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ORIGINAL PAPER

Influence of auxins, cytokinins, and nitrogen on production of rutin from callus and adventitious roots of the white mulberry tree (*Morus alba* L.)

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Abstract Rutin is an economically valuable flavone compound with anticancer activity, dietary effects, and anti-aging activity. In this study, callus and adventitious roots were induced from three *Morus* (mulberry) species. Among the three mulberry species tested for rutin production, roots of the Sugye (*M. alba* L.) had the highest levels (242.2 μg/g fresh tissue) of rutin. In addition, the mature leaves of this type of tree promoted higher levels of rutin compared to those of young leaves or those undergoing senescence. Adding auxins such as indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (NAA) not only enhanced the development of callus and adventitious roots but also increased the protein and rutin contents. In contrast, adding cytokinins such as 6-benzyladenine (BA) and kinetin (KN) retarded callus and adventitious root development as well as the protein and rutin contents. Callus in suspension culture in the presence of IAA produced more rutin than that in the absence of IAA. However, rutin secretion into a medium was greater in the absence of IAA. Different ammonium/nitrate (AM/NI) ratios in a root suspension culture also greatly affected rutin production and its secretion into a liquid medium. As a result, the highest level of rutin was produced when adventitious roots were grown in a 34/66 AM/NI full-strength standard MS medium containing 5 mg/l IAA.

Keywords Adventitious roots · Auxin · Callus · Cytokinin · Mulberry plants · NH_4^+-to-NO_3^- ratio · Plant growth regulator · Rutin · Secondary metabolites

Abbreviations

BA	6-Benzyladenine
2,4-D	2,4-dichlorophenoxyacetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KN	Kinetin
NAA	Naphthalene-1-acetic acid
PGR(s)	Plant growth regulator(s)

Introduction

Secondary metabolites, including flavonoids, are valuable plant compounds that are commercially and practically used as medicines, spices, dyes, insecticides, cosmetics, and foods (Zhong 2001). An increasing number of pharmaceutical industries are developing herbal-based supplements with many polyphenolic constituents. Nonetheless, a sufficient supply of these compounds is not available in nature, as they exist in trace amounts in a small group of plants; moreover, their production can be down-regulated by environmental factors such as climate, soil, and the

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presence of pests (Buitelaar and Tramper 1992). Slow plant growth rates and inefficient methods of purification also increase the difficulty in acquiring sufficient quantities of active compounds (Balandrin et al. 1985). Efforts to produce large quantities of physiologically active secondary compounds by organic synthesis are ongoing. However, nearly all active secondary compounds are structurally complex, and in many cases are impossible to synthesize. Alternatively, the yields are too low even when synthesis is possible. To overcome these difficulties, plant tissue culture techniques have been developed for the rapid, large-scale production of cells and their secondary compounds (Hirose et al. 1990).

Rutin (also known as vitamin P, rutoside, quercetin-3-rutinoside, or sophorin) is a citrus flavonoid glycoside. It has been well studied in buckwheat (Kreft et al. 1999). Rutin has many uses due to its beneficial biological activities: it has antioxidative (Ostrakhovitch and Afanas'ev 2001), anti-inflammatory (Guardia et al. 2001), anti-cancer, and anti-aging (Grinberg et al. 1994) effects. It induces apoptosis in a B-cell hybridoma cell line (Roseghini et al. 2009) and was shown to be a free radical scavenger (Bishnoi et al. 2007; Kamalakkannan and Prince 2006). In addition, rutin has been used to treat senile hyperlipemia (Xiping and Xianqiong 1995), Alzheimer's disease (Koda et al. 2008; Pu et al. 2004), and ischemia/reperfusion-induced apoptosis (Jeong et al. 2009).

Morus is a plant genus that comprises 10–16 species of deciduous trees, the majority of which are native to Asia and are widely cultivated in Korea, China, and India. Among these species, the white mulberry is raised in Eastern Asia for its foliage because the leaves are the sole food source of silkworms (*Bombyx mori* L.) (Vijayan et al. 1997). In addition, various parts of the mulberry are used as medicine in the countries of Korea, Japan, and China to treat diabetes, paralytic stroke, and beriberi (Kim et al. 2003). The fruit of the mulberry produces two pharmaceutically important ingredients, the antioxidant cyanidine-3-glucoside (C3G) rutin, which prevents hypertension, and 1-deoxynojirimycin (DNJ), an α-glucosidase inhibitor that lowers blood glucose levels. However, the total area available for mulberry cultivation is decreasing, and mulberry trees are susceptible to frost damage. Moreover, it is more difficult to extract and purify rutin from mulberry than from buckwheat. Therefore, it would be ideal to develop an efficient callus-inducing system for tissues from various parts of the tree to enable the mass production of rutin. Rutin has been biochemically analyzed using several methods (Kreft et al. 1999). However, mass extraction of rutin from either buckwheat or mulberry has not been studied intensively.

It is well known that diverse external factors, such as the temperature, light, pH, and salt concentration influence the production of secondary metabolites (Smetanska 2008). Internally, plant growth regulators (PGRs) play an important role not only in the growth of tissue cell lines but also in the production of secondary compounds. Auxins (IAA, NAA and 2,4-D) and cytokinins (BA, KN and zeatin) are the most important PGRs used in plant tissue cultures. 2,4-D and NAA are essential for inducing callus and shoots in *Leymus chinensis* (Sun and Hong 2010). BA increases the production of xanthones in *Gentianella austrica* shoot cultures (Vinterhalter et al. 2008). Interestingly, the types of auxin and cytokinin and the ratio between the two are also important not only for increasing the percentage of callus production but also for callus growth (Zenk 1978). In this context, selecting the most appropriate plant regulatory substances and determining their optimum concentrations is one of the most important steps in improving the production of secondary compounds.

