Journal of Medicinal Plants Research Vol. 5(33), pp. 7140-7145, 31 December, 2011
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPRx11.017
ISSN 1996-0875 ©2011 Academic Journals

Review

Research progress in somatic embryogenesis of Siberian ginseng (*Eleutherococcus senticosus* Maxim.)

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Accepted 1 November, 2011

Siberian ginseng (*Eleutherococcus senticosus*) Maxim. is a rare medicinal and perennial deciduous shrub, which is mainly distributed in vast mountain areas of northeast of China, far eastern region of Russia, Hokkaido area of Japan and Korea. Siberian ginseng is a raw material used to produce medicinal tablet, electuary, injection and ginseng tea. Recently, progresses in somatic embryogenesis of Siberian ginseng were reviewed. This paper investigated the pattern of somatic embryogenesis and physiological and biochemical changing in somatic embryo development, including auxin polar transportation, deoxyribonucleic acid (DNA) methylation and metabolic pathways regulation. Bioreactor culture of somatic embryos of Siberian ginseng was also discussed.

Key words: Siberian ginseng, somatic embryogenesis, bioreactor culture, secondary metabolism.

INTRODUCTION

Siberian ginseng (*Eleutherococcus senticosus* Maxim.) is a rare medicinal and perennial deciduous shrub, which belongs to Acanthopanax of Araliaceae, mainly distributed in vast mountain areas of northeast of China, far east region of Russia, Hokkaido area of Japan and Korea. Siberian ginseng is a raw material used to produce medicinal tablet, electuary, injection and ginseng tea. Since Brekhman (1960) firstly reported that Siberian ginseng "adaptogens" was similar to ginseng in the early 1960s, Siberian ginseng began to get attention and be widely used by people. According to market research of America which is the main Siberian ginseng consumer market in the year 2000, Siberian ginseng was listed as one of the best-selling medicinal plant in America market in 1995 and 1998. And a consequent excessive commercial harvest and destruction of nature environment induced that the number of wildness plants decreased rapidly. After 1990s, Siberian ginseng was listed as endangered plant species and the third-grade state protection plant. The state enhanced the protection of wild resource of Siberian ginseng. At the end of 1980s, artificial cultivation including seed reproduction and cutting has been researched. However, owing to poor quality of Siberian ginseng seed, long time of after-ripening in natural condition, low germination and cutting propagation rate restricted the development of artificial cultivation.

In recent years, protocol of tissue and cell culture used to propagate Siberian ginseng is getting more and more attention for this research. Somatic embryogenesis has numerous benefits such as rapid propagation and high reproduction coefficient without restriction of natural conditions. Besides, somatic embryogenesis replay the characteristic of zygote morphogenesis, and it is a perfect experimental system which makes a deep research of cell differentiation, development, morphogenesis and developmental mechanism of zygotic embryo (Yang and Zhang, 2010). Somatic embryogenesis and plant

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Abbreviations: MS, Murashige and Skoog; **2,4-D,** 2,4-Dichlorophenoxy acetic acid; **HPLC,** high-performance liquid chromatography; **MSAP,** methylation-sensitive amplified polymorphism; **SS,** squalene synthase; **GSH,** metabolism of glutathione; **SOD,** superoxide dismutase; **CAT,** catalase; **GSTs**, glutathione *S*-transferases; **GR,** glutathione reductase; **G-POD,** guaiacol peroxidase; **APX,** ascorbate peroxidase; **MDHAR,** monodehydroascorbate reductase; **DHAR,** dehydroascorbate reductase; **GSH-Px,** glutathione peroxidase; **LTB,** heat-labile toxin.

regeneration in Siberian ginseng was first reported by Gui et al. (1991). Few years later, Choi et al. (1999 a, b) researched the way of somatic embryogenesis and the induction of somatic embryos (SEs) from different explants. And then high frequency propagation through bioreactor culture was reported (Choi et al., 2002; Choi et al., 2003; Paek et al., 2005; Shohael et al., 2005). Researchers not only focused on development, but they also studied on regulation of metabolism pathway (Eeva et al., 2003; Kang et al., 2006; Lee et al., 2004; Shohael et al., 2006 a, b, 2007). These studies were helpful to achieve the optimal condition for induction and development of SEs, but what we known was not much on the mechanism of somatic embryogenesis of Siberian ginseng. There are not many of researches in mechanism of Siberian ginseng somatic embryogenesis reported in recent years. Recently, effects of auxin and methylation on development and induction of SEs were researched, and those processes were reported (Choi et al., 2001; Chakrabarty et al., 2003). In this paper, recent progresses in somatic embryogenesis of Siberian ginseng were summarized.

