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Regeneration of plantlet via somatic embryogenesis from hypocotyls of Tartary Buckwheat (*Fagopyrum tataricum*)

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Abstract

Buckwheat is a well-known minor cereal crops in South Asia. *In vitro* tissue cultures of buckwheat serve as an important mean for its improvement through genetic transformation as well as induced somaclonal variation. An experiment carried out to develop the efficient protocol for callus induction and plantlet regeneration for hypocotyls of tartary buckwheat. Twenty combinations of plant growth regulators (PGRs) such as 2, 4- Dichlorophenoxyacetic acid/ benzyladenine (2, 4-D/BA), 2, 4- Dichlorophenoxyacetic acid/ benzyladenine/ kinetin (2,4-D/BA/KIN), 2, 4- Dichlorophenoxyacetic acid/ 2-isopentenyl adenine/ kinetin (2,4-D/2ip/KIN), 2, 4-Dichlorophenoxyacetic acid/ zeatin (2, 4-D/ZA), 2, 4- Dichlorophenoxyacetic acid/ zeatin riboside/ thidiazuron (2,4-D/ZR, 2,4-D/ZR/TDZ) in MS basal medium were tested on hypocotyls for callus induction under light and dark condition. Callus formation was induced to all combination of PGRs except 2, 4-D 2 mg/L + ZR 1 mg/L + TDZ 2 mg/L under light condition. The best results for callus induction was obtained with 2, 4-D 4 mg/L + BA 1 mg/L under dark and 2,4-D 2.5 mg/L + BA 1.5 mg/L under light condition, which was 86.6 and 90% callus induction rate, respectively. Five combinations of PGRs in MS basal medium were used on somatic embryogenic callus for plantlet regeneration. Somatic embryos resulting regeneration of plantlet were achieved with IAA 1 mg/L + KIN 1 mg/L + BA 2 mg/L + TDZ 0.5 mg/L.

Keywords: Hypocotyls, Plantlet regeneration**,** Somatic embryogenesis, Tartary Buckwheat.
Abbreviations: 2 ip: 2-isopentenyl adenine; 2, 4-D: 2, 4-dichlorophenoxyacetic acid; ANOVA: Analysis of variance; BA: Benzyladenine; KIN: Kinetin; MS: Murashige and Skoog medium; PGR: Plant growth regulators; TDZ: Thidiazuron; ZA: Zeatin; ZR: Zeatin riboside.

Introduction

Buckwheat is gaining all-inclusive importance as a panorama of food crop due to highly nutritive value. Buckwheat is pseudo-cereal crops, which are good source for essential amino acid like lysine, and also has excellent protein quality in terms of essential amino acid composition. It is a multipurpose crop with variety of use such as food, feed, medicine and manure (Dutta et al., 2010). The tender shoots or sprouting are used as leafy vegetables while the flower, and also green leaves are used for the extraction of rutin (Marshall and Pomeranz, 1982), which is prosperous phenolic compound in buckwheat. The grain of tartary buckwheat has a high nutritive value and the flour contains relatively more functional compounds than that of common buckwheat (Bonafaccia et al., 2003). Tartary buckwheat grains were shown have 3-4 times higher anti-oxidative activity than common buckwheat grains, and the rutin content of tartary buckwheat was more than 100 times that of common buckwheat (Morishita et al., 2007). Tartary buckwheat is a wild self pollinating buckwheat species that has greater tolerance to environmental stresses than common buckwheat. Somatic embryogenesis is a procedure whereby a cell or group of cell from somatic tissue forms an embryo. The expansion of somatic embryos nearly replicates the process of zygotic embryo formation. Somatic embryo-genesis mostly occurs indirectly via a superseding callus phase or directly from early explants. Nowadays, there is a growing interest in tartary buckwheat biotechnology because *in vitro* techniques can provide the means for obtaining somaclones, induced variants, and genetically transformed plants, which can be used both in breeding and in biotechnological production of bioactive compounds. Somatic embryos have also proved to be excellent material for genetic transformation studies due to their competency in expressing incorporated DNA. The regeneration system is of

Table 1. Effect of various combinations of plant growth regulators on callus induction of hypocotyls of tartary buckwheat under dark and light condition after 2 weeks.