The production of secondary compounds in plant tissue cultures depends on cell proliferation and differentiation (George 2008). The growth of tissue cultures and amounts of secondary compounds that accumulate depend on the concentrations of growth regulators (Deus and Zenk 1982). The high levels of external auxins stimulate cell growth and division. However, the production of secondary metabolites is decreased at high auxin levels (Zenk et al. 1977). The type of auxin and its concentration also greatly influence the metabolic activities of various cell lines (Dougall 1987). Interestingly, diverse metabolites accumulate differentially depending on the plant growth and developmental stage. For example, bakuchiol (an antimicrobial meroterpene) is not detected in cell suspensions or hairy root preparations of *Psoralea drupacea*. In contrast, aerial parts of *P. Drupacea* grown in vitro accumulate up to 11% dry weight of the bakuchiol (Lystvan et al. 2010). Similarly, verbacscoside is the major phenylpropanoid produced in in vitro cultures (root, white and green callus) of *Buddleja cordata*, while linarin and hydroxycinnamic acid production is low in the same cultures. Linarin production is improved in cell suspension culture (Estrada-Zúñiga et al. 2009).

Nitrogen regulates the expression of specific proteins through mechanisms affecting transcription and/or mRNA stability (Sugiharto and Sugiyama 1992). Nitrogen is incorporated into amino acids and may also serve as a reprogramming signal for the metabolism of nitrogen and carbon, resource allocation, and root development (Wang et al. 2000). Nitrogen sources are important for secondary product synthesis of compounds such as alkaloids (Zhong 2001), anthocyanins, and shikonin from cell suspension cultures (Kim and Chang 1990). Interestingly, the NH_4^+-to-NO_3^- ratio in the medium affects not only the growth of plant cell cultures (Veliky and Rose 1973) but also the production of secondary compounds (Smetanska 2008). For example, the production of betacyanin in *Phytolacca americana* has been shown to be increased in a high NO_3^-/

NH_4^+ ratio medium (Sakuta et al. 1987). A low NO_3^-/NH_4^+ ratio in the medium was found to decrease the cellular syntheses of berberine in *Thalictrum minus* and ubiquinone in *Nicotiana tabacum* (Nakagawa et al. 1984; Ikeda et al. 1977) and of alkaloids in *Atropa belladonna* hairy roots (Bensaddek et al. 2001). The total nitrogen content is also a contributing factor (Sakamoto et al. 1994). The ammonium/nitrate ratio controls the pH of the growth media, stimulates morphogenesis and embryogenesis, and thus is important for inducing callus formation in many woody plant cultures. However, all the aforementioned effects of the culture medium differ from one species to another and from one compound to another (Aoki et al. 1997). Therefore, it is necessary to establish a reproducible external NO_3^-/NH_4^+ ratio condition for the stable production of large quantities of rutin.

The purpose of this study was to establish the optimal culture conditions for the mass proliferation of mulberry callus. To accomplish this, we evaluated the effects of auxins, cytokinins, and nitrogen on rutin production and optimized the conditions for the mass production of rutin from the leaf callus and adventitious roots of mulberry plants.

Materials and methods

Plant materials

Three species of mulberry tree, Sugye (*M. alba* L.), Subong (*M. bombycis* K.), and Jamsang 26 (*M. bombycis* K.), were kindly provided by the sericulture laboratory of the National Academy of Agricultural Science, Republic of Korea (www.naas.go.kr). They were grown in a greenhouse at 25 ± 1°C with a 16-h photoperiod.

Modification of nitrogen contents in MS (Murashige and Skoog) medium

For a careful assessment of the effects of nitrogen sources, a standard MS basal medium containing 1,650 mg/l NH_4NO_3 and 1,900 mg/l KNO_3 (Murashige and Skoog 1962) was modified as previously reported (Neidz 1994; Ziaratnia et al. 2009) by changing the amounts of inorganic nitrogen and altering the ammonium-to-nitrate ratio (AM/NI) in the medium (Table 1). Briefly, the 34/66 AM/NI MS medium is the standard MS basal medium. It contains the full strength of both nitrogen sources. The 41/59 AM/NI MS medium contains half-strength KNO_3 and full-strength NH_4NO_3, and the 27/73 AM/NI medium contains half-strength NH_4NO_3 and full-strength KNO_3. The 35/65 AM/NI medium contains KNO_3 and NH_4NO_3, both at half strength. The 0/100 AM/NI medium is the standard MS basal medium lacking NH_4NO_3.

Table 1 Total nitrogen concentrations and calculated ratios of NH_4^+ to NO_3^- in different MS media

Type of MS medium	Molar ratio of NH_4^+ to NO_3^-	Concentration of total nitrogen (mM)
34/66 AM/NI	34:66	60.0
41/59 AM/NI	41:59	51.0
27/73 AM/NI	27:73	39.4
35/65 AM/NI	35:65	30.0
0/100 AM/NI	0:100	18.7

Callus induction

Leaves with a diameter of less than 5 cm were used for the callus culture. They were washed thoroughly with water, placed in 70% ethanol for 30 s, transferred to a 0.25% hypochlorite solution for 20 min, and then rinsed with sterilized water three times. The middle leaf vein segments used in the callus culture were 0.2 cm × 0.2 cm in size and were incubated on autoclaved 1% 34/66 AM/NI MS agar media that contained 3% sucrose at pH 5.6–5.8. Several types of auxins (2,4-D, IAA, and NAA) and cytokinins (KN and BA) were added as growth regulators. The auxins were supplied at 0.1, 1, or 5 mg/l. For each auxin concentration, a cytokinin was provided at 0, 0.1, or 1 mg/l to determine the effects of different auxin-cytokinin combinations on callus induction. For callus induction, leaf segments on media were cultured in a chamber conditioned at 25 ± 1°C in the dark for 8 weeks. All chemicals used in this report were purchased from Sigma (St. Louis, MO, USA).

Root induction from callus

Middle vein segments from the Sugye mulberry were incubated on autoclaved 34/66 AM/NI MS media (pH 5.6–5.8) containing 3% sucrose, 1% agar, 5 mg/l IAA, and 0 or 0.1 mg/l BA. The incubation conditions were identical to those used for the callus induction process. For the root induction process, the segments were incubated for 56 days and the differentiated roots were used as materials for root suspension cultures.

