Regular Article
pISSN: 2288-9744, eISSN: 2288-9752
Journal of Forest and Environmental Science
Vol. 35, No. 1, pp. 41-46, March, 2019
https://doi.org/10.7747/JFES.2019.35.1.41

JFES
Journal of Forest and Environmental Science

Organogenesis from Callus Derived from *In Vitro* Root Tissues of Wild *Prunus yedoensis* Matsumura

Eun Ju Cheong*
Division of Forest Science, College of Forest and Environmental Science, Kangwon National University, Chuncheon 24341, Republic of Korea

Abstract

In vitro organogenesis system of the valuable ornamental species, *Prunus yedoensis* which is native to Korea, was established through callus culture derived from root tissues. Callus were induced on the medium supplemented with 2,4-D and BA or NAA and kinetin. Organogenesis was differ from the callus type, and NAA and kinetin combination was effective to induce organogenic callus. Growth of callus was influenced by sucrose concentrations. High level of sucrose (over 5%) had adverse effects such as decreased fresh weight and increased mortality of callus. Shoots developed from the callus when GA_3 was treated with BA in the medium. Results showed that GA_3 is essential for shoot development and elongation from callus in this species.

Key Words: *Prunus yedoensis*, callus culture, organogenesis, shoot development, root tissue

Introduction

In Korea, *Prunus* species are valued for their ornamental characteristics, as well as for timber and fruit. There are approximately 13 *Prunus* species native to Korea (Kim 1998). *Prunus yedoensis* Matsumura, which is native to the Jeju (Mt. Halla) region, is widely planted in parks and roadsides for its beautiful flowers in spring. The demand for this species has increased and become a most popular trees planted in landscape in many cities for years (Korea Forest Service 2012). Jinhae city is famous for the cherry blossom festival and planted thousands of trees all around the city. Most of trees were propagated by grafting and the donor plant is one or two clones of cultivated tree. There is an advantage using a clone to synchronize the flowering time. However, almost planted trees whether the old or young were infected by viruses that may cause problems of tree growth and longevity. These trees should be replaced with virus-free trees in the near future (Cheong et al. 2015). As the demand of the species increases, efficient mass propagation method beyond the conventional methods should be developed. Fortunately some wild *P. yedoensis* in natural habitats, Jeju are virus-free and have as good flowering characteristics as cultivated ones (Cheong et al. 2015). However, it is very difficult to apply *in vitro* technique to wild *P. yedoensis* because the old trees have less ability of organogenesis than young ones that result low efficiencies of micro-propagation even in *in vitro* culture (Lardet et al. 2009). In previous study of *P. yedoensis*, the physiological barrier could be overcome by tissue culture method (Cheong et al. 2000). The method could produce a lot more plants than cuttings or grafting, nevertheless it still need to

Received: November 21, 2018. Revised: January 2, 2019. Accepted: January 23, 2019.

Corresponding author: Eun Ju Cheong
Division of Forest Science, College of Forest and Environmental Science, Kangwon National University, Chuncheon 24341, Republic of Korea
Tel: 82-33-250-8312, Fax: 82-33-259-5617, E-mail: ejcheong@kangwon.ac.kr

be improved. Callus culture which proceed organogenesis or somatic embryogenesis is one of efficient propagation method and it is even better for mass production. Some commercially important coniferous species were extensively were target of somatic embryogenesis (Salaj et al. 2015). Immature embryos were mostly used in both conifers and deciduous (Koh et al. 1997; Tsukamoto et al. 2007; Salaj et al. 2015). For higher induction rate of somatic embryos, the collection time of seed, i.e. the development stages of seeds, was critical (Kim et al. 2007) and even the age of seed source tree also affected the embryogenesis efficiency (Lardet et al. 2009). Due to this reason, it is very time and physiological status restricted in somatic embryogenesis and there were very few researches on somatic embryogenesis from vegetative tissues. In case of flowering cherry, however, it is very important to maintain the true-to-type characteristics of donor plants for simultaneous flowering and uniform shape of trees. In this regard, organogenesis or somatic embryogenesis using vegetative tissues is proper than using immature seeds. In addition, the valuable individual cherry tree is infected with virus and it needs to be eliminated for healthy virus-free plants. Organogenesis from callus or somatic embryos would give virus-free plants even when the donor tree is infected with the virus because the plants arose from single cell which may virus-free (Wang and Hu 1980). In many aspects, organogenesis or somatic embryogenesis form vegetative tissues is necessary in this species. There were few report of somatic embryogenesis from vegetative tissues in *Prunus* species (Druart 1981; Cheong and Pooler 2004). *In vitro* induced root tissues of *P. yedoensis* were cultivated in for somatic embryogenesis and the optimum conditions were investigated in this study.