Treatment	Plant growth regulators	Mean ± SE (dark)	Mean ± SE (light)
T1	2, 4-D 1 mg/L + BA 0.1 mg/L	3.33 ± 0.33^{c}	
T2	2, 4-D 2 mg/L + BA 0.1 mg/L	4.00 ± 0.57^{c}	
T3	2, 4-D 4 mg/L + BA 0.1 mg/L	1.00 ± 0.57^{d}	
T4	2, 4-D 8 mg/L + BA 0.1 mg/L	1.33 ± 0.33^{dc}	
T5	2, 4-D 1 mg/L + BA 1 mg/L	6.66 ± 0.66^{b}	
T6	2, 4-D 2 mg/L + BA 1 mg/L	8.33 ± 0.33^{ab}	
T7	2, 4-D 4 mg/L + BA 1 mg/L	8.66 ± 0.88^{a}	
T8	2, 4-D 8 mg/L + BA 1 mg/L	2.66 ± 0.88^{cde}	
T9	2,4-D 2 mg/L +BA 1 mg/L	4.00 ± 0.57^{c}	4.00 ± 0.57^{cf}
T10	2,4-D 2 mg/L + BA 1 mg/L + KIN 0.2 mg/L	7.00 ± 0.00^{ab}	7.00 ± 0.57^{bc}
T11	2,4-D 2 mg/L +BA 1 mg/L + 2iP 10 mg/L	4.00 ± 0.57^{c}	2.66 ± 0.33^{fg}
T12	2,4-D 2 mg/L + 2iP 10 mg/L + KIN 0.2 mg/L	3.00 ± 0.57^{cd}	8.66 ± 0.33^{ab}
T13	2,4-D 2 mg/L + Zeatin 1 mg/L	8.00 ± 0.57^{ab}	7.33 ± 0.66^{abc}
T14	2,4-D 2 mg/L + Zeatin riboside (ZR) 1 mg/L	4.00 ± 0.33^{c}	2.00 ± 0.57^{g}
T15	2,4-D 2 mg/L + ZR 1 mg/L + TDZ 2 mg/L	3.00 ± 0.57^{cd}	0.00^{h}
T16	2,4-D 2 mg/L + BA 1.5 mg/L	3.00 ± 0.58^{cd}	7.00 ± 0.57^{bc}
T17	2,4-D 2 mg/L + BA 2 mg/L	4.00 ± 0.57^{c}	4.66 ± 0.33^{dc}
T18	2,4-D 1.5 mg/L + BA 2 mg/L	8.00 ± 0.58^{ab}	8.00 ± 0.57^{ab}
T19	2,4-D 2.5 mg/L + BA 1.5 mg/L	7.00 ± 0.58^{ab}	9.00 ± 0.57^{a}
T20	2,4-D 3 mg/L + BA 1 mg/L	7.00 ± 0.57^{ab}	6.00 ± 1.15^{cd}

ANOVA summary table

	Sources	df	Mean Square	F
Dark	Plant growth regulators	19	17.575	18.181*
	Error	40	.967	
	Total	59		
	Sources	df	Mean Square	F
Light	Plant growth regulators	11	24.444	24.444*
	Error	24	1.000	
	Total	35		

Values represent means ± SE of 10 segment of hypocotyls per treatment in three repeated experiments, Means in each column followed by same letters are not significantly different according to DMRT at α=0.05, * Significant at 5% probability level (*F* test).

prime importance for genetic transformation (Farzana et al., 2008). In our study, the effect of plant growth regulators on the culture of hypocotyls of tartary buckwheat was investigated, and plantlets were regenerated from hypocotyls directly as well as from calli.