Suspension cultures for callus and adventitious roots

Calluses and adventitious roots were induced in autoclaved 1% 34/66 AM/NI MS agar media that contained 3% sucrose at pH 5.6–5.8. They were then transferred to a liquid medium containing the same MS content in the presence or absence of 5 mg/l of IAA for induction of the

suspension culture. These samples were incubated in a rotary chamber rotating at 80 rpm at 25 ± 1°C in total darkness for 70 days. To assess the effects of the nitrogen sources, callus or the callus roots were incubated in the autoclaved MS media with various ratios of NH_4^+ to NO_3^- and 3% sucrose and were used as a suspension culture. IAA was provided at 0, 1, 2, or 5 mg/l, and NH_4^+ and NO_3^- were supplied at the following ratios: 34/66, 41/59, 27/73, 35/65, and 0/100 AM/NI MS media (Table 1).

Determination of protein concentration

The growth rates of the calluses and differentiated roots were determined by measuring their weights and protein contents. Briefly, calluses or differentiated adventitious roots were collected and homogenized in 1.5 ml of soluble protein extraction buffer (0.2 M Tris–Cl, pH 8.5, 1 M sucrose, 56 mM β-mercaptoethanol) including 1 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was centrifuged at 11,000*g* for 10 min and the supernatant was collected. Protein quantification was performed using Lowry's method, and bovine serum albumin (BSA) was used to construct a standard curve.

Rutin analysis

Three centimeters of the middle vein at the apex of young leaves, 3–5 cm of the middle vein at the third venation from the apex in mature leaves, and 7 cm of the middle vein on the bottom of senescing leaves were used to quantify the rutin (Fig. 1). For rutin extraction from callus tissue and its quantification, the calluses or the differentiated roots (1 g) were frozen in liquid N_2, ground to a fine powder using a mortar and pestle, and extracted with 10 ml methanol (high-performance liquid chromatography, HPLC grade; Merck) at 80°C for 1 h. The extract was then filtered through filter paper, sonicated for 10 min, and analyzed by HPLC. For rutin quantification by HPLC, the absorbance of rutin (Sigma; 95% purity) dissolved in methanol was determined via spectrophotometry (UV detector: Yong-Lin M720; pump: Yong-Lin M930). A Crespak C_{18} column was used for the HPLC analysis. The absorbances were the greatest at 208, 257, and 358 nm, and a wavelength of 358 nm was used for all subsequent experiments. A mixture of 2.5% acetic acid, methanol, and acetonitrile (35:5:10 in volume) was used for elution. The flow rate was 1 ml/min. A sample of 20 ml was injected for the HPLC process.

Repetition of experiments

We repeated each treatment or measurement three to five times, and in each replication, we used five to seven samples of each plant tissue (Fig. 1), eight to ten leaf disks (Fig. 2a), and six to eight calluses or adventitious roots (Figs. 2b, 3, 4, 5 and 6). Data were collected for all of these replicated experiments.

Results

The Sugye is the richest source of rutin among the three mulberry species evaluated

In this study, we aimed to identify which of three mulberry species is the richest source of rutin and to establish conditions for the mass production of rutin. As an initial step to this end, we analyzed the quantities of rutin produced by the leaves, stems, and roots of three Korean species of mulberry tree: the Sugye, Subong, and Jamsang 26 species. All three species produced some amount of rutin in the tissue types examined, especially in the roots, and the roots of the Sugye contained the highest amount of rutin (242.2 μg/g fresh tissue, which is three- to eightfold more than that found in the roots of the other two species; Fig. 1a). More rutin was detected in mature leaves than in young leaves or in those undergoing senescence (Fig. 1b).

Determination of PGR and nitrogen ratios for optimal induction of callus and adventitious roots from leaves

Based on our findings that roots were the richest source of rutin, we established the optimal induction conditions for leaf

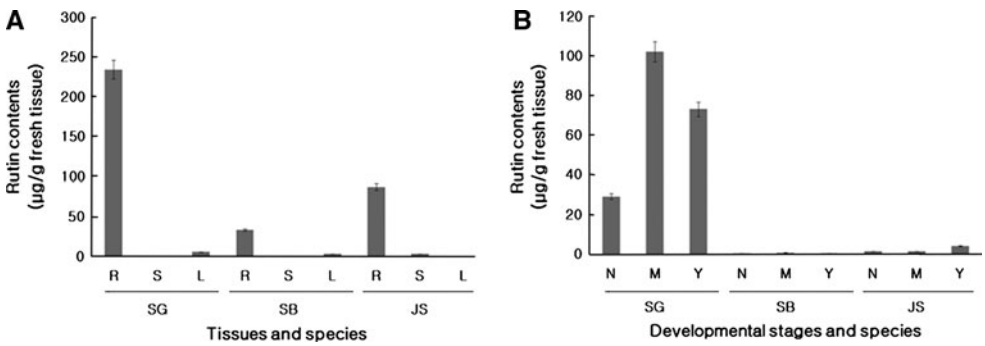

Fig. 1 Amounts of rutin in Sugye, Subong and Jamsang 26 species grown in a greenhouse. **a** Rutin contents in roots, stems, and leaves; **b** rutin contents in young, mature, and senescing leaves. *SG* Sugye, *SB* Subong, *JS* Jamsang 26, *R* root, *S* stem, *L* leaf, *N* necrotic leaf, *M* mature leaf, *Y* young leaf. *Error bars* standard deviations

