ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Shoot organogenesis and somatic embryogenesis in *Rehmannia glutinosa* Libosch. - An important medicinal plant

Prabhakaran Soundararajan[1], Abinaya Manivannan[1], Yoo Gyeong Park[2], Chung Ho Ko[1] and Byoung Ryong Jeong[1,2,3,*]

1. Division of Applied Life Science (BK21 Plus), Graduate School, Gyeongsang National University, Jinju, Korea 660-701,
2. Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, Korea 660-701,
3. Research Institute of Life Science, Gyeongsang National University, Jinju, Korea 660-701,

Manuscript Info

Manuscript History:

Received: 15 September 2014
Final Accepted: 22 October 2014
Published Online: November 2014

Key words

Medicinal plants, Micropropagation, Shoot induction, Somatic embryo, Rooting

Corresponding Author

Byoung Ryong Jeong

Abstract

Rehmannia glutinosa Libosch. is an important medicinal plant, listed as one of the 50 fundamental herbs used in Chinese medicine. In this study, callus mediated shoot organogenesis and somatic embryogenesis from leaf and petiole explants have been investigated. For shoot regeneration, Murashige and Skoog (MS) medium supplemented with different concentration of 6-benzyladenine (BA) such as 0, 1.0, 2.0, 3.0, 4.0, or 5.0 mg·L^{-1} along with 0.5 or 1.0 mg·L^{-1} indole-3-acetic acid (IAA) has been studied. For somatic embryogenesis, explants were cultured on MS medium containing 0, 1.0, or 2.0 mg·L^{-1} of 2, 4-dichlorophenoxyacetic acid (2, 4-D) in combination with 0 or 1.0 mg·L^{-1} of BA and/or gibberellic acid (GA_3). The highest number of shoots (4.4±0.30) per explants was observed on leaf explants cultured in 3.0 mg·L^{-1} BA with 0.5 mg·L^{-1} IAA medium. On the other hand, the petiole explant cultured on 2.0 mg·L^{-1} 2, 4-D medium induced the maximum (76.4%) embryogenic callus and 3.6±0.30 somatic embryos per explant. The conversion of plantlets from embryogenic callus was succeeded in half-MS medium supplemented with the 1.0 mg·L^{-1} BA and 1.0 mg·L^{-1} GA_3. Plant growth regulators (PGRs) free-MS medium was used for in vitro rooting. Approximately 90% of plants were successfully survived in the greenhouse. Thus, we concluded from our results that leaf might be the best explant for shoot induction and multiplication whereas petiole can be the effective explant for somatic embryogenesis. Therefore, the developed protocol from our studies can be utilized for the large scale clonal propagation, germplasm conservation, and genetic manipulation of *R. glutinosa*.

Introduction

Rehmannia glutinosa Libosch. is one of the pharmacologically important medicinal plants belongs to Scrophulariaceae family distributed widely in Korea, China, and Japan. It is listed in 50 fundamental and important medicinal plants list used in traditional Chinese medicine (Zhang et al., 1993). The extracts and dried root of *R. glutinosa* highly constitutes the phytochemicals such as catalpol, danmelittoside, leonuride, aucubin, melittoside, rehmaglutin etc., (Albach et al., 2007; Liu et al., 2002). These valuable secondary metabolites are used as anti-anemic, anti-pyretic, anti-inflammatory, anti-hypoglycemic, anti-hypertensive, and anti-senescence agents (Zhang et al., 2008). Similarly, its raw extract has the ability to purify blood system, immune system, endocrine system,

cardiovascular system, and nervous system (Liu et al., 2002; Zhang et al., 2008). Therefore, the extract of *R. glutinosa* is highly demanded in the market (Zhou et al., 2010).

Conventionally *R. glutinosa* has been vegetatively propagated by tuberous root segments. Generally, the roots of *R. glutinosa* are highly susceptible to virus and soil borne fungus which affects the yield and quality. Besides, the seed based propagation of *R. glutinosa* is impracticable due to poor quality, dormancy, and low propagation rate (Park et al., 2009; Zhang et al., 2008). Due to the above mentioned difficulties, an alternative measure of mass propagation by means of plant tissue culture approaches is necessary to meet the growing need of *R. glutinosa* roots. Tissue culture is the vital technique extensively used to obtain clonal plants with homogenous secondary metabolite contents. In vitro shoot multiplication gives a rapid and year round production of *R. glutinosa* plants for commercial cultivation and high yield production. Meanwhile, somatic embryogenesis helps to produce embryos from the somatic/vegetative part of the plants similar to the zygotic embryo (Bhojwani and Razdan, 1996). Especially, for plants like *R. glutinosa* producing infertile seeds, somatic embryos could be an alternative for producing artificial and healthy seeds (Parimalan et al., 2011; Kong and von Aderkas, 2011). In addition, somatic embryos help to produce the improved traits with disease and pest resistance. Hence, true-to-type plant developed from somatic embryos can provide a suitable/appropriate material for genetic manipulation, cryopreservation and embryogenesis studies.