Materials and Methods

Plant materials

Initial cultures were established using winter buds from wild *P. yedoensis* trees (ca 140 years old) growing in Mt. Halla. Multiple shoots were induced from the apical and axillary buds in WPM medium (WPM, Lloyd and McCown 1980) supplemented with 10 μM of gibberellic acid (GA_3) and 4 μM of 6-benzylaminopurine (BA) and *in vitro* shoots were transferred to 1/10 concentrations of plant hormones (1 μM of GA_3 and 0.4 μM of BA) for root induction. Roots were induced within 2 weeks and roots were collected from the shoots. Root tips were remove to prevent further root production or elongation and explants were cut 1 cm in length.

Media and plant growth regulators

Callus was induced from the root tissues on MS medium (Murashige and Skoog 1962) supplemented with 10 μM 2,4-D and 0.4 μM of BA. Various concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) (5, 10 and 15 μM) alone or with 0.4 μM of BA, and 1-Naphthaleneacetic acid (NAA) (5, 10 and 15 μM) alone or with 0.4 μM of kinetin in MS medium with 30g/L of sucrose. Friable and granular callus were selected and propagated in MS medium supplemented with 15 μM of NAA and 0.4 μM of kinetin with different concentration of sucrose (10, 20, 30, 40, 50 and 100 g/L). Callus were transferred to a MS medium with two cytokinins (BA and zeatin) and GA_3 alone in different concentrations (5, 10, 15, 20 and 25 μM) each or combination of BA and GA_3 (10 μM of GA_3 and 2 μM of BA, 5 μM of GA_3 and 1 μM of BA, or 1 μM of GA_3 and 0.2 μM of BA) which were the most effective for shoot multiplication with 30 g/L of sucrose in bud culture. Media were adjusted to pH 5.7 before autoclaving and solidified 2 g/L of gel-rite. 20 mL of media were dispensed in petri dishes (9 cm in diameter) in laminar hood. Cultures were conducted under 16 hour-photoperiod of fluorescent light and 23±2°C in all experiments. Five explants (root tissues in callus induction and 6 g of callus in callus culture) were placed in each treatment and replicated five times. Callus formation, root formation, fresh weight of callus and shoot regeneration within 5 weeks of culture. Data analyzed by the Duncan's multiple test ($p=0.05$) using SPSS (ver. 18).

Results and Discussion

Callus induction

In culture to induce callus from root, root tissues were placed on MS medium containing 2,4-D and BA. Callus were induced within 3-4 weeks of culture. It was hard to determine that the callus is embryogenic or organogenic because the induced callus had no appearances such as white

and granular. Most of callus colored yellowish white, friable and some are very wet especially in the medium with 2,4-D only. In order to induce the organogenic callus, callus transferred in different combinations of auxins and cytokinins and different concentrations. The responses to the plant growth regulators of callus were assessed by measuring fresh weight and root formation (Table 1). Among the tested PGRs, roots were induced on single use of auxins, both 2,4-D and NAA, along with the callus. However, appearance of callus grown on medium containing NAA were different from the one with 2,4-D. Callus have less water and small granules (cells) on the surface although root induction rate was still high on 15 μM of NAA regardless of the existence of kinetin. Callus grown on medium containing 2,4-D are heavier in fresh weight but it resulted from the moisture in callus. It is considered that callus grown on NAA containing medium had characteristics of embryogenic or organogenic callus. The auxin, 2,4-D, is the mostly used PGR for somatic embryogenesis from immature embryos in many species (Salaj et al. 2015). It was also used in the experiment of somatic embryogenesis in *Prunus* species (Druart 1981; Koh et al. 1997; Cheong and Pooler 2004). However, NAA was effective on inducing embryogenic callus in this study. Moreover, combination with low concentration of kinetin increased the embryogenic callus formation. Based on the observation of the callus grown on the medium with 2,4-D at initial induction stages from root tissues, it was not effective PGR for somatic embryogenesis through root culture in this species. Compared to other *Prunus* species, the process of embryogenesis was different from this study. While somatic embryos were directly induced from the root tissues in *Prunus incisa* cultivars (Druart 1981; Cheong and Pooler 2004), any somatic embryos were developed from the root tissues. The appearances of callus were changed after the subcultures on the medium containing NAA and kinetin from 2,4-D medium. Generally auxins stimulate cell division and differentiation in somatic embryogenesis and somatic embryogenesis is prohibited when auxin inhibiters were treated in coniferous species (Vondráková et al. 2011) although there was no report on the specificity of auxins in somatic embryogenesis. It was an important finding that this species responded better to NAA not 2,4-D to induce embryogenesis or organogenesis.