SOMATIC EMBRYOGENESIS, REGENERATION AND BIOREACTOR CULTURE IN SIBERIAN GINSENG

Induction of SEs

The ways of somatic embryogenesis includes indirect formation that SEs differentiated from callus, and direct formation that SEs generated from explants directly has been developed (Yang and Zhang, 2010). The latter is the main way for Siberian ginseng. Gui et al. (1991) reported that SEs can be induced from cotyledons and hypocotyls directly. Authors reported that the initiated SEs were small cylindrical tubers with very smooth surface. After long direct elongation, there were lobed leaves emerged on the top, then SEs developed into torpedo stage, the whole SE presented bulb horn resulted in hypocotyls elongating and cotyledons combining. The slice observation indicated that SEs originated from the epidermal or subepidermal layer of the cotyledons or hypocotyls. Such proximal SEs joined to gather into a mass so that it was difficult to separate and they lost regeneration capacity (Gui et al., 1991). Choi et al. (1999a) cultured SEs of Siberian ginseng on MS (Murashige and Skoog, 1962) medium supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) to induce embryogenic callus. During the process, plenty of synchronizing SEs harvested from embryogenic callus via solid or liquid culture.

This result was important for mass breeding, physiological and biochemical research of embryo development and molecular biology study. They found out that types of explants affected normal development and plantlet conversion for SEs significantly. Choi et al. (1999a) compared three different parts (cotyledon, hypocotyl and root) of one week-old seedlings of Siberian ginseng to test the ability of somatic embryogenesis. These explants were cultured on MS medium with 1.0 $mg \cdot l^{-1}$ 2, 4-D. The result showed that SEs was formed directly from the surface of explants, and the frequency of direct SE formation was the highest in the hypocotyl segments. And hypocotyl explants from 3 different stages of seedlings (0, 1, 3 week old) were cultured on MS medium with 1.0 $mg \cdot l^{-1}$ 2,4-D, the frequency of SE formation rapidly declined as the zygotic embryos germinated. Many embryos are polyembryony, which cannot grow into whole plantlet, however, after germinated for 1 to 3 weeks, single embryo formed from different tissues of seedlings, and grew normally and was finally developed into the whole plant. Therefore researchers indicated that hypocotyl from one week-old seedlings was the optimal explants material for direct somatic embryogenesis.

Plant production via somatic embryogenesis from suspension and bioreactor culture

Germination from seeds needs stratification treatment (for maturation of zygotic embryos) and chilling treatment (to break dormancy) required for 18 months (Choi et al., 1999a), and the seeds germinate after about 3 years in natural condition, so that gaining explants such as hypocotyl from seeds to induce SEs is difficult. Choi et al. (1999a) induced SEs abundantly in suspension culture. They cultured hypocotyl segments on MS medium with 4.5 µM 2,4-D, and SEs appeared directly, and friable embryogenic calli were formed mainly from radical tips of SEs. These calli were maintained on MS agar or liquid medium with 4.5 µM 2,4-D, after 5 weeks, they were transferred to MS medium without plant growth regulators (PGRs) to promote embryos development. The result showed that the frequency of SE induction from cell suspension was much higher than which from callus culture. But most SEs (97%) formed on agar medium converted into normal plantlets with both shoots and roots, but only 76% of embryos cultured in liquid medium developed into normal plantlets.

The appearance of abnormal embryos and subsequent low frequency of plantlet conversion are severe constraints preventing practical application (Stasolla and Yeung, 2003). Abnormal SE development in Siberian ginseng has been previously reported (Gui et al., 1991; Choi et al., 1999a). Gui et al. (1991) induced SEs directly from immature zygotic embryos of Siberian ginseng. However, the radical portion of SEs was tightly fused to parent explants, and could not develop into normal plantlets. As a result, plant regeneration was obtained by secondary somatic embryogenesis. Secondary somatic embryogenesis is a process in which new SEs are initiated from primary SEs. This process has certain

advantages compared to primary somatic embryogenesis, such as independence from explants, ease of maintenance, high induction frequency, and repeatability (Yang et al., 2010). Choi et al. (1999b) reported developmental stage of explants were also important for normal development of induced SEs in Siberian ginseng.