Previously, direct somatic embryogenesis from petiole and leaf explants were reported on *R. glutinosa* in liquid culture (Park and Chae, 1997). Although, few notable studies reported the adventitious shoot induction from leaf (Park et al., 2009), shoot tip (Paek et al., 1995) and root (Jeong et al., 2002) explants of *R. glutinosa* However, till now it is a challenging task to develop an efficient shoot induction and proliferation with exact concentration and combination of auxin and cytokinin on *R. glutinosa*. Likewise, no reports are available on the possibility of shoot regeneration from petiole explants. In addition, this is the first study on indirect somatic embryogenesis of *R. glutinosa* L. in solid medium. Thus, the aim of the present study is to develop an efficient protocol for the rapid mass propagation and somatic embryogenesis of the *R. glutinosa*.

Materials and methods

Shoot Organogenesis

Explants such as leaf and petiole was excised from the in vitro grown plants were cut into 1.0-1.5 cm segments. Five explants per petri dishes were cultured on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar containing different concentration of 6-benzyladenine (BA) such as 0, 1.0, 2.0, 3.0, 4.0 ,or 5.0 $mg \cdot L^{-1}$ in combination with 0.5 or 1.0 $mg \cdot L^{-1}$ indole-3-acetic acid (IAA). The pH of the medium was adjusted to 5.75 before autoclaving at 121 ºC for 15 min. All the cultures were maintained under a 16 h photoperiod provided by cool-white fluorescent light (45 $\mu mol\ m^{-2}\ s^{-1}$) at 25±1 ºC in the growth chamber with 70% relative humidity (RH). For shoot elongation, induced adventitious shoots were cultured in MS medium containing various concentration of gibberellic Acid (GA_3) such as 0, 0.5, 1.0, 1.5 or 2.0 $mg \cdot L^{-1}$. After three weeks, elongated shoots were cultured in hormone-free MS medium for in vitro root induction.

Somatic embryogenesis

For embryogenic callus induction, leaf and petiole explants were excised from in vitro grown plants and cultured on MS medium containing 0, 1.0, or 2.0 $mg \cdot L^{-1}$ of 2,4-D in combination with 0 or 1.0 mg L^{-1} of BA or GA_3. For somatic embryo development, germination, and plantlet conversion, the embryogenic callus was transferred to half strength-MS medium supplemented with combination of 0 or 1.0 mg L^{-1} BA and/or GA_3. After plantlet conversion, explants were cultured in MS medium and acclimatized.

Hardening and acclimatization

All well-rooted healthy plants were hardened in a growth chamber for two weeks in 50 square plug tray containing commercial Tosilee medium (Tosilee medium, Shinan Precision Co., Jinju, Korea). The day/night temperature was maintained at 24/17±2 °C with 80% of RH. In addition, trays were covered with plastic cover to maintain RH and irrigated with quarter-strength nutrient solution. The nutrient solution was prepared according to the Soundararajan et al. (2014). After the emergence of new leaves, the plantlets were transferred to 10 cm pots and acclimatized in the greenhouse at Gyeongsang National University, Jinju, Korea, with the day/night temperature of 27/19±2 ºC and 70% RH under a normal day-light condition. The full-strength nutrient solution was supplied once a day through by ebb- and flow-method. After five weeks survival rate of acclimatized plant have been measured.

Statistical analysis

All treatments were set up in a completely randomized design with five replicates per treatment containing five explants in each petridish. Significant differences among the treatments were determined by analysis of variance (ANOVA) followed by Duncan multiple range tests at a 5% probability level by using SAS computer package (SAS Institute Inc., Cary, NC, USA).

Results and discussion

Shoot organogenesis

After one week of inoculation, both leaf and petiole explants were bulged in PGR supplemented medium. Subsequent callus formation was noted after two weeks and the adventitious shoot buds were emerged from the organogenic callus after third week of culture (Fig 1A). However the explants inoculated on the hormone-free MS medium become brown and failed to develop shoots. Shoot induction percentage and number of shoots were recorded after six weeks of culture (Table-1). Mostly shoot regeneration of *R. glutinosa* was accompanied with callus formation at the cut ends of the explants (Fig 1B). This effect might be due to the accumulation of auxin and this response stimulates the cell proliferation especially in the presence of cytokinin (Marks and Simpson, 1994). Among the various concentration of BA along with 0.5 or 1.0 $mg \cdot L^{-1}$ IAA experimented, the maximum number of 4.4±0.30 shoots were induced from the leaf explants in MS medium supplemented with 3.0 $mg \cdot L^{-1}$ BA and 0.5 $mg \cdot L^{-1}$ IAA. However, previous reports using TDZ at 1.0 $mg \cdot L^{-1}$ only produced 2.1±0 shoots per leaf explants (Park et al., 2009). Generally, low auxin concentration along with high cytokinin level induced the maximum frequency of shoot organogenesis than the medium containing cytokinin alone. Auxin and cytokinin ratio is vital for redifferentiation of callus into organized tissue (Wang et al., 2008). On the other hand, at the concentration of 3.0 $mg \cdot L^{-1}$ BA with increased IAA (1.0 $mg \cdot L^{-1}$) produced more roots instead of shoots. This result is in agreement with the synergistic effect of BA and IAA concentration (Beena et al., 2003). Patnaik and Debata (1996) also observed the similar response in *Hemidesmus indicus*.