Effect of sucrose on embryogenic callus growth

Somatic embryogenesis is needed very delicate conditions in all stages, initiation, development, maturation and germination. Among these conditions, exogenous carbon sources also play a critical role in many species, conifers (Lipavská and Konrádová 2004), herbaceous plants (Karami et al. 2006), grape (Compton and Gray 1996) and *Prunus* species (Cheong and Pooler 2004). Sucrose was the most frequent carbon source in somatic embryogenesis and

Table 1. Callus formation, fresh weight of callus and root formation rate on different combination of PGRs[1]

PGR Concentration (μM)	Callus formation (%)	Fresh Weight (mg)	Root formation (%)
NAA 5	70.0	24.6 ± 2.5e[2]	11
NAA 10	93.3	54.9 ± 2.2d	37
NAA 15	90.0	85.9 ± 11.6b	83
NAA 5+kinetin 0.5	73.3	43.4 ± 10.8	0
NAA 10+kinetin 0.5	96.7	50.1 ± 1.9d	7
NAA 15+kinetin 0.5	96.7	96.1 ± 26.1b	70
2,4-D 1	60	69.8 ± 25.4c	60
2,4-D 5	70	80.4 ± 17.9bc	70
2,4-D 10	90	110.0 ± 24.6b	100
2,4-D 1+BA 0.5	100	85.2 ± 35.2b	10
2,4-D 5+BA 0.5	100	130.4 ± 81.4a	20
2,4-D 10+BA 0.5	100	161.2 ± 72.2a	40

Culture period was 5 weeks under 16 h photoperiods.
[1]Plant Growth Regulator; [2]Same letters are not significantly different at p=0.05 by Duncan test.

other monosaccharide such as fructose and glucose had an effect on embryogenesis. Various concentrations of sucrose on the optimum PGRs condition (NAA 15 μM and kinetin 0.5 μM) were tested to improve the capability of organogenesis from callus. Fresh weight of callus were significantly influenced by the sucrose concentration (Table 2). Fresh weight was decreased as high as 50 g/L and the fresh weight of callus on 100 g/L sucrose was lower than that on all other medium even a medium without sucrose. More critical effect of sucrose was mortality of callus (Fig. 1). This indicated that there was a detrimental osmotic effect by high concentration of sucrose. More than half of callus turned dark in color, cell clusters became small and consequently died. Optimum concentration of sucrose ranges from 20 to 30 g/L but statistically 30 g/L showed a maximum growth without dead callus. The effect of sucrose on organogenesis and maturation stage of embryogenesis is very species or cultivar dependent. Some coniferous species required high concentration of sucrose for somatic embryogenesis for osmotic activities as well as differentiation signaling (Lipavská and Konrádová 2004). In case of *Solanum* species, somatic embryos developed at only 90 g/L of sucrose in medium (Criollo et al. 2014). However, excessive sucrose inhibited somatic embryogenesis and cell division in this species, and the result was similar to other *Prunus* species (Cheong and Pooler 2004; Tsukamoto et al. 2007). Exogenous sucrose had an effect on organogenesis such as shoot or root induction. Brar et al. (2015) reported the low concentration (1-2%) was optimum to induce shoot but 5% sucrose retarded callus growth and shoot induction in bamboo.

Table 2. Effect of sucrose on embryogenic callus growth

Sucrose (mg/L)	Fresh weight (mg)	Growth[1]	Mortality (%)
0	106.4 ± 15.9c[2]	+	28.0 ± 22.8
10	165.9 ± 27.7c	++	0
20	581.7 ± 70.6b	+++	0
30	493.7 ± 183.3a	++++	0
50	261.1 ± 68.2b	++	20.0 ± 16.3
100	99.5 ± 17.8c	+	56.0 ± 16.7

[1]+, very poor; ++, fair; +++, good; ++++, excellent.
[2]Same letters are not significantly different at p=0.05 by Duncan test.

Organogenesis from callus

Friable and granular callus were transferred to media with different PGRs and concentrations for organogenesis.