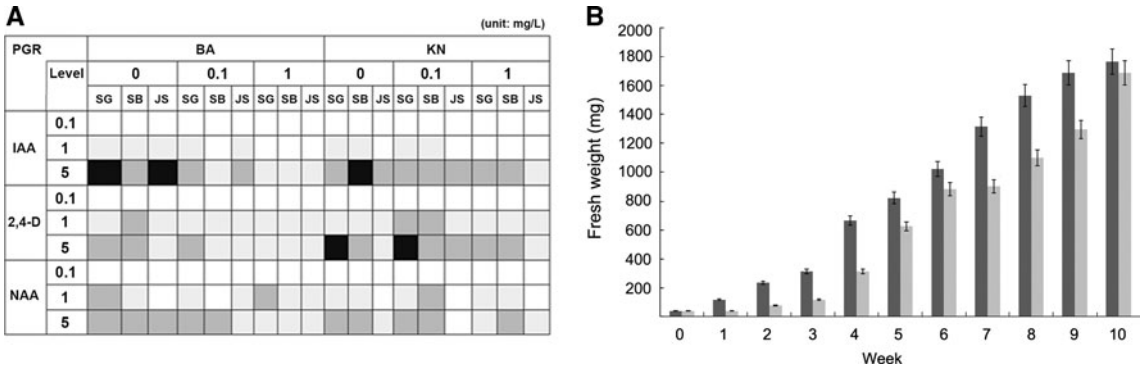

Fig. 2 Effects of PGRs on growth of callus and adventitious roots in mulberry species. **a** Effects of auxins and cytokinins on callus formations in the Sugye, Subong, and Jamsang 26 species. Different auxin-to-cytokinin ratios were tested for their induction of callus in the 34/66 AM/NI full-strength MS media. *White square* no induction, *light gray square* rare induction, *gray square* good induction, *black square* excellent induction of callus. **b** Growth of adventitious roots of the Sugye cultured in liquid MS medium supplemented with or without IAA. Adventitious roots induced from IAA-induced callus were further subjected to 34/66 AM/NI full-strength MS suspension media with or without IAA and were grown for the indicated periods. *Dark gray* –IAA, *light gray* +IAA. *Error bars* standard deviations

calluses and adventitious roots for the mass production of rutin under tissue culture conditions. Calluses were induced from the middle veins of the three different species of mulberry by incubating leaf disks on the 34/66 AM/NI standard full-strength MS medium containing various amounts of auxins and cytokinins. Five to seven days after the leaf disks were incubated in the MS medium, there was a noticeable outgrowth of cells from the middle veins. After 10 days, calluses were visible on the cut surface. Calluses began to form on medium containing 1.0 mg/l auxins of IAA, 2,4-D and NAA, and were most efficiently induced in media supplemented with 5 mg/l 2,4-D or 5 mg/l IAA without any cytokinins. The addition of a cytokinin, BA or KN, lowered the induction efficiency in a concentration-dependent manner (Fig. 2a). Interestingly, at the highest concentration of IAA (5 mg/l), the formation and growth of adventitious roots also increased markedly, especially in the Sugye species (data not shown).

Once adventitious roots were induced in the medium containing 3% sucrose, 1% agar, and 5 mg/l IAA, auxin no longer appeared to be a significant regulating factor for root growth. We induced adventitious roots from the Sugye species, as described above, and transferred adventitious roots longer than 4 cm to a 34/66 AM/NI MS suspension medium containing 3% sucrose with or without 5 mg/l IAA. These roots were then incubated for 10 weeks to measure the fresh weights of the induced roots (Fig. 2b). In MS media containing 5 mg/l IAA, the growth and development of adventitious roots was enhanced as the incubation period increased. Hence, 10 weeks after the transfer,

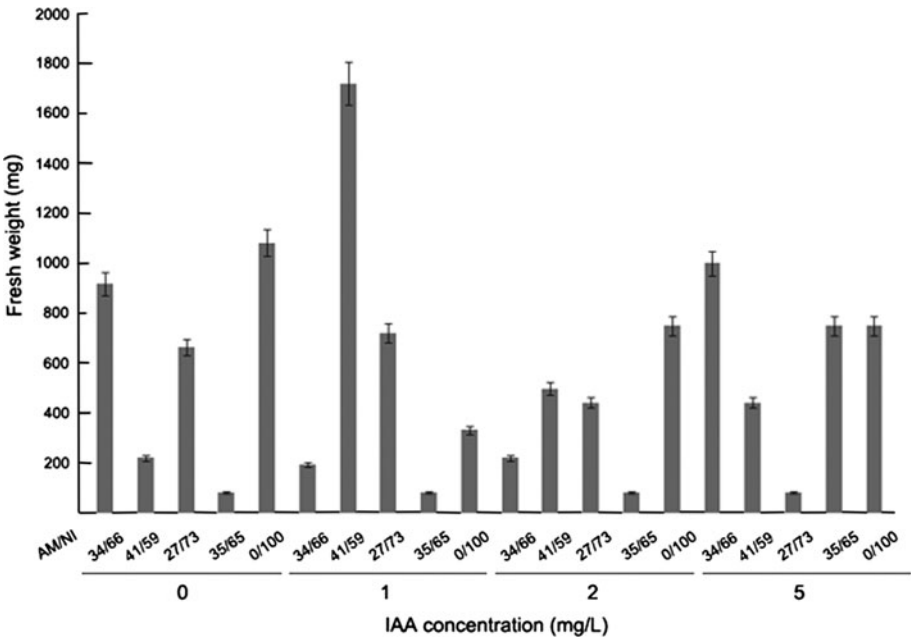

Fig. 3 Growth of adventitious roots of Sugye species cultured in modified MS media supplemented with different amounts and concentration ratios of NH_4NO_3 and KNO_3 in the presence or absence of IAA. The adventitious roots were induced in the 34/66 AM/NI full-strength MS medium containing 3% sucrose, 1% agar, and 5 mg/l IAA and were then transferred to media containing different concentrations and/or ratios of IAA and nitrogen sources. They were then incubated for 4 weeks. The ratios of NH_4^+ to NO_3^- and total nitrogen concentrations in the indicated AM/NI media are described in Table 1. *Error bars* standard deviations