Though petiole explants showed similar response to leaf explants in callus induction, the number of shoots induced per explants was lesser than the leaf explants (Table. 1) i.e., maximum 3.2±0.53 shoots per explants were achieved in 4.0 $mg \cdot L^{-1}$ BA and 0.5 $mg \cdot L^{-1}$ IAA. This indicates the difference in regeneration potential of explants attributed to the physiological state, vascular tissue intensity, and cellular differentiation response of the constituent cells (Ghimire et al., 2010). Importantly, this is first report describing the callus-mediated shoot regeneration from petiole explants. Similarly, frequency and number of shoots from the petiole were lesser than from leaves. Similar observations were documented by Venkataiah and Subhash (2001) in Capsicum.

For the shoot elongation, callus clumps containing microshoots were isolated and cultured in MS medium supplemented with 0, 0.5, 1.0, 1.5 or 2.0 mg L^{-1} GA_3 (Fig 1C and D). Upon GA_3 concentration increase in MS medium, shoots are elongated well but the shoots were fragile (Fig 1D). This might be due the stimulation of endogenous auxin in the axillary bud by GA_3, which directs the elongation of internode region (Ross et al., 2003). In addition, shoots are hyperhydric in GA_3 added medium. Hence, the PGRs were devoid from the medium for further in vitro root induction. About 95% of root induction was achieved in hormone-free MS medium (Fig 1E).

Somatic embryogenesis

Somatic embryogenic callus induction was observed after three weeks of culture in 2, 4-D medium (Fig 2A and D). On contrary 2, 4-D medium along with BA and/or GA_3 produced - callus was necrotized (Fig 2B and C). In general 2, 4-D is the most potent auxin for the somatic embryo induction (Pradeep et al., 2009). This particular synthetic auxin is concerned for somatic embryogenesis due to its capability to induce the endogenous auxin. It can act as a stress-inducer for accelerating and triggering the reprogramming mechanism of somatic embryogenesis (Ragavan, 2004). Compared with leaf explant, petiole possessed highest percentage of somatic embryogenesis induction percentage in 2, 4-D medium (Table 2). Significantly maximum 76.4±1.86 % of embryogenic callus was induced at 2.0 $mg \cdot L^{-1}$ 2, 4-D medium. Whereas, very less and non-significant embryogenic callus induction was observed in leaf explants (Table 2). In contrast, leaf explants produced more somatic embryos than the petiole in liquid medium (Park and Chae, 1997). Rhizogenic callus was observed on the 2, 4-D medium along with GA_3 (Fig 2 D and H) and 2, 4-D with both BA and GA_3 proliferated green colored callus (Fig 2C and G). As mentioned earlier, cellular constituents of explants determines its embryogenic potential according to the type/level of PGRs supplemented in the culture medium.

Auxin level is one of the crucial factors for the somatic embryo germination (Thorpe and Stasolla, 2001). Normally, auxin-devoid medium are used for germination of somatic embryos (Jimenez, 2005). Secondary somatic embryogenic structures (data not shown) are observed in callus subcultured in GA_3 medium (Fig 3A). Meanwhile, BA alone produced direct shoot induction (Fig 3B). Analogously, Wang et al. (2008) reported the same effect in *Pogotherum paniceum*. Importantly, half-MS medium supplemented with 1.0 mg L^{-1} BA and 1.0 mg L^{-1} GA_3 developed the somatic embryos i.e., the formation of globular structure is shown in Fig 3C. Similar to *Crocus heuffelianus* (Demeter et al., 2010), bipolar embryos were directly differentiated from the globular stage (Fig 3D). The presence of two meristems such as root and shoot poles (Fig 3D-E) confirmed the somatic embryo structure (Thompson et al., 2001). Addition of GA_3 along with the cytokinin enhanced the embryo maturation and germination (Ali et al., 2010). Development of somatic embryo depends on the fine temporal and spatial regulation of cell division, enlargement, and differentiation by PGR(s) supplemented to the medium (De Garcia et al., 1995).