Results and discussion

Embryogenesis induction

Explants which developed on free MS hormone medium did not exhibit any growth. They became brown and necrosed after a few days of culture. On medium containing growth regulators, explants after 2 weeks of culture formed calluses differing by their coloration and aspect. Most of them were whitish and compact but not embryonic. Some of them were yellowish which is more friable. They continue to grow and did not exhibit any neoformation, when transferred into fresh free growth regulator medium or into medium containing sorts of the growth regulators concentration. After 2 weeks, some of calluses were whitish to yellowish, friable and showed at their surface globular yellow light structures which developed into somatic embryos. In embryogenesis stages, calluses were showed as globular, torpedo (Fig. 1). In buckwheat, somatic embryos were get hold of using immature embryos (Neskovic et al., 1987, Rumyantseva et al., 1989), protoplasts (Adachi et al., 1989), cotyledons (Woo et al., 2000), and young seedling hypocotyls (Lachmann et al., 1991) as primary explants in common buckwheat. Above all cases of indirect somatic embryogenesis, when somatic embryos enlarge from callus cells formed from the primary explants. As compared to the direct embryo induction in the explants tissues, the indirect somatic embryogenesis takes much longer period for the induction and development of embryos and subsequent plant regeneration in buckwheat (Lachmann et al., 1991). The indirect embryogenesis also includes the development of somatic embryos via the stage of the development of pro-embryogenic cell complexes (PECC). PECC develop both in callus tissues and directly in the explants tissues (Gumerova et al., 2003). In our study, we used 20 different combinations of growth regulators with MS medium. Most of them treatment exhibit the callus except T_{15} under light condition. Under dark condition, some hormonal combinations were exhibited highly callus formed. T_7 were exhibited highest callus percentage (86.6%) followed by 83.3% in T6; 80% in $T_{13,18}$; 70% in $T_{10,19,20}$; 66.6% in T_5 ; 40% in $T_{2, 9, 11, 14, 17}$; 33.3% in T_1 ; 30% in $T_{12, 15, 16}$; 26.6% in T_8 ; 13.3% in T_4 ; 10% in T_3 (Table 1, Fig. 1 and 2). Under the light condition, T_{19} were showed highest callus induction (90%) followed by 86.6% in T_{12}; 80% in T_{18}; 73.3% in T_{13}; 70% in $T_{10, 16}$; 60% in T_{20}; 46.6% in T_{17}; 40% in T_9; 26.6% in T_{11}; 20% in T_{14} (Table 1, Fig. 1 and 2). Interestingly, T_{15} were not exhibited any callus under the light condition. Compared between light and dark condition, T_{19} (2, 4-D 2.5 mg/L + BA 1.5 mg/L) were showed excellent performance