They found out that mature zygotic embryos induced multiple embryos for 3 week-old seedlings and could only induced single embryos when transferred to regeneration medium, multiple embryos produced only multiple shoots without roots, whereas all of the single embryos regenerated into normal plantlets. To date, Siberian ginseng plantlets have been obtained through somatic embryogenesis using semisolid and suspension culture, 2,4-D supplementation is necessary for induction of embryogenic caluus in all studies (Choi et al., 1999 a, b; Gui et al., 1991). Unfortunately, cotyledonary-stage SEs of Siberian ginseng will not germinate even with repeated subculture on PGR-free medium, whereas gibberellic acid supplement is required to stimulate SE germination (Choi et al., 1999a, 1999b). The similar results appeared in the *E. sessiliflorus* (Shohael et al., 2005). In this study, PGR-free medium induced embryogenic callus derived cotyledonary-stage SEs could germinate without additional dormancy or gibberellic acid treatment. The results suggest that the arrested germination of mature SEs of Siberian ginseng on PGR-free medium may be because the SEs originated from embryogenic callus cultured on 2,4-D–containing medium, and this problem could be overcome by using PGR-free medium induced embryogenic callus.

For mass propagation of SEs, bioreactor technology which is an important part of plant tissue culture and has several advantages including large volume, high production capacity per unit volume, easy controlling condition of physics and chemistry is used widely. Automation of micropropagation via organogenesis or somatic embryogenesis in a bioreactor has been advanced as a possible way of reducing costs (Paek et al., 2005). Meanwhile, because of advancements of the bioreactor culture technology, synchronizing germination and rapid growth of high density SEs of medicinal plants such as Siberian ginseng (Choi et al., 2002), *E. sessiliflorus* (Shohael et al., 2005) and *Eleutherococcus koreanum* (Park et al., 2005) were successfully cultured in bioreactor. The multiplication rate and production of Siberian ginseng SEs specially has reached cell culture level (Choi et al., 2002; Paek et al., 2005). The production of SEs in bioreactors from embryogenic callus cultured on 2,4-D–containing medium has been reported for Siberian ginseng (Choi et al., 2002) and some closely related species (Shohael et al., 2005).

Unfortunately, unlike shaking flasks, SEs germinated in bioreactors cannot convert to normal plants (Shohael et al., 2005). There have some reports that 2,4-D can promote abnormal SE development, resulting in the arrested plant conversion (Hussain et al., 2009). Recently, we achieved high conversion frequency (64.7%) of bioreactor-cultured SEs by using PGR-free medium induced embryogenic cells as the initial material (Unpublished, Submitted to Plant Cell, Tiss. Org. Cult). Similarly, for *Panax ginseng* (Choi et al., 1998), SEs induced on PGR-free medium produce more normal plantlets than SEs induced by 2,4-D treatment. Thus, the poor conversion ability of bioreactor-cultured, germinated SEs may be due to the fact that the SEs originated from embryogenic callus cultured on 2, 4-D–containing medium. Therefore, elimination of 2, 4-D during the culture process, even from the initiation medium, may be helpful for promoting normal development and conversion of SEs into plantlets.

THE EFFECTS OF EXTERNAL INFLUENCE FACTORS ON SOMATIC EMBRYOGENESIS OF SIBERIAN GINSENG

Optimum medium and plant growth regulators

SEs cannot be induced from Siberian ginseng explants on MS medium without PGRs. It was found that 2,4-D plays a central role in the induction of SEs. SEs can be induced by 2,4-D obviously, but removing it make SEs develop and grow. Element nitrogen has a great impact on somatic embryogenesis. Choi et al. (2003) cultured cotyledons explants of *P. ginseng* on solid hormone-free MS medium with high concentration of NH_4NO_3, and embryogenic callus were induced. They indicated that embryogenic callus production through the modification of NH_4NO_3 can be an efficient method to induce ginseng callus without exogenous auxin supply.

Plasmolyzing pretreatment induce SEs

You et al. (2006) pretreated Siberian ginseng zygotic embryos for plasmolyzing pretreatment with 1.0 M mannitol for 3 to 24 h, this pretreatment resulted in a high frequency of SE formation on medium without plant growth regulator, and all the SEs formed directly and independently from single epidermal cells on the surface of zygotic embryos. Fluorometric analysis detected that callose concentration increased sharply in zygotic embryos after plasmolyzing pretreatment, which resulted in heavy accumulation of callose between the plasma membrane and cell walls, but it remain low in the untreated control, suggesting that plasmolyzing pretreatment of zygotic embryos induced the accumulation of callose, interruption of cell-to-cell communication, this might stimulate the reprogramming of epidermal cells into embryogenically competent cells and finally induce somatic-embryo development from single cells. We have successfully induced SEs of

Siberian ginseng from mature zygotic embryos through starvation and high temperature treatment, and secondary embryos maintain the proliferation ability over 8 years on hormone-free medium (unpublished).