Furthermore, after embryo maturation in BA and GA_3 medium, incorporation of auxin or cytokinin can interfere with the embryo germination (Deo et al., 2010). Hence, the newly emerged cotyledonary stage plantlets were cultured in full strength-MS medium for rooting (Fig 4A-B).

Acclimatization

After four weeks, well rooted plantlets derived from both organogenesis and somatic embryogenesis were transferred to 50 square plug tray and kept in the growth chamber for hardening. Plants were transferred to 10 cm pots in the greenhouse for acclimatization after emergence of new leaves (Fig 1E and Fig 4C). About 90% of survival rate was achieved after five weeks of acclimatization.

Table 1: Shoot organogenesis from leaf and petiole explants of *Rehmannia glutinosa* in different concentration of BA with combination of 0.5 or 1.0 mg·L^{-1} IAA.

Treatments (mg·L^{-1})		Number of shoots per explant		Shoots induction percentage	
IAA (A)	BA (B)	Leaf	Petiole	Leaf	Petiole
0.5	1.0	2.0±0.31e[z]	1.4±0.24c	52.5±1.11f	40.6±0.40e
	2.0	2.8±0.24cde	2.2±0.20bc	65.0±2.23de	41.8±1.28e
	3.0	4.4±0.30a	2.4±0.24bc	81.8±1.22a	48.4±0.49d
	4.0	3.8±0.20ab	3.2±0.53a	67.0±0.67cd	53.8±0.34cd
	5.0	3.2±0.30bcd	2.9±0.20a	62.0±1.22e	32.8±1.15f
1.0	1.0	2.4±0.24de	1.8±0.37bc	62.0±1.22e	52.0±1.75d
	2.0	3.4±0.74abcd	2.2±0.25c	70.0±1.50bc	58.8±0.50b
	3.0	3.8±0.80ab	2.5±0.27ab	79.6±1.60a	63.8±1.25a
	4.0	3.6±0.65abc	2.8±0.50ab	78.0±2.50a	59.0±1.00b
	5.0	3.5±0.30abc	2.6±0.40ab	72.0±1.25b	56.0±1.5bc
F-test	A	NS	NS	***	***
	B	***	**	***	***
	A x B	NS	NS	**	***

Data are the mean ± SE from three replicates.
[z]Mean separation within columns by Duncan's multiple range test at p=0.05.
NS,*,**,***Nonsignificant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively.

Table 2: Effect of different concentrations of 2,4-D on somatic embryogenesis of *Rehmannia glutinosa* alone or with BA and/or GA_3 in petiole and leaf explants.

Treatments ($mg \cdot L^{-1}$)			Petiole			Leaf		
2,4-D (A)	BA (B)	GA_3 (C)	Embryogenic callus induction (%)	Callus Induction (%)	No. of somatic embryos per explants	Embryogenic callus induction (%)	Callus Induction (%)	No. of somatic embryos per explants
1	0	0	54.8±1.77b[z]	00.0±0.00e	2.2±0.25a	33.34 ±0.00b	00.0±0.00e	01.0±0.00a
	1	0	00.0±0.00e	74.0±1.87c	0.0±0.00d	00.0±0.00d	93.0±1.20b	00.0±0.00c
	1	1	00.0±0.00e	83.7±1.85b	0.0±0.00d	00.0±0.00d	100.0±0.00a	00.0±0.00c
	0	1	38.0±4.00d	52.0±3.70d	1.0±0.00c	12.0±2.00c	38.0±1.20d	00.4±0.40c
2	0	0	76.4±1.86a	00.0±0.00e	3.6±0.30a	67.0±2.50a	00.0±0.00e	01.5±0.50a
	1	0	00.0±0.00e	86.0±0.63b	0.0± .00d	00.0±0.00d	100.0±0.00a	00.0±0.00c
	1	1	00.0±0.00e	100.0±0.00a	0.0± .00d	00.0±0.00d	100.0±0.00a	00.0±0.00c
	0	1	45.0±2.30c	56.0±4.00d	0.5±0.50c	16.0±1.90c	54.0±2.45c	00.0±0.00c

Table 2. Continues

Treatments (mg·L^{-1})			Petiole			Leaf		
2,4-D (A)	**BA (B)**	**GA_3 (C)**	**Embryogenic callus induction (%)**	**Callus Induction (%)**	**No. of somatic embryos per explants**	**Embryogenic callus induction (%)**	**Callus Induction (%)**	**No. of somatic embryos per explants**
	A		***	***	***	***	***	***
	B		***	***	***	***	***	***
	C		***	***	***	***	***	***
F-test	A x B		**	**	**	**	**	**
	B x C		**	**	**	**	**	**
	A x C		***	***	***	***	***	***
	A x B x C		***	***	***	***	***	***

Data are the mean ± SE from three replicates. [z]Mean separation within columns by Duncan's multiple range test at p=0.05. NS,*,**,***Nonsignificant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively.