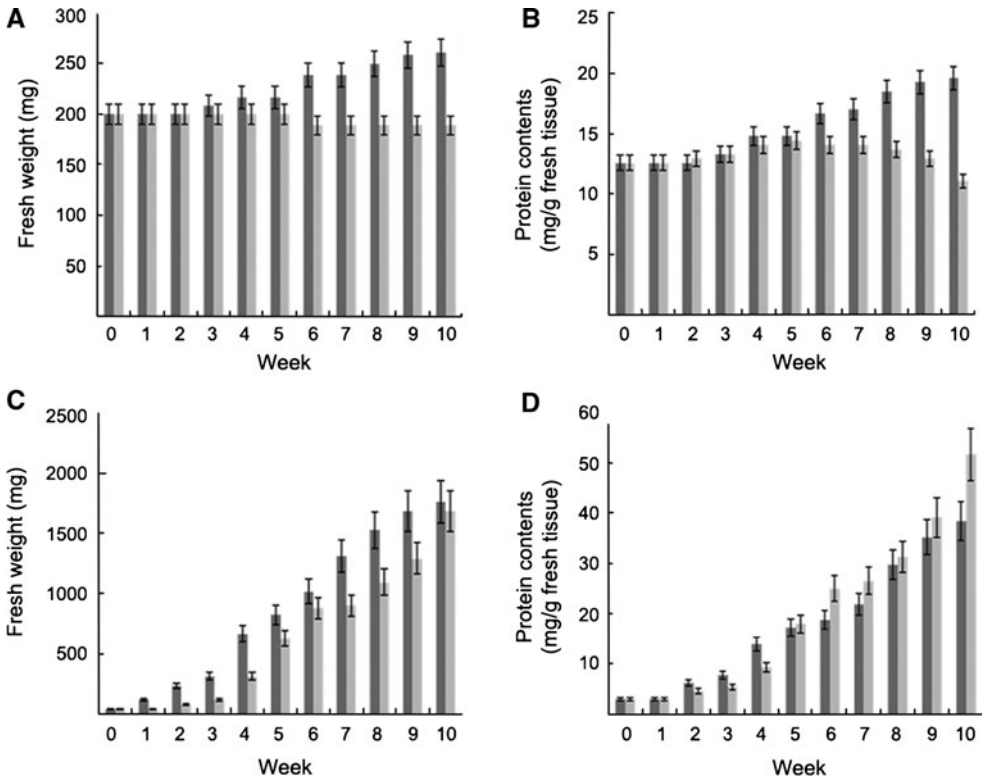

Fig. 4 Fresh weights and amounts of protein present in the calluses and adventitious roots of the Sugye, cultured in 34/66 AM/NI full-strength MS liquid media with 5.0 mg/l IAA (*dark gray*) and without IAA (*light gray*) for 10 weeks. Calluses and roots grown for 10 weeks on a solid 34/66 AM/NI full-strength MS medium containing 5 mg/l of IAA were transferred to liquid MS media containing 5 mg/l of IAA or lacking IAA. Medium was changed every 4 weeks. **a** Fresh weight of callus; **b** protein content of callus; **c** fresh weight of adventitious roots; **d** protein content of adventitious roots. *Error bars* standard deviations

the fresh weight of the roots was almost 45 times greater than the initial weight (an increase from 40 to 1,780 mg). This tendency of the fresh weight to increase during incubation was also observed for roots grown in a medium without auxin; however, during most of the time period, the growth rate was less than that of the roots grown in the auxin-supplemented medium. Nonetheless, root growth in the presence or absence of auxin was not significantly different at 10 weeks.

The ratio of NH_4^+ to NO_3^- in a growth medium has a significant influence on the types and amounts of secondary metabolites in tissue cultures (Dodds and Roberts 1985). To determine the optimal ratio of NH_4^+ to NO_3^- (the NH_4NO_3/KNO_3 ratio) in MS suspension media for the growth of adventitious roots, we incubated the adventitious roots of the Sugye species for 4 weeks in MS suspension media containing 3% sucrose with or without IAA and with different ratios of NH_4NO_3 to KNO_3 as nitrogen sources (Fig. 3). Interestingly, different auxin concentrations in the media required different NH_4^+/NO_3^- ratios for optimal root growth. For example, the highest production of roots in a medium containing 0 or 2 mg/l IAA was obtained when 0/100 AM/NI MS medium was used, while roots formed in a medium containing 1 mg/l or 5 mg/l IAA showed the maximum fresh weight increase when 41/59 or 34/66 AM/NI MS medium was used for growth. The lowest production of roots for each IAA-supplemented MS medium, except for the medium containing 5 mg/l IAA, was noted when the 35/65 AM/NI MS medium was used. Collectively, the greatest quantity of fresh roots was obtained when the 41/59 AM/NI MS medium supplemented with 1 mg/l of IAA was used.

Establishment of PGR ratio for the optimal induction of total proteins

To assess the viability and productivity of calluses and adventitious roots, we quantified the amount of total proteins extracted from the induced calluses and roots grown for 10 weeks on solid 34/66 AM/NI MS media containing different concentrations of PGRs, with IAA as an auxin source and BA as a cytokinin source. As expected, the growth of the calluses and adventitious roots were the most efficient when the media was supplemented with 5 mg/l of IAA without any cytokinin and when the Sugye was used as the source of the callus and the adventitious roots. Interestingly, the production of total protein from calluses and roots was also highest when they were grown under the same media conditions (data not shown). Next, we transferred the adventitious roots and calluses grown on the solid media to liquid MS media containing 5 mg/l of IAA or lacking IAA to measure the changes in the fresh weight and total protein content for each incubation period. Both the protein content and the fresh weight of the calluses increased slightly depending on the incubation period, showing fresh weight and protein content increases of 25