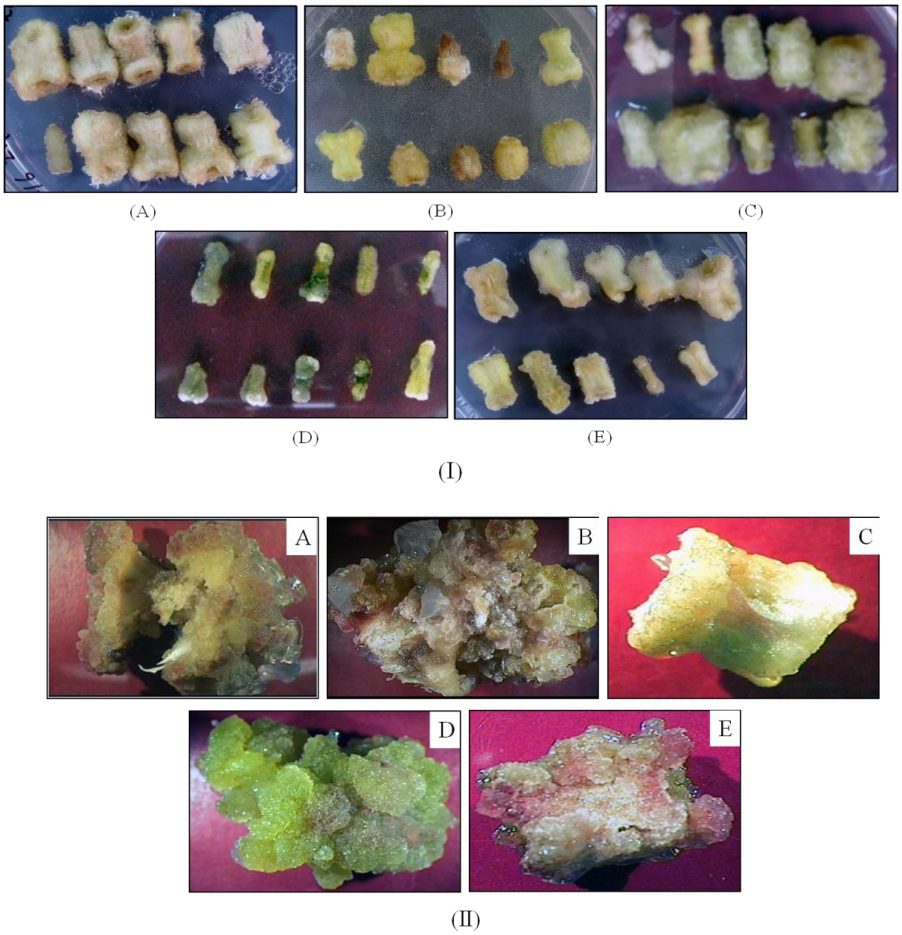

Fig 1. Callus initiation from segments of hypocotyls of tartary buckwheat at (A) 2, 4-D 4 mg/L + BA 1 mg/L under dark, (B) 2,4-D 2 mg/L + 2iP 10 mg/L + KIN 0.2 mg/L under light, (C) 2,4-D 2 mg/L + Zeatin 1 mg/L under dark, (D) 2,4-D 1.5 mg/L + BA 2 mg/L under dark, (E) 2,4-D 2.5 mg/L + BA 1.5 mg/L under light condition after 2 weeks (I), and after 4 weeks (II).

for embryogenic callus induction. Different concentration of MS supplemented hormone such as 2, 4-D, KIN, and BA showed good callus formation in cotyledon of common buckwheat, which is similar to our results (Woo et al., 2000).

Influence of Growth Hormone

Explants as hypocotyls were culture on MS basal medium containing different auxin and cytokinin combinations for callus induction (Table 1). Nineteen and twenty treatment out of 20 was exhibited callus under light and dark condition couple to different supplemental growth regulators, respectively. In our experiment, 2, 4-D and BA is the most common growth hormone as used. Different combinations of 2, 4-D and BA were showed various results, which is resulted different callus percentage. According to our finding, 2, 4-D showed vital responsibility with BA for callus induction. 2, 4-D increased the number of explants which caused bud forming and embryogenic tissue, but was not essential for morphogenesis (Neskovic et al., 1987). Auxins, in particular 2, 4-D, are known to promote the induction of somatic embryogenesis, whereas embryo development proceeds on the hormone depleted or hormone-free medium (Steward et al., 1970). The cytokinin as BA requirement for somatic embryo development is known to be species specific (Kohlenbach et al., 1978). Cytokinins are important for accelerating the maturation of somatic embryos and especially for cotyledon development (Ammirato et al., 1983). Whereas, KIN and 2 ip with 2, 4-D were exhibited good performance for callus induction between ZR and TDZ under light condition. In addition, zeatin and 2, 4-D was demonstrated good performance for callus induction under dark condition with BA (Table 1, Fig. 1).