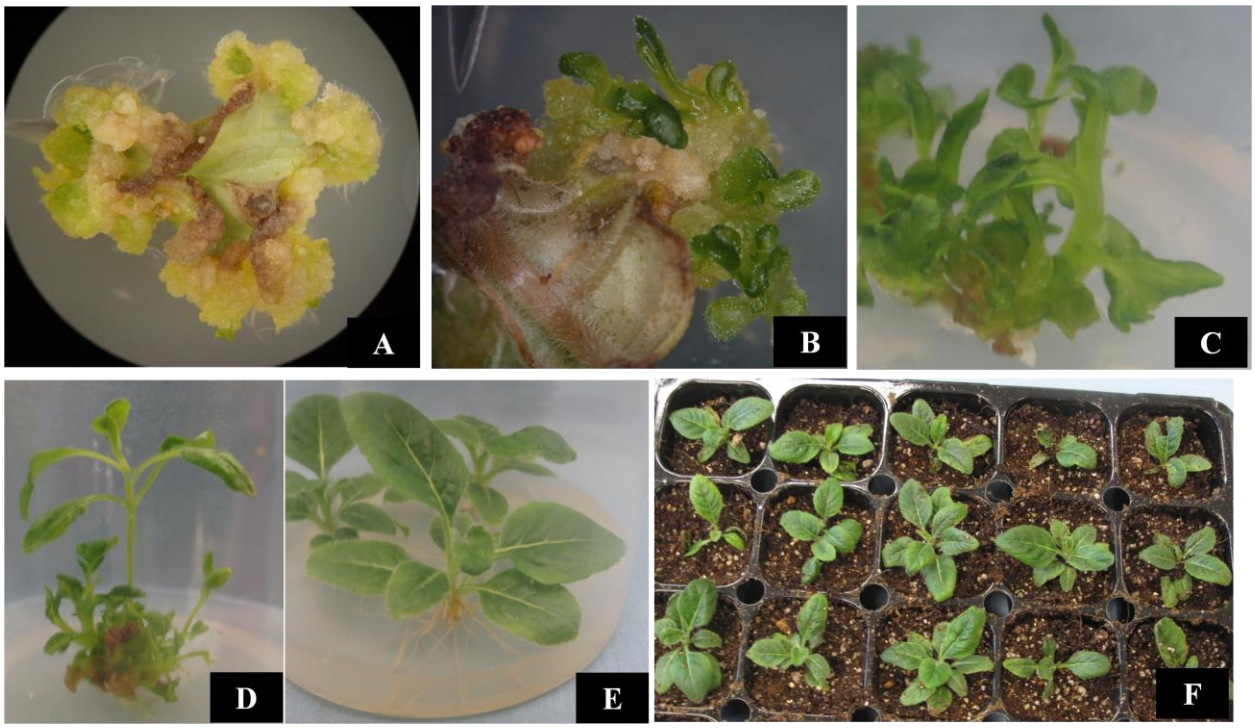

Figure 1. Shoot multiplication and proliferation of *Rehmannia glutinosa* from leaf explants. MS medium supplemented with 1.0 mg·L^{-1} BA and 0.5 mg·L^{-1} IAA, after 3 weeks (A) and five weeks (B), Shoot elongation in 0 (C) and 0.5 mg·L^{-1} GA_3 (D) in MS medium, Shoot development and rooting in MS (E) medium and Acclimatization (F).