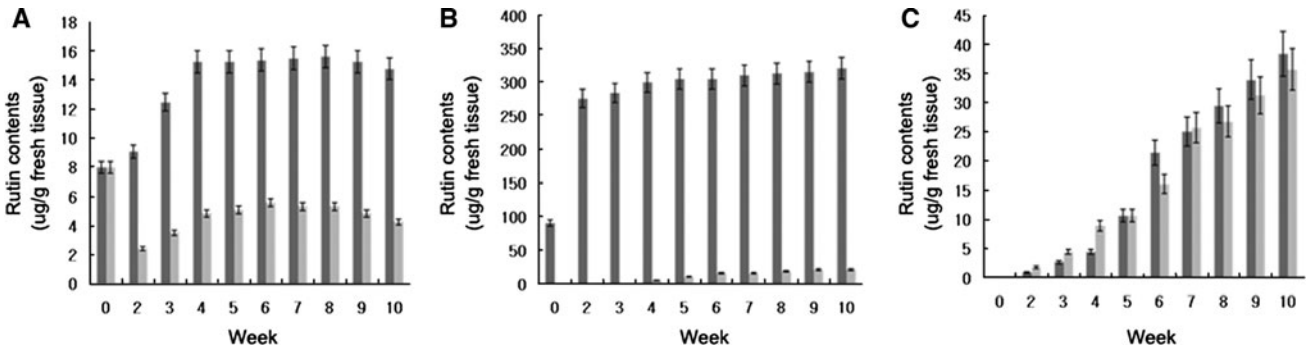

Fig. 5 Amounts of rutin in the calluses, adventitious roots, and secreted rutin in the cultured liquid medium of the Sugye species. Calluses and roots grown for 10 weeks in solid 34/66 AM/NI MS medium containing 5 mg/l of IAA were transferred to solid MS media (in the case of callus) or liquid media (in the case of adventitious roots) containing 5 mg/l of IAA (*dark gray*) or lacking IAA (*light gray*). The medium was changed every 4 weeks. **a** Rutin compound in the callus; **b** rutin compound in the adventitious roots; **c** secreted rutin compound in the liquid culture media in (**b**). *Error bars* standard deviations

and 40% after 10 weeks, respectively, compared to those of the 0-week control (Fig. 4a, b). However, in the media lacking IAA, the fresh weight and protein content did not change. Surprisingly, incubation of adventitious roots in suspension media resulted in different outcomes with respect to the amount of total protein and fresh weight. The weights of adventitious roots increased dramatically in medium either containing IAA or lacking IAA, so that after 10 weeks of incubation, the weights had increased by 40-fold (from 45 to 1,800 mg) in medium containing IAA and by 33-fold (from 50 to 1,650 mg) in a medium lacking IAA (Fig. 4c). A similar tendency was observed for the root total protein abundance; it increased by 10-fold (from 3.6 mg/g fresh weight to 36 mg/g fresh weight) in the presence of IAA or by 14.3-fold (from 3.5 mg/g fresh weight to 50 mg/g fresh weight) in the absence of IAA in the medium (Fig. 4d).

In summary, the total amount of protein obtained from 45 mg of initial callus after 10 weeks of incubation in an IAA-containing liquid 34/66 AM/NI MS media was increased by 400-fold, from 0.162 mg (45 mg fresh weight × 3.6 mg proteins/g fresh weight) to 64.8 mg (1,800 mg fresh weight × 36 mg proteins/g fresh weight). In medium lacking IAA, there was a 471-fold increase in total proteins, from 0.175 mg (50 mg fresh weight × 3.5 mg proteins/g fresh weight) to 82.5 mg (1,650 mg fresh weight × 50 mg proteins/g fresh weight).

Establishment of PGR and nitrogen ratios for the optimal induction of rutin production from calluses and adventitious roots

Our major research goal was to establish the optimum tissue culture conditions for the mass production and efficient purification of rutin from mulberry plants. We found that the growth of both calluses and adventitious roots and the production of total proteins from them were highest when 5 mg/l of IAA without any cytokinin was added to solid or liquid MS media. Interestingly, the same growth conditions greatly increased the production of rutin by 87.5% during the first 4 weeks after the callus was induced (an increase from 8 μg/g to 15 μg/g fresh callus) on the solid 34/66 AM/NI standard full-strength MS media, and the production was stabilized thereafter (Fig. 5a). Surprisingly, for calluses grown in medium lacking IAA, the rutin content decreased dramatically to 31.7% of that of the 0-week control in the first 2 weeks (8 μg/g to 2.5 μg/g fresh callus). It thereafter recovered to 62.5% of the amount of the 0-week control. In our experiment, the production of calluses and roots and their protein contents were much higher when they were grown in a liquid suspension medium rather than in a solid agar medium. Specifically, the roots of mulberry trees were the most productive source of rutin (Fig. 1a). We measured the production of rutin in adventitious roots of Sugye grown in 34/66 AM/NI MS liquid media containing or lacking 5 mg/l IAA. In this experiment, we measured endogenous rutin in the roots in addition to the rutin secreted into the suspension medium. In the presence of IAA, rutin in the roots was greatly increased in the first 2 weeks of incubation (a two-fold increase from 90 to 270 μg/g roots); after this point, the production was maintained around this level until 10 weeks of incubation (Fig. 5b). Interestingly, about 10.7% of the total rutin was secreted into the suspension medium (37 μg/g fresh tissue, divided by the total rutin, which is the sum of 37 μg/g fresh tissue in the medium and 310 μg/g fresh tissue in the roots) at 10 weeks of incubation. In the absence of IAA, the amount of rutin in the roots was greatly reduced compared to that in roots grown in the same incubation time period in IAA-containing media. For example, the total rutin (the endogenous rutin in roots, 20 μg/g fresh tissue, and the secreted rutin in suspension medium, 32 μg/g fresh weight) produced by roots grown in media lacking IAA at the incubation time point of 10 weeks was 52 μg/g root weight,

