolite production, as shown in Fig. 6. Particularly, the highest accumulation of riccionidin A was obtained in the absence of NO_3^-, although the growth of roots was strongly suppressed. The best culture condition for both growth and polyphenol production (GGs, 15.5 mg/flask; riccionidin A, 3.3 mg/flask) was achieved in the medium with 30 mM NH_4^+ and 30 mM NO_3^-.

2.3. Conclusion

Adventitious root culture of *R. javanica* which produced GGs (penta- to undeca-GGs) and an anthocyanidin, riccionidin A was established. Adventitious root cultures of *Quercus glauca* (Nishikawa, Tanaka, Nakanishi, Shimomura & Ishimaru, 1997) and *Cornus capitata* (Tanaka, Shimomura, Kamiya, Kayano & Ishimaru, 1997; Tanaka, Kamiya, Shimomura & Ishimaru, 1998) were previously reported to produce mono- to penta-GGs and flavan-3-ols. However, no report of the production of any GGs of larger molecular weight than penta-GG in such tissue system has hitherto appeared. It is thus emphasized that the present study provides the first example of production of GGs with six or more galloyl groups by adventitious roots.

The pigment in the cultured roots was shown to be localized mainly in the cell wall of epidermal cells, and the main pigment was identified as riccionidin A (**1**). However, most anthocyanidins are localized in vacuoles in glycosidic form. Thus it is interesting that the anthocyanidin isolated here does

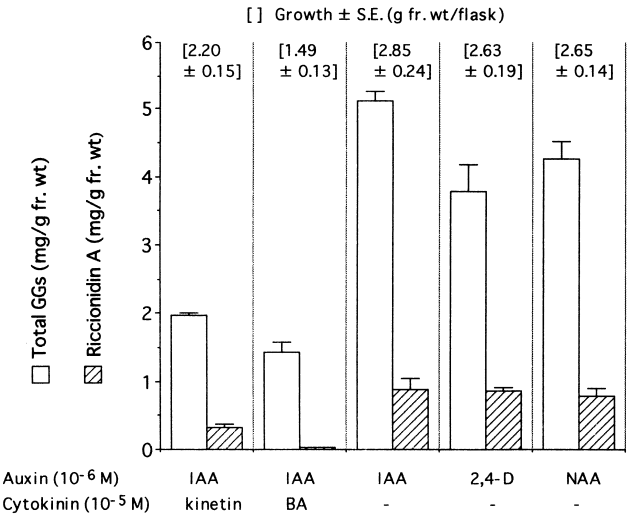


Fig. 4. Effects of plant hormones on cell growth and GGs and riccionidin A contents and in the adventitious roots of *Rhus javanica*. Root tissues were cultured in LS liquid medium containing various plant hormones and 3% (w/v) sucrose at 25° in the dark, and harvested three weeks after inoculation. The bars indicate standard errors of the means of three replicates.

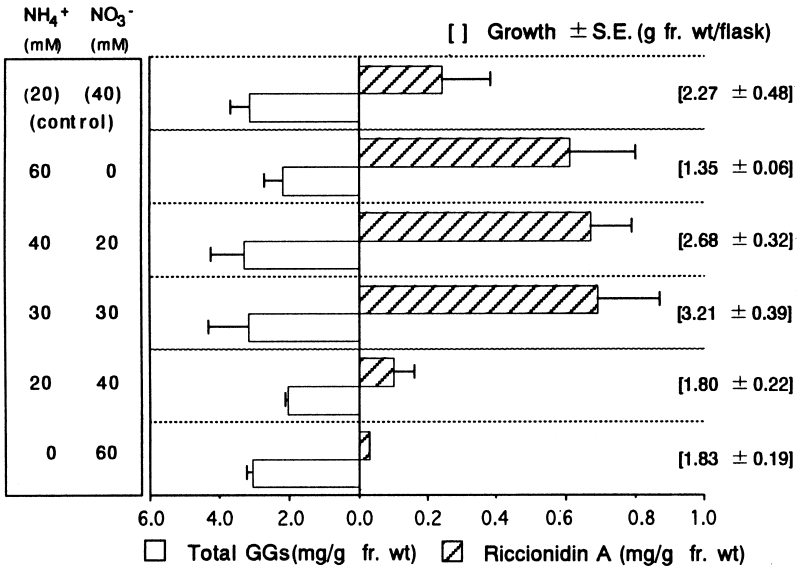


Fig. 5. Effects of NH_4^+/NO_3^- ratio as nitrogen sources on cell growth and GGs and riccionidin A contents in the adventitious roots of *Rhus javanica*. Root tissues were cultured in modified LS liquid medium containing 10^{-6} M IAA, 10^{-5} M kinetin and 3% (w/v) sucrose at 25° in the dark and harvested three weeks after inoculation. Various ratios of NH_4Cl and KNO_3 (60 mM in total) were added to the nitrogen free LS medium. The bars indicate standard errors of the means of three replicates.

