Growth Characteristics and Ginsenosides Production of In Vitro Tissues of American Ginseng, *Panax quinquefolius* L.

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Abstract. American ginseng (*Panax quinquefolius* L.) is an economically important perennial herb whose root is highly valued in the Orient for its medicinal properties. The root grows into different morphotypes, notably “bulb or round” (BLB), “man-like” (ML), and “straight or stick” (STK), and these roots are valued differently by consumers because they are assumed to have different medicinal qualities. Currently, wild-growing and field-cultivated plants are the major source of ginseng roots available on the market; however, because of declining wild populations and the lengthy time required in field culture to produce marketable root size, in vitro propagation has been sought as a potential alternative to supply ginseng’s bioactive components (ginsenosides). The objectives of this study were: 1) to evaluate how explants derived from the three root morphotypes (lines), BLB, ML, and STK, responded to in vitro callus induction and growth; 2) to compare ginsenosides profiles and content among stock roots and their callus tissues; and 3) to assess genetic diversity among stock roots. Root explants were cultured on solid Murashige and Skoog medium supplemented with 1.0 mg L⁻¹ 2,4-D and 0.1 mg L⁻¹ kinetin for 12 weeks. Explants from the three lines exhibited varied callus induction response, growth, and ginsenosides production. Explants from the ML line induced callus faster, were prolific in growth, and accumulated more biomass compared with explants from BLB and STK lines. ML lines (both stock roots and calluses) had significantly higher total ginsenosides content than either BLB or STK lines. There were positive and highly significant correlations between total ginsenosides content of stock roots and callus tissues and callus dry weights. Ginsenosides profiles varied among lines. ML lines exclusively exhibited low Rg1/high Re ginsenosides profiles, whereas BLB and STK lines exhibited mixed Rg1/Re profiles. Random amplified polymorphic DNA (RAPD) analysis of stock roots showed genetic variations within and among lines; however, there was no clear link between DNA bands or band patterns and ginsenosides profiles or content. Overall, these results showed that ginsenoside content of stock roots directly influenced callus induction response and subsequent callus biomass and ginsenoside content. These results provide information that could be useful in selecting suitable stock plants for in vitro production of ginsenosides. Also, because there are no ginseng cultivars, this information would be useful in advancing breeding efforts toward selecting superior cultivars for this species. Chemical names used: 2,4-dichlorophenoxyacetic acid (2,4-D)

American ginseng (*Panax quinquefolius* L., Araliaceae) is a perennial herb that is native to the eastern deciduous woodlands of North America (Catling et al., 1994). For many years it has been harvested from the wild as well as cultivated in several regions in the United States and Canada for its highly valued root (Pritz, 1995). Ginseng root is renowned for its medicinal properties and is often used in herbal therapy to reduce stress, lower blood pressure, and boost the body’s immunity (Vuksan et al., 2001). The pharmacological properties of ginseng are attributed to a group of secondary metabolites called ginsenosides (Attele et al., 1999). Besides their use as therapeutic agents, ginsenosides are also marketed as dietary supplements and are often included in health foods and energy drinks (Qu et al., 2009; Shen et al., 2003).

Most of the *P. quinquefolius* roots supplied to the market are harvested from the wild. Recent demographic studies, however, have reported that abundance of wild populations is declining as a result of overharvesting, habitat fragmentation, and degradation (Nantel et al., 1996; Robbins, 1998). On the other hand, cultivation of *P. quinquefolius* is on the rise in response to declining wild populations and increasing demand for ginseng from nutraceutical and beverage industries. Field cultivation of *P. quinquefolius* is labor-intensive and time-consuming as a result of the slow-growing nature of this species (Proctor, 1996). It takes between 5 and 7 years from seedling to final marketable root size, and during that time, caring for plants against various pathogens and pests is needed (Proctor, 1996). In addition, there are no improved cultivars of *P. quinquefolius*, although this species is reported to be genetically diverse (Bai et al., 1997; Boehm et al., 1999; Cruse-Sanders and Hamrick, 2004; Schluter and Punja, 2002).

To circumvent the lengthy duration and difficulties associated with traditional cultivation, and to meet the increasing demand for ginseng products, researchers have attempted to produce ginsenosides using in vitro cultures (Wu and Zhong, 1999). With in vitro culture, ginsenoside production is more controlled with regard to quality and quantity because desirable cell lines can be selected (Wu and Zhong, 1999). Since the first report on in vitro culture of *P. quinquefolius* by Jhang et al. (1974), there has been few reports on its vitro culture compared with numerous reports on other species of the genus *Panax*. The few reports on in vitro culture of *P. quinquefolius* have basically focused on optimizing conditions either for cell growth and ginsenoside production using suspension cultures or plantlet regeneration through somatic embryogenesis (Mathur et al., 1994; Punja et al., 2004; Wang, 1990; Wang et al., 1999; Zhong et al., 1996; Zhou and Brown, 2005).