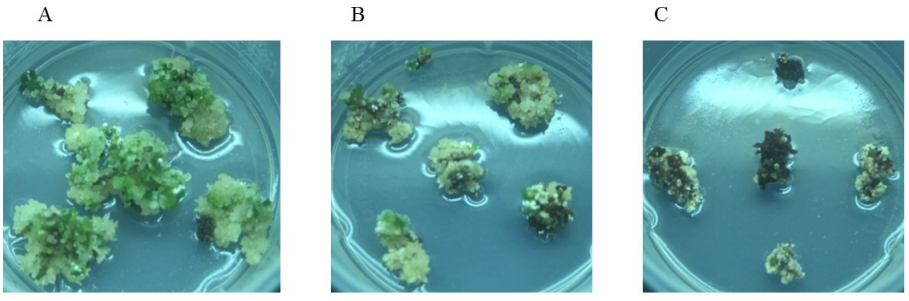

Fig. 1. Growth of callus on different concentrations of sucrose (A) 30 g/L sucrose. (B) 50 g/L sucrose, (C) 100 g/L sucrose.

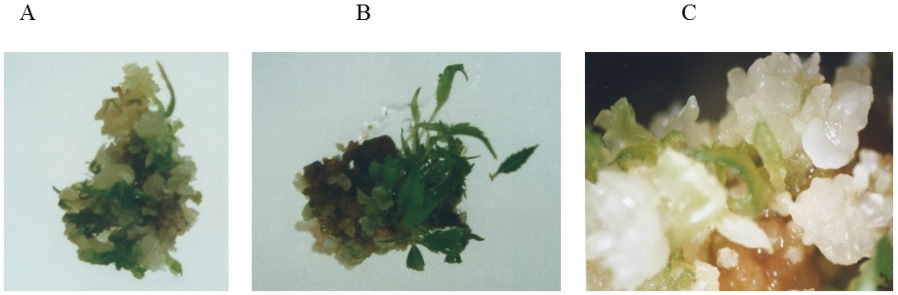

Fig. 2. Organogenesis (leaves and shoot development) from callus on different culture medium. (A) BA only, (B) GA_3 5 μM+BA 1 μM, (C) close up of callus on GA_3 10 μM+BA 2 μM.

Table 3. Shoot induction from the callus

PGR Concentration (μM)	Shoot induction (%) (mean ±SD)	No. of shoots induced (mean± SD)
GA_3 1+BA 0.2	12.0 ± 11.0c[1]	1.3 ± 0.6c
GA_3 5+BA 1	76.0 ± 16.7a	4.0 ± 2.5a
GA_3 10+BA 2	52.0 ± 11.0ab	2.5 ± 1.4ab

[1]Same letters are not significantly different at p=0.05 by Duncan test.

As culture period extended, the surface of callus became bumpy that has granular cells. The appearance of some cells were similar to the somatic embryos (Fig. 2). However cells aggregated and hardly separated then leaves and shoots arose from the callus instead go through the germination stages. Each treatment of PGRs had different effect on organogenesis from the callus. No shoots or leaves were developed from the callus cultivated on the media supplemented with zeatin and GA_3 only (data not shown). While some leaves were induced from the callus in the medium supplemented with BA only, these failed to develop shoot elongated later. Consequently, leaves and shoots developed from the callus on the media supplemented with BA and GA_3. In the combination of 5 μM of GA_3 and 1 μM of BA, 76 % of callus induced shoots and 52% of callus induced shoots on the medium with 10 μM of GA_3 and 2 μM of BA. Number of shoots induced per callus were also greatest on 5 μM of GA_3 and 1 μM of BA, and followed by 10 μM of GA_3 and 2 μM of BA. Although there were very few shoots induced in the medium of 1 μM of GA_3 and 0.2 μM of BA, it was better than the medium containing BA only (Table 3). In *Prunus* species, adventitious shoots were induced from leaf tissues and generally used TDZ with NAA (Takashina et al. 2003; Bhagwat and Lane 2004) and TDZ or BA with IBA (Ainsely et al. 2000). Gibberellic acid was rarely used for the shoot induction from callus in this species. Given the present results, this species required BA for organogenesis and GA_3 for shoot elongation. In the previous study, BA was very effective to flush bud and induce leaves however, shoot could elongated when GA_3 was added with BA. It suggests that for effective organogenesis in this species, the combination of GA_3 and BA is essential.

The optimal condition for shoot organogenesis through callus induction from root tissues was investigated in this study. Callus induced on the medium supplemented with NAA and kinetin had capability of organogenesis in the subsequent culture for shoot induction. High concentrations above 50 g/L of sucrose inhibited callus growth and 30 g/L of sucrose gave the best result. In the shoot organogenesis, BA was effective to induce leaves but GA_3 was essential for shoot development from callus which was novel finding in this study. Compare to the multiplication from shoot cultures, slightly lower concentrations of BA and GA_3 was effective on shoot induction and elongation.

Acknowledgement

This study was supported by 2016 Research Grant from Kangwon National University and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2017R1D1A3B03032994).