Plantlet Regeneration

For regeneration of plantlet, we selected some high exhibited calluses, which are influenced by different hormone combinations. Calluses from the combinations of T_7 (2, 4-D 4 mg/L + BA 1 mg/L), T_{12} (2,4-D 2 mg/L + 2iP 10 mg/L + KIN 0.2 mg/L) under light, T_{13} (2,4-D 2 mg/L + Zeatin 1 mg/L) under dark, T_{18} (2,4-D 1.5 mg/L + BA 2 mg/L) under dark, and T_{19} (2,4-D 2.5 mg/L + BA 1.5 mg/L) under light condition were transferred into MS basal medium with different hormone concentration and combinations for plantlet regeneration under light conditions. Among those calluses, IAA 0.1 mg/L+ KIN 1 mg/L + BA 2 mg/L + TDZ 0.5 mg/L were induced superior shoot and root under light condition (Fig. 3) followed by IAA 2 mg/L+ KIN 1 mg/L + BA 1 mg/L, IAA 1 mg/L+ KIN 1 mg/L + BA 5 mg/L, respectively. Additionally, IAA 2 mg/L + BA 1 mg/L, IAA 2 mg/L+ BA 5 mg/L were not induced any shoot and root under light condition (Table 2). When medium contained sucrose alone, kinetin significantly stimulated subsequent plant regeneration in rice. Because kinetin did not necessarily benefit both the quantity and regenerative capacity for callus, the decision to include kinetin is based on genotype and the culture purpose, which is massive callus or regenerative callus (Al-Khayr et al., 1996). Combination with 2, 4-D IAA, BA, and 2ip were displayed good performance for plant

Table 2. Effect of different concentration of plant growth regulators for plantlet regeneration from callus of hypocotyls of tartary buckwheat after 8 weeks on MS medium.

IAA (mg/L)	KIN (mg/L)	BA (mg/L)	TDZ (mg/L)	Plantlet (%)
2.0	0	1.0	0	–
2.0	0	5.0	0	–
2.0	1.0	1.0	0	++
2.0	1.0	5.0	0	+
0.1	1.0	2.0	0.5	+++

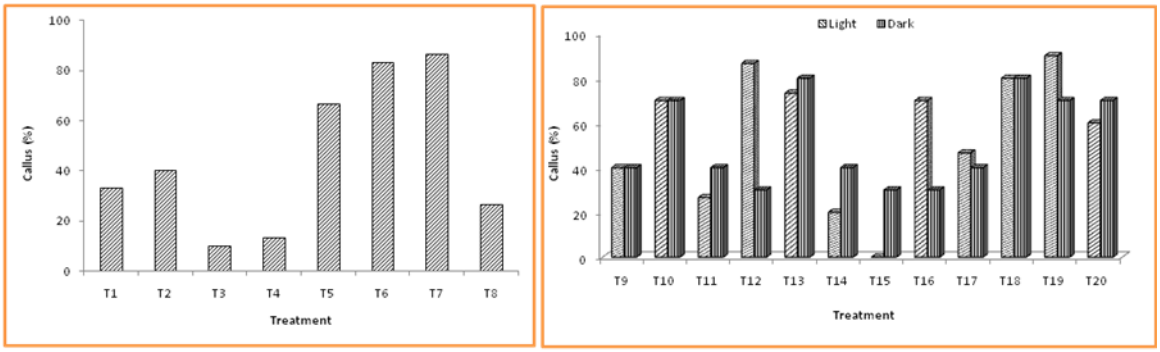

Fig 2. Effects of plant growth regulators on callus percentage from hypocotyls of tartary buckwheat under (A) dark, and (B) light and dark condition.

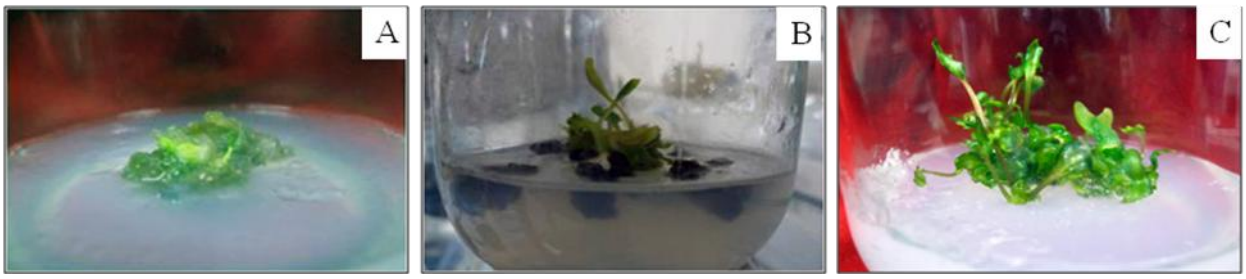

Fig 3. Regeneration of plantlet from callus of hypocotyls of tartary buckwheat after (A) 6 weeks, (B) 8 weeks, and (C) 10 weeks.