Although in vitro production of ginsenosides has been successful in the genus *Panax*, fluctuation of ginsenoside content among in vitro cultures is often reported (Wu and Zhong, 1999). This has been attributed to use of different plant growth hormone combinations and concentrations (Zhong et al., 1996); however, the influence of genotype has not been examined in *P. quinquefolius*. Naturally, *P. quinquefolius* plants grow roots of different morphotypes (Roy et al., 2003), and such variation of root morphology could be the result of underlying genetic differences among plants. Most common root morphotypes of *P. quinquefolius* are BLB, a compact, round, or bullet-shaped root; ML, a branched root resembling a human shape; and STK, a slender elongated taproot without lateral roots (Fig. 1). The roots of different morphotypes

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Steel cylindrical core borer and cut into small discs of equal sizes (6 × 6 mm). The explants were then transferred into borosilicate glass culture tubes (25 × 150 mm) containing 20 mL of sterilized culture medium. The culture medium was Murashige and Skoog (Murashige and Skoog, 1962) supplemented with 30 g L⁻¹ sucrose, 2 mL L⁻¹ Plant Preservation Mixture (Plant Cell Technology, Washington, DC), 1.0 mg L⁻¹ 2,4-D, 0.1 mg L⁻¹ kinetin, and 8 g L⁻¹ agar. The pH of the medium was adjusted to 5.8 before adding agar and autoclaving (121 °C and 105 kPa for 20 min). Explants were cultured in the dark for 12 weeks at 24 ± 1 °C and were transferred to fresh medium every 4 weeks. There were six replicates per ginseng line with five explants per replicate.

**In vitro data collection and analysis.** Average time (days) it took explants to induce callus and percentage of explants of each ginseng line with callus tissues at Days 14, 21, and 28 was tabulated. After 12 weeks of culture, callus tissues were harvested, lyophilized, and their individual dry weights measured and averaged for each root replicate across each ginseng line. Also, the growth index of callus tissues was computed as:

\[ \text{Growth index} = \frac{\text{Final} - \text{Initial}}{\text{Initial}} \times 100 \]

Data were analyzed using one-way analysis of variance in SAS (SAS Institute, Cary, NC) and Tukey’s Studentized range test (\( P \leq 0.05 \)) was used for mean separation. DNA extraction. DNA was isolated from leaf tissues using GenElute Plant Genomic DNA Mini Prep Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer’s instructions. Quantity of isolated DNA was determined using a spectrophotometer, and DNA quality was assessed by gel electrophoresis before RAPD analysis. Only non-degraded DNA samples with 260/280 absorbance ratio of 1.8 or greater were used in RAPD analysis.

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**Polymerase chain reaction and gel electrophoresis.** HotStarTaq® Master Mix kit (Qiagen, Germantown, MD) was used for polymerase chain reaction (PCR). Each 25-μL reaction mixture contained 12.5 μL Hotstart master mix, 11 μL of RNase-free water, 0.5 μL of primer (0.2 μM), and 1 μL of DNA template (≥20 ng). Six primers were used for RAPD analysis (Table 1). PCR was done using GeneAmp® PCR System 9700 (Applied Biosystems, Carlsbad, CA) following Schlag and Punja’s (2002) protocol with slight modifications. The modification included heating the reaction mixture for an additional 15 min at 94 °C during the initial cycle to activate the HotStarTaq DNA polymerase as recommended by the manufacturer. Ten microliters of PCR product was loaded.
into 1.5% agarose gels alongside a 1-kb DNA ladder (Promega, Madison, WI) and electrophoresed in 0.5x Tris borate ethylenediaminetetraacetic acid buffer at 105 V for 40 min. Gels were stained with Ethidium bromide to enable visualization of DNA fragments on ultraviolet transilluminator. Gel pictures were taken using a mounted digital Canon Power-shot G6 camera (Canon USA).

Random amplified polymorphic DNA analysis. DNA fragments on gel pictures were manually scored as either present (1) or absent (0) at specific size locations in reference to the DNA ladder. Genetic relationship among stock plants was determined based on Nei’s (1978) genetic distance measure using TFPGA software (Miller, 1998). A phenogram was generated using unweighted pair group method with arithmetic averages and 1000 bootstraps to measure robustness of different clusters in the phenogram.