Research in secondary metabolite

Plant secondary metabolisms are micromolecule organic compounds that are not necessary for growing development in plants but are important to achieve source of medicines such as vinca alkaloids, industrial chemicals such as rubber, great economic benefits and social benefits with significant influence for human production and lives. In recent years, more and more researchers have realized that synergistic reaction of each organ in a plant play an important role in secondary metabolites synthesis (Luczkiewicz and Kokotkiewicz, 2005; Iwase et al., 2005). Although undifferentiated cell cultures mainly have been studied, a large interest has been shown in organ cultures. Plant organs use to produce secondary metabolites including hairy roots, shoots (Bourgaud et al., 2001), SEs and plantlings (Paek et al., 2005). Moreover, at present, the research about gene regulation of secondary metabolism biosynthesis of medical plants has been an active front field of molecular biology. Phytosterol and triterpene saponins are of secondary metabolites have functions of regulating organism immunity, reducing glycemia, fighting cancer and so on, and their contents and components depend on key enzymes in biosynthesis and expression level of these enzymes in cells (Haralampidis et al., 2002). Squalene synthase (SS) is a key enzyme for synthesizing triterpenes and sterols. SS of *P. ginseng* in the biosynthetic route of triterpenes and sterols has a strong regulation function, and this is proved by research of transgenic ginseng (Lee et al., 2004).

On the basis of the experiment, transgenic Siberian ginseng plants were generated by introducing an SS-encoding gene derived from *P. ginseng* (PgSS1) through *Agrobacterium*-mediated transformation. The result demonstrated that, although squalene levels were similar in transgenic and wild-type cells, the content of β-sitosterol and stigmasterol in both transgenic SEs and plants had enhanced about one fold (Seo et al., 2005), suggesting that productions of phytosterols and triterpenoids were successfully achieved by enhancing enzymatic activities. In recent times, researches studies on secondary metabolites processivity of SEs via bioreactor culture technique have received extensive attention (Eeva et al., 2003; Shohael et al., 2006 a, b). Main physics and chemistry factors such as temperature and light influence biomass accumulation, induction of antioxidant, metabolism of glutathione (GSH) and synthesis of secondary metabolites in bioreactor for Siberian ginseng SEs culture gaining more and more attention. Shohael et al. (2005) cultured Siberian ginseng SEs in MS medium in balloon type bubble bioreactor to investigate the effect of light on biomass, secondary metabolites, stress levels and changes of antioxidant enzymes (Shohael et al., 2006b). Results showed that red light induced higher oxidative stress levels and increased antioxidant activities in Siberian ginseng embryos compared with other kind of lights.

It further indicates that activities of superoxide dismutase (SOD), catalase (CAT), glutathione *S*-transferases (GSTs), glutathione reductase (GR) and guaiacol peroxidase (G-POD) activities increased and ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) decreased compared with other kind of light treatments, while the production of eleutheroside E and E1 increased. Fluorescent light produced higher phenolic, flavonoid and chlorogenic acid contents compared to the wild type control embryos. These results indicated that different light sources induced different plant resistance mechanisms, leading to different level of antioxidases activities and various secondary metabolites produced. SEs of Siberian ginseng were cultured under different temperature conditions in bioreactor to research effects of such treatments on the production of secondary metabolites and antioxidases activity (Shohael et al., 2006b). The results revealed that low (12℃) and high (30℃) temperature inhibited SEs growth, and concentration of phenolics, flavonoids, chlorogenic acid and eleutheroside decreased. Low (12℃) temperature significantly increased SOD, CAT, GR, APX, MDHAR and DHAR activities.

Both low and high temperatures denature intracellular proteins and then restrain the growth of SEs irreversibly. Besides, low temperature increased antioxidases activity, suggesting that it may be a self-protection mechanism for oxidative stress. Higher level of Glutathione disulfide (GSSG)/GSH produced in embryogenic callus of Siberian ginseng compared with non-embryogenic callus, and it reached maximum at heart-stage embryo period, and dropped sharply at torpedo-stage embryo periods, then decreased minimum at cotyledon-stage (Shohael et al., 2007). During maturation period of Siberian ginseng SEs, GST, G-POD, CAT and glutathione peroxidase (GSH-Px) activities increased significantly, and antioxidases including APX, MDHAR, DHAR etc were also induced (Shohael et al., 2007). To produce transgenic plant by bioreactor culture is also a hot point research in molecular biology and biotechnology area, and it has been used to produce important medicinal protein, such as antibody, blood substitute and bacterin and so on. The B subunit of *Escherichia coli* heat-labile toxin (LTB) is a potent mucosal immunogen and immunoadjuvant for coadministered antigens (Kang et al., 2006). Producing large scale of LTB can promote the development of edible vaccine. Kang et al. (2006) cultured transgenic SEs of Siberian ginseng in 130 L air-lift type bioreactor, the mature SEs contained approximately 0.36% LTB of

the total soluble protein (Kang et al., 2006).