not accumulate in vacuoles but is instead secreted into the cell wall.

Investigation on the regulatory elements such as hormones and nitrogen sources on the polyphenol production in the root cultures showed the following features: (1) Light irradiation to the *R. javanica* root cultures suppressed production of GGs and riccionidin A. (2) Production of GGs and riccionidin A was stimulated in the absence of cytokinins. (3) The variation of the NH_4^+/NO_3^- ratio in the medium did not gave a clear effect on GG production, while riccionidin A production was significantly affected. The presence of ammonium ion is essential for high riccionidin A production. (4) The highest production of GGs and riccionidin A as well as the growth of cultured roots was achieved in medium containing 30 mM NH_4^+ and 30 mM NO_3^- in the presence of 10^{-6} M IAA.

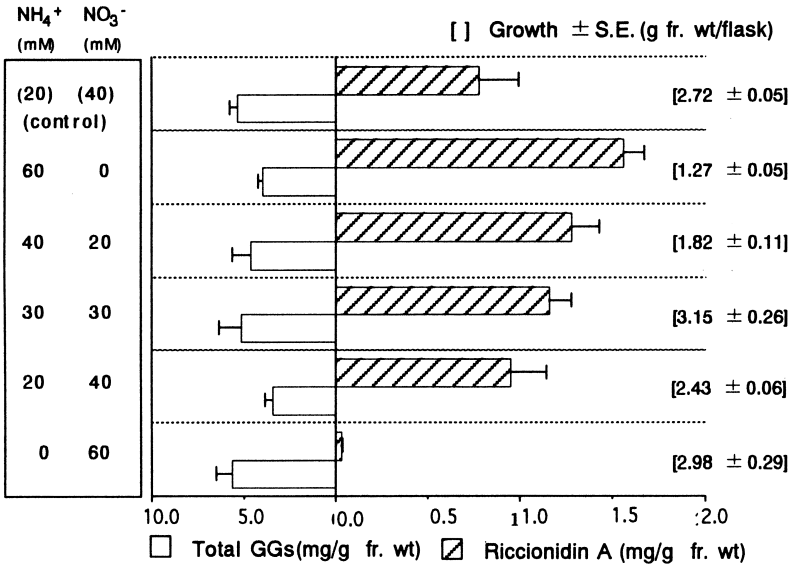
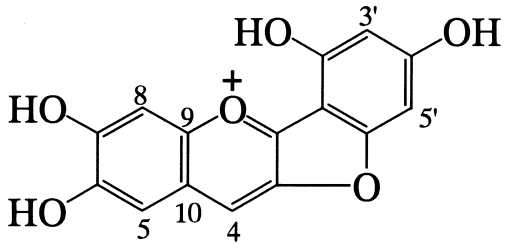


Fig. 6. Effects of NH_4^+/NO_3^- ratio as nitrogen sources on cell growth and GGs and riccionidin A contents in the adventitious roots of *Rhus javanica*. Root tissues were cultured in modified LS liquid medium containing 10^{-6} M IAA and 3% (w/v) sucrose at 25° in the dark and harvested three weeks after inoculation. Various ratios of NH_4Cl and KNO_3 (60 mM in total) were added to the nitrogen free LS medium. The bars indicate standard errors of the means of three replicates.



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3. Experimental

3.1. Plant material and culture conditions

Adventitious root tissue formation was induced from surface-sterilized leaves of *R. javanica* on LS agar medium containing 10^{-5} M IAA and 10^{-5} M kinetin. There were then subcultured in LS liquid medium containing 10^{-6} M IAA and 10^{-5} M kinetin at intervals of three weeks on a rotary shaker (100 rpm) in the dark for over four years.