regeneration in wheat (Rashid et al., 2009). Various concentrations of BA and KIN has been shown efficient performance in shoot organogenesis in common buckwheat (Lee et al., 2009, Woo et al., 2000), which is proved our findings. MS basal medium containing different concentration of BA, KIN and TDZ has been displayed plantlet regeneration in common buckwheat (Park et al., 2001).

Materials and methods

Plant Materials

Seeds of Tatary buckwheat (*Fagopyrum tataricum*) were collected from the field at Chungbuk National University, Cheong-ju 361-763, Korea. Plants had been grown during the summer when the temperature was 28 to 30°C. The physiology of this species is timed so that plants will bloom and set seed when the hot, dry weather is over. Seeds were harvested and stored at 10°C. An experiment of somatic embryogenesis was conducted in the Laboratory of Biotechnology, Chungbuk Agricultural Research and Extension Services, Chungcheongbuk-do 363-883, Korea. Surface sterilization of mature seeds was carried out under laminar air flow cabinet. Seeds coats were removed by surgical blades then sterilized with 70% ethanol (v/v) for 5 min followed by three times washed for 5 min each in sterile distilled water. Further, washed seeds were transferred to 0.3% (w/v) sodium hypo-chloride solution containing a drop of Tween 20 detergent for 30 min followed by five times washed for 3 min each in sterile distilled water.

Seed Germination

Seeds were germinated on half-strength MS medium (Murashige and Skoog, 1962). The medium contained sucrose which is solidified with 5.5% agar (Sigma Chemical Co.,). The pH of the medium was adjusted to 5.8 with 1 M NaOH before autoclaving at 121ºC for 20 min. Seeds were germinated in Flux containing 10 ml of the half-strength MS medium. Seeds were incubated in light at 25±2ºC for 7 days.

Callus Initiation and Plantlet Regeneration

The 7-day-old seedlings were removed from the culture medium and transversal. Hypocotyls were obtained from germinated seedlings by cutting with surgical blades into 2-3 cm. Ten hypocotyls sections were placed in 20 differential combinations of auxin and cytokinin concentration in

medium supplanted with MS salts (Murashige and Skoog 1962), myo-inositol (100 mg/L), sucrose (3%), and different combinations of benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (KIN), 2-isopentenyl adenine (2 ip), zeatin, zeatin riboside (ZR), thidiazuron (TDZ) (Table 1). Cultures were incubated in the dark and light (~1200 lux) at 25±2ºC and sub-cultured onto fresh medium at every 2 weeks for callus initiation. For the plantlet regeneration, small fragment of friable callus transferred to MS basal medium contained supplemented indo-acetic acid (IAA), KIN, BA, and TDZ (Table 2). For plantlet regeneration, a culture with friable callus was incubated in the light (~1200 lux) at 25±2ºC for 3 weeks.

Statistical Analysis

All experiments were set up in completely randomized block design and repeated 3 times with 10 replicates per treatments. Statistical difference among the means was analyzed by Duncan's multiple range test using PASW ver. 17 (SPSS inc.,). The results were expressed as the means ± standard error (SE) of three independent experiments. Data were also subjected to analysis of variance (ANOVA).

Conclusion

In our best knowledge, this is first somatic embryogenesis reports of tartary buckwheat from hypocotyls in this world. Differentiation of adventitious bud requires relatively high level 2, 4-D and it should be combined with the application of BA for callus formation. These experiments demonstrate that plant regeneration from hypocotyls of tartary buckwheat can be obtained enthusiastically and consistently at a moderate frequency. The fabrication of buckwheat regenerated plants could be used as a possible transformation protocol will creates new opportunities to study the molecular and metabolic regulation of useful compounds in tartary buckwheat.