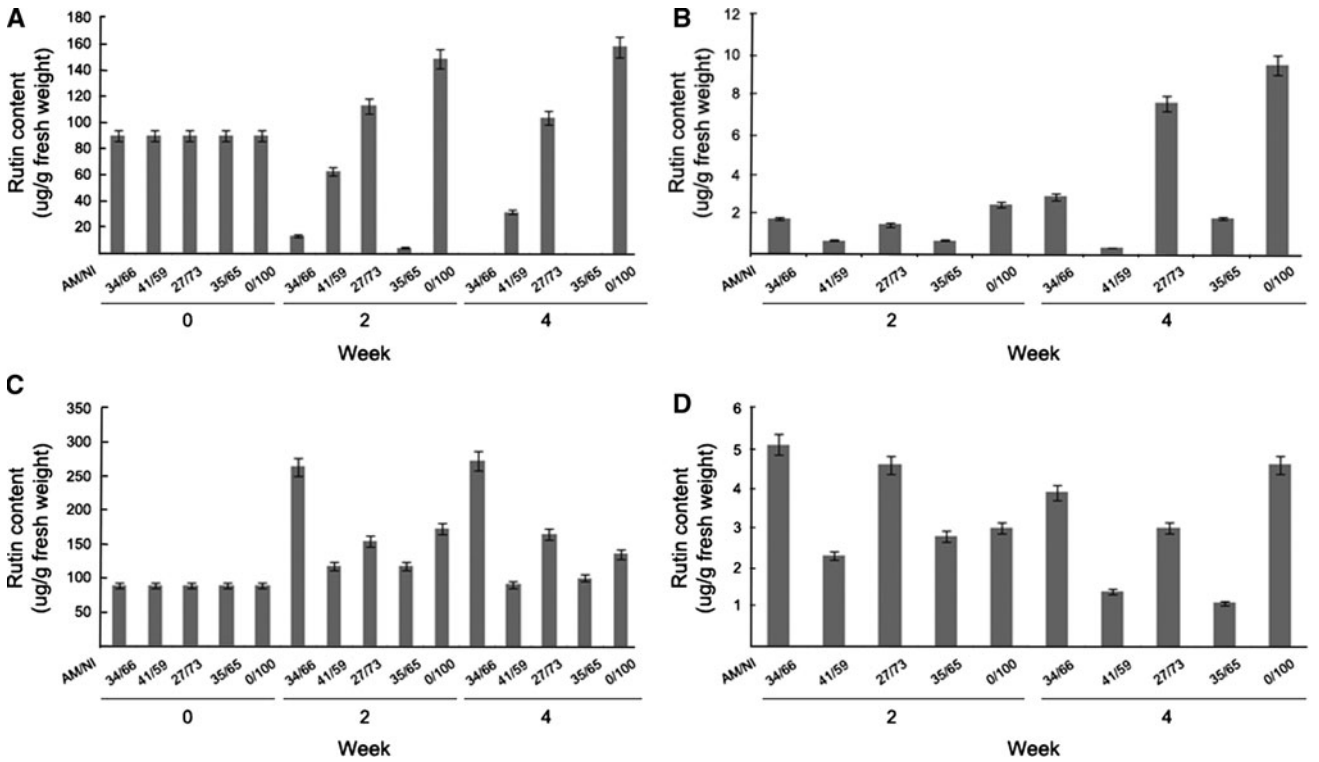

Fig. 6 Amounts of rutin in the adventitious roots and cultured liquid medium of the Sugye species after incubation in modified liquid MS media supplemented with different concentrations and ratio of NH_4^+ to NO_3 in the presence or absence of 5.0 mg/l IAA. Adventitious roots were induced in the 34/66 AM/NI full-strength MS medium containing 3% sucrose, 1% agar, and 5 mg/l IAA and were then transferred to a medium containing different concentrations and/or ratios of IAA and nitrogen sources to incubate for 4 weeks. **a** Rutin compound in adventitious roots grown in a medium lacking IAA and containing the indicated ratio of NH_4^+ to NO_3; **b** rutin secreted into the medium in (**a**); **c** rutin in adventitious roots grown in a medium containing IAA and the indicated ratio of NH_4^+ to NO_3; **d** rutin secreted into the medium in (**c**). The ratios of NH_4^+ to NO_3^- and total nitrogen concentrations in the indicated AM/NI media are described in Table 1. *Error bars* standard deviations

while that from the roots grown in IAA-containing media was 347 μg/g root weight (Fig. 5b, c). Interestingly, secretion was prominent when the roots were grown for 10 weeks in medium lacking IAA, so that almost 61.5% of the total rutin produced was actively secreted into the suspension medium (Fig. 5c).

The highest production of adventitious roots in medium containing 5 mg/l IAA was observed when the standard 34/66 AM/NI MS medium was used for incubation (Fig. 3). To evaluate the effects of different ratios of nitrogen sources on rutin production, we modified the amounts of NH_4NO_3 and/or KNO_3^- in the 5 mg/l IAA-lacking or -containing liquid media and measured the amount of rutin either in the Sugye roots or the culture medium. The incubation of adventitious roots in medium lacking IAA for 2 or 4 weeks increased the amount of rutin produced when 0/100 AM/NI MS media was used (i.e., from 90 to 140 μg/g roots after 4 weeks of culture in a 0/100 liquid medium containing no IAA), while rutin production was reduced to nearly zero when roots were cultured in 34/66 AM/NI full-strength or 35/65 AM/NI half-strength MS medium (Fig. 6a). Secretion of rutin into the suspension medium was also relatively high when roots were grown in 0/100 AM/NI MS medium (Fig. 6b). Interestingly, an addition of 5 mg/l IAA to the medium changed the response to nitrogen nutrition so that roots grown in 34/66 AM/NI full-strength MS medium containing 5 mg/l IAA showed a dramatic increase in the amount of rutin produced, from 80 to 260 μg/g roots after 4 weeks of culture in a liquid medium. In contrast, roots grown in other (0/100, 41/59, 27/73 or 35/65 AM/NI) MS media containing 5 mg/l IAA produced rutin at almost the same levels with the 0 h control or slightly more (i.e., increased from 80 to 80–160 μg/g roots after 4 weeks of culture; Fig. 6c). Secretion into the medium was also relatively high when roots were grown in a 34/66 AM/NI full-strength or 0/100 AM/NI MS medium (Fig. 6d).