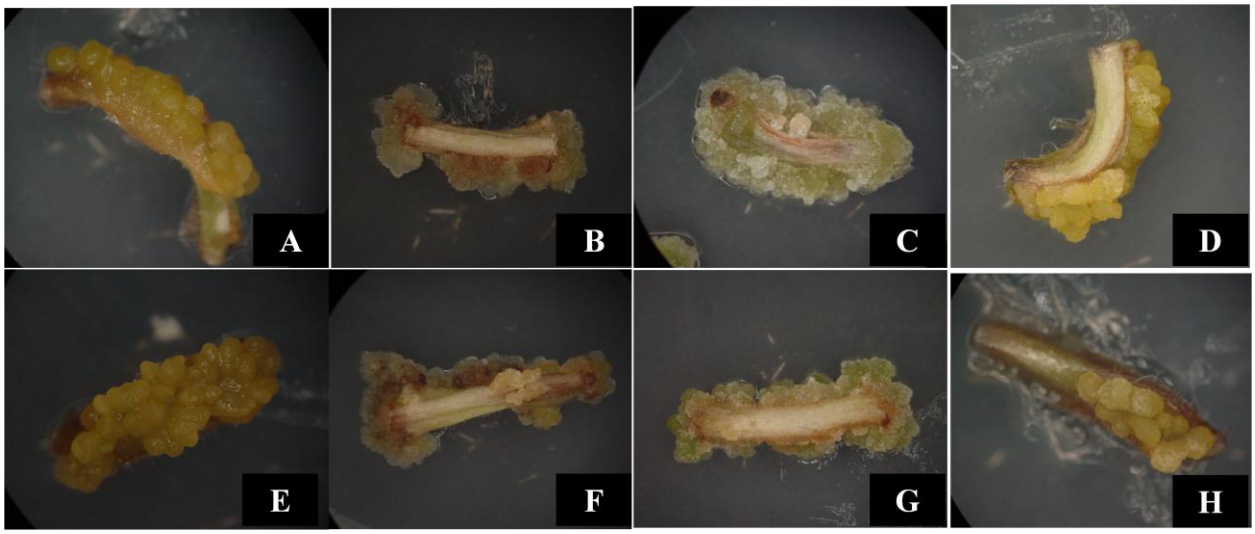

Figure 2. Somatic embryogenesis on petiole explant of *Rehmannia glutinosa*. Somatic embryogenic callus in (A) 1.0 mg·L^{-1} 2, 4-D and (E) 2.0 mg·L^{-1} 2, 4-D. Callus induction in (B) 1.0 mg·L^{-1} 2, 4-D + 1.0 mg·L^{-1} BA, (C) 1.0 mg·L^{-1} 2, 4-D + 1.0 mg·L^{-1} BA + 1.0 mg·L^{-1} GA_3, (F) 2.0 mg·L^{-1} 2, 4-D +1.0 mg·L^{-1} BA, and (G) 2.0 mg·L^{-1} 2, 4-D + 1.0 mg·L^{-1} BA + 1.0 mg·L^{-1} GA_3. Rhizogenic callus induction in (D) 1.0 mg·L^{-1} 2, 4-D + 1.0 mg·L^{-1} GA_3 and (H) 1.0 mg·L^{-1} 2, 4-D + 1.0 mg·L^{-1} GA_3 supplemented on MS medium medium.

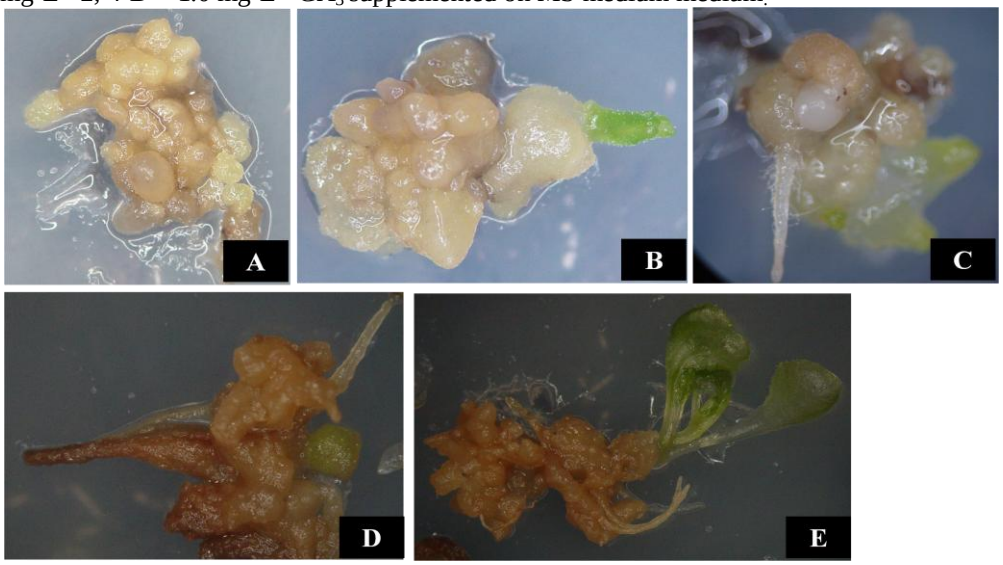

Figure 3. Maturation and germination of somatic embryos of *Rehmannia glutinosa*. Embryogenic callus cultured in half-MS medium supplemented with (A) 1.0 mg·L^{-1} GA_3, (B) 1.0 mg·L^{-1} BA; (C-E) 1.0 mg·L^{-1} GA_3 + 1.0 mg·L^{-1} BA. (A) Secondary somatic embryo-like structure, (B) Direct shoot induction from embryogenic callus. (C) Globular stage, (D) Bipolar-structure and (E) Cotyledonary-stage of somatic embryo.