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References

Adachi T, Yamaguchi A, Miike Y, Hoffmann F (1989) Plant Regeneration from Protoplasts of Common Buckwheat (*Fagopyrum esculentum*). Plant Cell Rep 8:247-250.

Al-khayri JM, Shamblin CE, Mcnew RW, Anderson EJ (1996) Callus induction and plant regeneration of U.S. Rice genotypes as affected by medium constituents. In Vitro Cell Dev Biol-Plant. 32:227-232.

Ammirato PV (1983) Embryogenesis, *Handbook of Plant Cell Culture*, vol. 1, Techniques for Propagation and Breeding, Evans, D.A. et al., Eds., New York: Macmillan, p 82-123

Bonafaccia G, Marocchini M, Kreft I (2003) Composition and technological properties of the flour and bran from common and tartary buckwheat. Food Chem 80:9-15.

Dutta MNM, Shah S, Kumar P (2010) Assessment of agro-morphological. Physiological and genetic diversity among buckwheat cultivars. Proc of the 11th intl. sym. on Buckwheat, Orel, Russia, p 94-101.

Farzana ARF, Palkadapala PGVN, Meddegoda KMMN, Samarajeewa PK, Eeswara JP (2008) Somatic embryogenesis in papaya (*Carica papaya* L. cv. Rathna). J Nat Sci Found Sri Lanka 36:41-50.

Gumerova EA, Galeeva EI, Chuyenkova SA, Rumyantseva NI (2003) Somatic embryogenesis and bud formation on cultured *Fagopyrum esculentum* hypocotyls. Rus J Plant Physiol 50: 640-645.

Kohlenbach HW (1978) Comparative somatic embryogenesis. In: Thorpe TA (Ed) Frontiers of Plant Tissue Cultures, Calgary Univ Press, Calgary

Lachmann S (1991) Plant Cell and Tissue Culture in Buckwheat: An Approach towards Genetic Improvements by Means of Unconventional Breeding Techniques. Proc ICOBB, Miyazaki, p 145-154.

Lee SY, Kim YK, Uddin MR, Park NI, Park SU (2009) An efficient protocol for shoot organogenesis and plant regeneration of buckwheat (*Fagopyrum esculentum* Moench.). Roman Biotech Lett 14:4524-4529.

Marshall HG, Pomeranz Y (1982) Buckwheat description, breeding, production and utilization. In. Y. Pomeranz (ed) Advances in cereal science and technology. Am Assoc Cereal Chem St Paul, MN, p 157-212.

Morishita T, Yamaguchi H, Degi K (2007) The Contribution of Polyphenols to Antioxidative Activity in Common Buckwheat and Tartary Buckwheat Grain. Plant Prod Sci 10:99-104.

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

Neskovic M, Vujicic R, Budimir S (1987) Somatic embryogenesis and bud formation from immature embryos of buckwheat (*Fagopyrum esculentum* Moench.). Plant Cell Rep 6:423-426.

Park SU, Park CH (2001) Multiple shoot organogenesis and plant regeneration from cotyledons of buckwheat *(Fagopyrum esculentum* Moench.) The proc of the 8th ISB, p 427-430

Rashid U, Ali S, Ali GM, Ayub N, Masood MS (2009) Establishment of an efficient callus induction and plant regeneration system in Pakistani wheat (*Triticum aestivum)* cultivars. Elec J Biotech 12:1-12.

Rumyantseva NI, Sergeeva NV, Khakimova LE, Salnikov VV, Gumerova EV, Lozovaya VV (1989) Organogenesis and somatic embryogenesis in tissue culture of two buckwheat species. Fiziol Rast 36:187-194 (in Russian).

Steward FC, Ammirato PV, Mapes MO (1970) Growth and development of totipotent cells. some problems, procedures and perspectives. Ann Bot 34:761-787.

Woo SH, Nair A, Adachi T, Campbell C (2000) Plant regeneration from cotyledon tissue of common buckwheat (*Fagopyrum esculentum* Moench.). In Vitro Cell Dev Biol 36:358-361.