3.2. Isolation and identification of riccionidin A

Frozen tissues of cultured roots (330 g, fresh mass) were homogenized in 1% HCl–MeOH (330 ml × 4). The filtrate of the homogenate was concentrated in vacuo to 120 ml and extracted with EtOAc (60 ml × 3). The aqueous layer was concentrated and chromatographed on Dia ion HP-20 (30 I.D. × 220 mm) column with aqueous MeOH containing 0.5% HCl as eluant. Fractions containing pigments were combined and further rechromatographed over a MCI-gel CHP-20P (11 I.D. × 235 mm) column, eluted with $H_2O \rightarrow$ aq. MeOH (20% → 40→ 60%) → MeOH → MeOH containing 0.1% HCl; riccionidin A (**1**) (17 mg) was isolated from the MeOH eluate.

Riccionidin A (**1**): UV–Vis-λ_{max} (MeOH containing 0.01% HCl) nm (log ε): 497 (4.24), 330 (3.25), 281 (3.62), 240 (4.03) 205 (4.16) [M/15 phosphate buffer pH 7.0–MeOH (9:1)] nm (log ε): 548 (4.08), 420 sh (3.46), 293 (3.69), 243 sh (3.93), 210 (4.10) [M/15 phosphate buffer pH 4.5–MeOH (9:1)] nm (log ε): 513 (4.05), 288 (3.75), 240 sh (4.03), 213 (4.11), ESI–MS: m/z 285 $(M)^+$, ^{1}H-NMR (500 MHz, DMSO-d_6 + DCl): δ 6.64, 6.73 (each br s, H-3′, H-5′), 7.53 (*s*, H-8), 7.62 (*s*, H-5), 9.01 (*s*, H-4), (500 MHz, MeOH-d_4 + TFA-*d*): δ 6.47, 6.66 (each *d*, $J = 1.6$ Hz, H-3′, H-5′), 7.49 (*s*, H-8), 7.57 (*s*, H-5), 8.78 (*s*, H-4), ^{13}C-NMR (125 MHz, DMSO-d_6 + DCl): δ 91.7, 100.2 (C-3′, C-5′), 100.1 (C-1), 103.4 (C-8), 112.2 (C-5), 116.1 (C-10), 127.0 (C-4), 144.3, 157.3 (C-2, C-3), 149.5 (C-9), 148.0, 155.8, (C-6, C-7), 158.1, 163.7, 170.6 (C-2, C-4, C-6). This compound was identified as riccionidin A by comparison of the ^{1}H- and ^{13}C-NMR spectral data with those reported in the literature (Kunz et al., 1994).

3.3. Quantitative analysis of GGs and riccionidin A

Cultured roots (0.5 g, fresh mass) were chopped and homogenized in 0.1% HCl–MeOH (5 ml). After removal of the cell debris by centrifugation, an aliquot (0.2 ml) of each supernatant was evaporated to dryness. The residue was dissolved in MeOH, and subjected to normal phase HPLC [YMC PACK SILA-003 (4.6 × 250 mm) eluted with *n*-hexane–MeOH–THF–HCOOH (60:45:15:1) containing oxalic acid (450 mg/l), at a flow rate of 1.5 ml/min at room temperature with detection at 280 nm]. The quantity of each GG in the cultured root was estimated from the peak area based on that of penta-GG. The content of riccionidin A was determined by reversed phase HPLC [Wakosil-II 5C 18AR (4.6 × 150 mm) eluted with H_2O–MeOH–AcOH–H_3PO_4 (16:2:1.7:0.3 v/v) at a flow rate of 1.0 ml/min at 40° with detection at 500 nm]. Intact roots and leaves were treated in an analogous way.

3.4. Time-course experiments

Precultured root tissues (1.7 g) were inoculated in LS liquid medium (30 ml in 100 ml Erlenmeyer flasks) and were cultured in the dark. The tissues were harvested every seven days by Miracloth filtration (Calbiochem) with three replicates.

3.5. Influence of culture conditions on growth and galloylglucose and riccionidin A content

Three replicates of the precultured roots were inoculated (1.7 g) in each liquid medium (30 ml). They were cultured at 25° on a rotary shaker (100 rpm) and harvested after three weeks. The LS medium containing 3% sucrose was used as basal medium. In experiments examining the effects of the nitrogen sources, both NH_4Cl and KNO_3 (60 mM in total) were added to the nitrogen free LS medium.

Acknowledgements

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