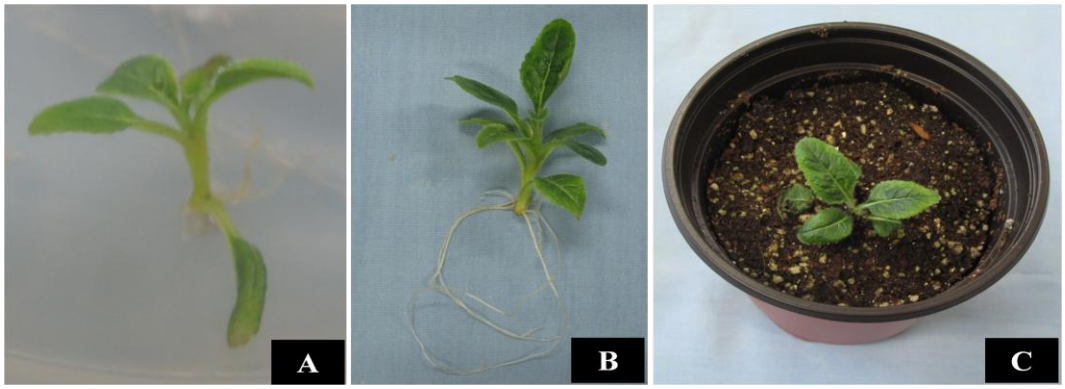

Figure 4. Rooting and acclimatization of somatic embryo derived-plantlets of *Rehmannia glutinosa*. (A) Plantlet in MS medium, (B) Well-rooted, and (C) Acclimatized plantlet.

Conclusion

The established mass propagation protocol can be efficient for the large-scale multiplication of the pharmaceutically important plant, *R. glutinosa*. To best of our knowledge this is the first work describing the adventitious shoot induction from petiole explants and somatic embryogenesis in *R. glutinosa*. The current study can be utilized for further artificial seed production, germplasm conservation and genetic manipulation.

Acknowledgements:

This work was supported by the 'Development of Basic Technologies for the Domestic Cultivation of Herbal Medicine Resources' (K14417) funded by the Ministry of Science, ICT and Future Planning (MSIP) of Korea to the Korea Institute of Oriental Medicines (KIOM). Prabhakaran Soundararajan, Abinaya Manivannan, and Chung Ho Ko were supported by a scholarship from the BK 21 Plus Program, the Ministry of Education, Korea. We would like to thank Dr. Sowbiya Muneer, Institute of Agriculture and Life Science, Gyeongang Naitonal University, Korea on valid discussion for proofreading the manuscript.

References

Albach, D.C., Li, H.Q., Zhao, N. and Jensen, S.R. (2007): Molecular systematics and phytochemistry of *Rehmanni*a (Scrophulariaceae). Biochem. Systematics Ecol., 35: 293-300.

Ali, S.B.G.M., Kourosh, V., Hassan, B.S., Siamak, K. and Charles, L. (2010): Enhancement of maturation and germination of somatic embryos in *Persian walnut* (*Juglans regia* L.) using osmolites, hormones and cold treatments. Afr. J. Food Sci., 12: 735-743.

Beena, M.R., Martin, K.P., Kirti, P.B. and Hariharan, M. (2003): Rapid in vitro propagation of medicinally important Ceropegia candelabrum. Plant Cell Tissue Organ Cult., 72: 285-289.

Bhojwani, S.S. and Razdan, M.K. (1996): Plant tissue culture: Theory and practice. Elsevier, Amsterdam.

De Garcia, E., Martinez, S. and De Garcia, E. (1995): Somatic embryo genesis in *Solanum tuberosum* L. cv. Desiree from stem nodal sections. J. Plant Physiol., 145: 526-530.

Demeter, Z., Gyula, S.V., Molnar, A., Sramko, G., Beyer, D. and Konya, Z. (2010): Somatic embryogenesis and regeneration from shoot primordia of *Crocus heuffelianus*. Plant Cell Tissue Organ Cult., 100: 349-353.

Deo, P.C., Tyagi, A.P., Taylor, M., Harding, R., and Becker, D. (2010). Factors affecting somatic embryogenesis and transformation in modern plant breeding. South Pacific J. Nat. App. Sci. 28: 27-40.

Ghimire, B.K., Seong, E.S., Ki, E.H., Kabir, L., Yu, C.Y. and Chung, I.M. (2010): Direct shoot organogenesis from petiole and leaf discs of *Withania somnifera* L. Dunal. Af. J. Biotechnol., 44: 7453-7461.

Jeong, J.H., Yu, K.W., Chakrabarty, D., Kim, S.J. and Peak, K.Y. (2002): In vitro regeneration and plantlet formation from adventitious roots of *Rehmannia glutinosa* Liboschits. Propag. Ornam. Plants, 2: 19-23.

Jimenez, V.M. (2005): Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regulation, 47: pp. 91-110.

Kong, L. and von Aderkas, P. (2011): A novel method of cryopreservationwithout a cryoprotectant for immature somatic embryos of conifer. Plant Cell Tiss. Organ. Cult., 106: 115-125.