References

Ainsley PJ, Collins GG, Sedgley M. 2000. Adventitious shoot regeneration from leaf explants of almond (*Prunus dulcis* mill.). In Vitro Cell Dev Biol Plant 36: 470-474.

Bhagwat B, Lane WD. 2004. In vitro shoot regeneration from leaves of sweet cherry (*Prunus avium*) 'Lapins' and 'Sweetheart'. Plant Cell Tiss Org Cult 78: 173-181.

Brar J, Anand M, Sood A. 2015. Low sucrose and high gibberellic acid levels positively regulates relative growth rate and organogenesis in *D. membranaceus* Munro. Biochem Anal Biochem 4: 198.

Cheong EJ, Kim CS, Kinard G, Li R. 2015. Evaluation of the status of the virus and viroid infection in flowering cherry (*Prunus yedoensis*) collections in Korea and the U.S. J Plant Pathol 97: 155-160.

Cheong EJ, Kim CS, Yi JS. 2000. Differentiation of winter buds of *Prunus yedoensis* Matsumura from Jeju Island depending on the collection time and media conditions. J Korean Soc For Sci 89: 522-526.

Cheong EJ, Pooler MR. 2004. Factors affecting somatic embryogenesis in *Prunus incisa* cv. February Pink. Plant Cell Rep 22: 810-815.

Compton ME, Gray DJ. 1996. Effects of sucrose and methylglyoxal bis-(guanylhydrazone) on controlling grape somatic embryogenesis. Vitis 35: 1-6.

Criollo H, Perea M, Toribio M, Muñoz J. 2014. Effect of the combination of NAA, kinetin and sucrose on the induction of somatic embryogenesis in lulo (*Solanum quitoense* Lam.). Agron Colomb 32: 170-179.

Druart P. 1981. Somatic embryogenesis and plantlets obtained in-vitro from *Prunus incisa serrula* (GM 9). Bull Rech Agron Gembloux 16: 205-220.

Karami O, Deljou A, Esna-Ashari M, Ostad-Ahmadi P. 2006. Effect of sucrose concentrations on somatic embryogenesis in carnation (*Dianthus caryophyllu*s L.). Sci Hortic 110: 340-344.

Kim CS. 1998. Distribution and taxonomic study of *Prunus yedoensis* Matsumura (Rosaceae). MS thesis. Jeju National University, Jeju, Korea. (in Korean)

Kim YW, Park SY, Park IS, Moon HK. 2007. Somatic embryogenesis and plant regeneration from immature seeds of *Magnolia obovata* Thunberg. Plant Biotech Rep 1: 237-242.

Koh JK, Park YC, Yang DY, Kim ES, Oh MY, Koh SC. 1997. Plant regeneration and somatic embryogenesis from zygotic embryo-derived callus of native *Prunus yedoensis* in Mt. Halla. Korean J Plant Tiss Cult 24: 345-349.

Korea Forest Service. 2012. Reports on landscape species planting in Korea in 2012. Korea Forest Service, Urban Forest and Landscape Division, Daejeon. (in Korean)

Lardet L, Dessailly F, Carron MP, Montoro P, Monteuuis O. 2009. Influences of aging and cloning methods on the capacity for somatic embryogenesis of a mature *Hevea brasiliensis* genotype. Tree Physiol 29: 291-298.

Lipavská H, Konrádová H. 2004. Somatic embryogenesis in conifers: The role of carbohydrate metabolism. In Vitro Cell Dev Biol Plant 40: 23-30.

Lloyd G, McCown BH. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Comb Proc Int Plant Propag Soc 30: 421-427.

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

Salaj T, Matusova R, Salaj J. 2015. Conifer somatic embryogenesis- an efficient plant regeneration system for theoretical studies and mass propagation. Dendrobiology 74: 69-76.

Takashina T, Nakano H, Kato R. 2003. Efficient plant regeneration culture from leaf explants of in vitro-grown sweet cherry. Acta Hortic 622: 123-127.

Tsukamoto T, Gao M, Negoro K, Hanada H, Tao R, Kawabe M, Yonemori K. 2007. Somatic embryogenesis and plant regeneration from immature cotyledons of *Prunus mume* 'Nanko'. Acta Hortic 738: 697-701.

Vondráková Z, Eliášová K, Fischerová L, Vágner M. 2011. The role of auxins in somatic embryogenesis of *Abies alba*. Cent Eur J Biol 6: 587-596.

Wang PJ, Hu CY. 1980. Regeneration of virus-free plants through in vitro culture. In: Advances in Biomedical Engineering, Volume 18 (Fiechter A, ed). Springer, Berlin, Heidelberg.