Discussion

Rutin is a flavone compound that has beneficial effects on diet and blood pressure control. It also has anti-cancer, anti-aging, and anti-oxidative activities. The main purposes

of this study were (1) to investigate and analyze the effects of PGRs and nitrogen sources on the growth of calluses and roots of mulberry trees under tissue culture conditions, and (2) to determine the optimal culture conditions for stable and long-lasting mass rutin production.

We examined the rutin content in three different tissues of three mulberry species, the Sugye, Subong, and Jamsang 26, and found that the roots of the Sugye species contained the greatest amount of rutin (Fig. 1a). Callus induction itself was active in all species examined, and the Sugye was the best rutin source for our purposes (Fig. 1a). To determine the conditions for the maximum production of rutin, we induced callus formation in the three mulberry species on media containing various amounts of auxins and cytokinins. The Sugye showed the highest callus and adventitious root formation in the media containing 5 mg/l IAA and lacking a cytokinin (Fig. 2a). Cytokinin has been reported to stimulate callus induction (Wu et al. 2003), but we found that both BA and kinetin negated IAA-induced callus induction under our conditions. This contradictory observation may be explained by a report demonstrating that the efficiencies of auxin and cytokinin for callus induction depend on the age of the source, the type of organ used, and the location of the tissue within the organ, as different tissues have different sensitivities to plant hormones, even in the same species (Wilson et al. 1971).

IAA is involved in many plant growth and development processes, and its homeostasis in a cell is established through the accurate coordination of its metabolism, synthesis, conjugation, hydrolysis, oxidation, and transport (Normanly et al. 1995). In general, auxins inhibit the metabolism of secondary compounds. However, the opposite response was observed in this study. Adding IAA to incubation medium not only enhanced the development of callus and adventitious roots but also increased protein and rutin content. Interestingly, the secretion of rutin into the media was more efficient when there was no IAA in the media. The amount of rutin released into the culture medium reached its maximum at 4 weeks when adventitious roots were grown in 0/100 AM/NI MS medium containing no NH_4NO_3 and full-strength KNO_3 in the absence of IAA (Fig. 6b). Similar to our results, a high concentration of IAA increased the production of ajmalicine in shoot cultures of *Catharanthus roseus* but inhibited its extracellular secretion. In contrast, shoots released high levels of ajmalicine into the culture medium when the medium was supplemented with a low concentration of IAA and a high concentration of BA (Satdive et al. 2003). Other than BA, methyl jasmonate has been reported to increase the production and the root exudation of secondary metabolites, including ajmalicine, in the hairy roots of *C. roseus* (Ruiz-May et al. 2009). *Arabidopsis* has a large number of ABC transporter proteins involved in the efflux of plant-derived secondary metabolites (Yazaki, 2006), but it is not clear how plant hormones affect the secretion of secondary metabolites.

The NH_4^+/NO_3^- ratio in the medium affects not only the growth of plant cell cultures (Veliky and Rose 1973) but also influences the types and production of secondary compounds (Smetanska 2008). We showed that different ratios and amounts of NH_4^+ and NO_3^- were required for optimal root growth under different auxin concentrations in the media. The addition of auxin increased the amount of total endogenous rutin but inhibited secretion of rutin to the liquid media. Interestingly, in the absence of IAA, the amounts and ratios of NH_4^+ to NO_3^- greatly affected rutin production and its secretion into the liquid medium. Maximum rutin production and secretion occurred when NH_4NO_3 was not supplied in a medium lacking IAA (Figs. 6a, b). Growing roots in a medium containing a lower NH_4^+/NO_3^- ratio (<0.5; i.e., 27/73 and 0/100 AM/NI MS media) resulted in greater rutin production than growth in a medium containing a higher ratio of nitrogen sources (≥ 0.5; i.e., 34/66, 41/59, and 35/65 AM/NI MS media). Collectively, NO_3^- appeared to stimulate root growth and rutin production more efficiently than NH_4^+.

Due to the harmful effects of PGRs on cells, it is necessary to determine the conditions for mass production of rutin using the minimal amount of IAA and the appropriate ratio of NH_4^+ to NO_3^-. IAA and horseradish peroxidase (HRP) produce free radicals and reactive oxygen species (ROS) due to the decarboxylation of IAA (Folkes and Wardman 2001; Kawano et al. 2001). These free radicals and ROS are detrimental to cells. Based on our results, it may be possible to increase rutin production by adjusting the NH_4^+ to NO_3^- ratio in the absence of IAA. Our results showed that NO_3^- is an important nitrogen source when the IAA concentration is low, whereas NO_3^- and NH_4^+ are equally important when the IAA concentration is high. Although we were not able to achieve a high yield of rutin without IAA, the data we obtained are nevertheless useful for further studies. Manipulating nitrogen supplies, for example, by increasing the concentration of NO_3^- and decreasing that of NH_4^+ in a bioreactor, would allow the establishment of an optimal ratio of nitrogen compounds for the mass production of rutin.

Establishing a protocol for the large-scale production of rutin in a bioreactor is highly desirable, and the optimization of conditions for the efficient purification of rutin from plant tissues or from a suspension medium is equally important for the use of rutin for commercial purposes. If rutin is secreted efficiently into the suspension media, then the downstream extraction process could be simplified because there would be no need to extract rutin directly from the biomass. From this perspective, cell immobilization is another special technique for cell cultures to produce

and purify secondary compounds more economically. Immobilized cells lengthen the production time, stabilize the product without changes, and release the product into the media (Smetanska 2008). Diverse immobilization matrices such as foam, calcium alginate, natural glass, polyurethane foam, and gel have been used to establish high-producing cell lines using artificial plant tissue culture techniques (Kim and Chang 1990). Therefore, we intend to investigate this approach in the near future to evaluate its effectiveness, productivity, and feasibility. Another efficient means of mass production is to use state-of-the-art biotechnology, such as molecular farming. To use this method, genes encoding proteins that catalyze rutin biosynthesis could be cloned and engineered. In this way, it may be possible to obtain rutin from the field by bioengineering cereal plants that carry a rutin transgene.

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