Liu, C.H., Zhang, L.J. and Li, G.S. (2002): Determination of rehmannioside A in root tuber of *Rehmannia glutinosa*. Chinese Traditional Herbal Drugs 33: 706-707.

Liu, H.R., Tang, X.Y., Dai, D.Z. and Dai, Y. (2008): Ethanol extracts of *Rehmannia* complex (Di Huang) containing no *Corni fructus* improve early diabetic nephropathy by combining suppression on the ET-ROS axis with modulate hypoglycemic effect in rats. J. Ethnopharmacol., 118: 466-472.

Marks, T.R. and Simpson, S.E. (1994): Factor affecting shoots development in apically dominant Acer cultivars in vitro. J Hortic Sci., 69:543–551

Murashige, T. and Skoog, F. (1962): A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Paek, K.Y., Yu, K.J., Park, S.I., Sung, N.S. and Park, C.H. (1995): Micropropagation of *Rehmannia glutinosa* as medicinal plant by shoot tip and root segment culture. ISHS international symposium on medicinal and aromatic plants. Acta Hortic., 390:120-123

Parimalan, R., Venugopalan, A., Giridhar, P. and Ravishankar, G.A. (2011): Somatic embryogenesis and *Agrobacterium*-mediated transformationin *Bixa orellana* L. Plant Cell Tiss. Organ. Cult., 105: 317-328.

Park, J.H. and Chae, Y.A. (1997): Effect of growth regulators and explants on direct somatic embryogenesis in liquid culture of *Rehmannia glutinosa*. J. Medicinal Crop Sci. 5: 289-193.

Park, S.U., Kim, Y.K. and Lee, S.Y. (2009): Improved *in-vitro* plant regeneration and micro- propagation of *Rehmannia glutinosa* L. J. Medicinal Plants Res., 3(1): 031-034.

Patnaik, J. and Debata, B.K. (1996): Micropropagation of *Hemidesmus indicus* (L.) R. Br. through axillary bud culture. Plant Cell Rep., 15: 427-430.

Pradeep, C.D., Robert, M.H., Mary, T., Anand, P.T. and Douglas, K.B. (2009): Somatic embryogenesis, organogenesis and plant regeneration in taro (*Colocasia esculenta* var. esculenta). Plant Cell Tiss. Organ. Cult., 99: 61-71.

Ragavan, V. (2004): Role of 2,4-dichlorophenoxyacetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. American J. Bot., 91: 1743-1756.

Ross J.J., O'Neill D.P. and Rathbone, D.A. (2003): Auxin-gibberellin interactions in pea: Integrating the old with the new. J. Plant Growth Regul., 22: 99-108.

Soundararajan, P., Sivanesan, I., Jana, S. and Jeong, B.R. (2014): Influence of silicon supplementation on the growth and tolerance to high temperature in *Salvia splendens.* Hort. Environ. Biotechnol. 55:271-279.

Thompson, D., Harrington, F., Douglas, G., Hennerty, M.J., Nakhshab, N. and Long, R. (2001): Vegetative propagation techniques for oak, ash, sycamore and spruce. COFORD, Dublin.

Thorpe, T.A. and Stasolla, C. (2001): Somatic embryogenesis. In Bhojwani SS, Soh WY (eds) Current trends in the embryology of angiosperms, Dordrecht, Netherlands. Kluwer Academic Publishers, pp. 279-336.

Venkataiah, P. and Subhash, K. (2001): Genotype, explant and medium effects on adventitious shoot bud formation and plant regeneration in *Capsicum annuum* L. J. Genet. Breed., 55: 143-149.

Wang, W., Zhao, X., Zhuang, G., Wang, S. and Chen, F. (2008): Simple hormonal regulation of somatic embryogenesis and/or shoot organogenesis in caryopsis cultures of *Pogonatherum paniceum* (Poaceae). Plant Cell Tiss. Organ. Cult., 95: 57-67.

Zhang, G.J., Ji, J.Y., Liu, Q., Zhang, L.H., Gong, G.Y., Jin, Z.X. and Ren, S.Z. (1993): Collected Edition of Identification and Assessment of Common TCD. Harbin, China. Heilongjiang Science and Technique Press, pp. 301-302.

Zhang, R.X., Li, M.X. and Jia, Z.P. (2008): *Rehmannia glutinosa*: review of botany, chemistry and pharmacology. J. Ethnopharm., 117: 199-214.

Zhou, Y., Gu, F., Zhou, C.H., Yao, H., Duan, H., Wang, F., Liu, Y., Xing, Y. and Chu, S. (2010): Genetic diversity of *Rehmannia glutinosa* cultivars based on sequence-related amplified polymorphism markers. Sci. Hort., 125: 789-794.