RESEARCH IN MECHANISM OF SOMATIC EMBRYOGENESIS

Auxin polar transportation

The growth and development of higher plants are regulated widely by hormone, and the auxin function is obviously unique. The relative content of hormone in plant tissues determines the development of these tissues. A known hormone that characterized by polar transportation at present is only auxin. Thus, obviously, plant physiological phenomena such as polar development, differentiation and growth are linked with auxin polar transportation, including apical dominance, development of vascular tissue, tropism growth, and so on (Estelle, 1998). Somatic embryogenesis of Siberian ginseng demands definite concentration gradient (Choi et al., 2001).When Siberian ginseng embryogenic cells cultured on medium containing 2, 3, 4-triiodobenzoic acid (TIBA), an auxin polar transportation inhibitor, embryogenic cells formation was suppressed but did not affected cell division. Transferring SEs at different stages to medium containing TIBA, development of axial and bilateral polarity was restrained in a stage specific manner, and further development of shoot, root apical meristems and vascular differentiation was also suppressed. The shoot and root apical meristems formation and internals differentiation happened during the period that global embryos change to heart-shape embryos, it demonstrated that auxin polar transportation played a very important role during the status of Siberian ginseng embryos changed from global axial symmetry to heart-shape bilateral symmetry (Choi et al., 2001).

DNA methylation

DNA methylation is a DNA modification process that add methyl group from S-adenosylmethionine to the 5 position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring. DNA methylation involves in regulation of gene expression, and it is important for normal development in plants (Finnegan et al., 2000). Methylation level of genome DNA in embryogenic callus was detected through two methods: High-performance liquid chromatography (HPLC) and methylation-sensitive amplified polymorphism (MSAP) technology. Chakrabarty et al. (2003) used HPLC and MSAP technology to assess the extent and pattern of cytosine methylation compared embryogenic callus with non-embryogenic callus during somatic embryogenesis. The HPLC analysis result showed that global DNA methylation rates were significantly lowered in embryogenic calli. MSAP revealed that the cytosine methylated rate of 5'-CCGG-3 sites in the genome of embryogenic callus was 11.20%, which was lower than the rate of non-embryogenic callus tissue that reached 16.99%. Therefore, these results proved that the level of DNA methylation in embryogenic callus was lower than non-embryogenic callus. Besides, hypermethylation of DNA in non-embryogenic callus compared with embryogenic callus reflects the marked expression of this molecular feature, which may well contribute to the developmental gene expression (Chakrabarty et al., 2003).

PROSPECTS

In recent years, the research of Siberian ginseng SEs has considerably developed but still faces many problems: (1) Majority of research in SEs use zygotic embryos as explants. Although SEs of some forest trees has been already induced from their mature tissues, the induction rate is low. Besides, Siberian ginseng SEs induced from mature tissues has not been reported. (2) Incomplete system of somatic embryogenesis. Although the frequency of abnormal embryo formation of Siberian ginseng is lower than other plants, the problem still baffle mass production. (3) Less study in molecular level. The understanding of the regulatory mechanism of Siberian ginseng SEs is rare. (4) The few studies of histology, plant physiology and molecular biology are restriction for reforming bioreactor culture condition of Siberian ginseng and controlling production of secondary metabolites in bioreactor to induce Siberian ginseng SEs. The development of biotechnology brought new chances for research of somatic embryogenesis. To understand the mechanism of somatic embryogenesis and better resolve the problem (browning problem) in Siberian ginseng, such as interior mechanism and control mechanism during SE development, respects of growth status, physiological and biochemical changes and molecular level of Siberian ginseng SEs cultured in bioreactor, etc, need further study. For increasing culture efficiency, extending the scope of bioreactor culture and use it into industrial manufacture by the greater extent is essential. Thus, automated cell culture system needs to be use widely which is still necessary to improve.

ACKNOWLEDGEMENTS

This work was supported by the 948 Project of State Forestry Administration of China (2009-4-26) and Fundamental Research Funds for the Central Universities (DL11EA02) and the postgraduate degree dissertation in Northeast Forestry University (Gram09).

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