Table 2. Induction time, growth index, and dry weight of callus tissues from different ginseng lines after 12 weeks of culture on Murashige and Skoog medium supplemented with 1.0 mg L⁻¹ 2,4-D and 0.1 mg L⁻¹ kinetin.

| Ginseng line | Induction time (d) | Growth index (%) | Dry wt (mg) |
|--------------|--------------------|------------------|-------------|
| BLB          | 19                 | 294              | 104.4 b     |
| ML           | 14                 | 395              | 131.1 a     |
| STK          | 23                 | 168              | 70.9 c      |

BLB = “bulb or round”; ML = “man-like”; STK = “straight or stick” root morphotypes.

Average number of days explants took to induce callus.

Growth index = [final – initial] explant dry weight/initial explant dry weight × 100.

Mean of six replications per ginseng line.

Values followed by different letters within column are significantly different (P ≤ 0.05, Tukey’s Studentized range test).

Table 3. Ginsenoside content and profiles of stock roots and 12-week-old callus tissues from root explants of different morphotypes (ginseng lines).

| Ginseng line | Rg1 (mg g⁻¹ dry wt) | Re | Rb1 | Rb2 | Rd | Total | Rg1/Re profile |
|--------------|----------------------|----|-----|-----|----|-------|----------------|
| Stock roots  |                      |    |     |     |    |       |                |
| BLB          | 5.55 ± 0.20          | 2.20 b | 11.29 b | 5.21 a | 0.28 a | 1.41 a | 25.93 b | 1 |
| ML           | 2.69 ± 0.40          | 11.13 a | 15.17 a | 4.62 a | 0.20 a | 1.27 a | 25.78 b | 1 |
| STK          | 5.20 ± 0.20          | 3.29 b | 9.03 b | 4.18 a | 0.20 a | 1.25 a | 23.16 b | 1 |

BLB = “bulb or round”; ML = “man-like”; STK = “straight or stick” root morphotypes.

Mean of six replications per ginseng line.

Sum of six preceding individual ginsenosides.

1 = high Rg1/low Re profile; 2 = low Rg1/high Re profile.

Values for stock roots followed by different letters within column are significantly different (P ≤ 0.05, Tukey’s Studentized range test).

Values for callus tissues followed by different letters within column are significantly different (P ≤ 0.05, Tukey’s Studentized range test).

Results and Discussion

Callus induction and growth. Callus induced on root explants at different times across ginseng lines. On average, explants from the ML line developed callus in 14 d, whereas those from BLB and STK lines took 19 and 23 d, respectively (Table 2). Callusing frequency was variable among explants over time. Between Days 7 and 14, 52% of explants from the ML line had developed callus compared with 17% in the BLB line and zero in the STK line. At Day 21, 100%, 83%, and 38% of explants from ML, BLB, and STK lines, respectively, had callused, and at Day 28, all explants had developed callus. Similar variability in callusing frequency has been reported on leaf and stem explants from different ginseng lines for this species (Punja et al., 2004) and therefore could be attributable to underlying genetic variations.

Table 1. Primer code, nucleotide sequence, total number of fragments, number of polymorphic fragments, and percentage of polymorphic fragments generated by each primer used for random amplified polymorphic DNA analysis of ginseng lines.

| Primer | Sequence (5’→3’) | Total fragments | Polymorphic fragments | Polymorphic fragments (%) |
|--------|------------------|-----------------|-----------------------|--------------------------|
| OPD-05 | TGAGCGGACA       | 8               | 8                     | 100.00                   |
| OPH-04 | GGAATCGGCC       | 5               | 4                     | 80.00                    |
| OPH-05 | AGTCGTCGCC       | 4               | 4                     | 100.00                   |
| OPO-15 | TGGCTCATTT       | 9               | 9                     | 100.00                   |
| OPAD-11 | CAATCCGGG        | 6               | 5                     | 83.33                    |
| UBC-81 | GAGCACAGG       | 5               | 3                     | 60.00                    |

*Primer references: 1 = Boehm et al. (1999); 2 = Schluter and Punja (2002).*

Ginsenosides content of stock roots. Total ginsenoside content (sum of six ginsenosides) was highest in roots of the ML line (35.08 mg g⁻¹) followed by the BLB line (25.93 mg g⁻¹) and STK line (23.16 mg g⁻¹) (Table 3). Total ginsenoside content was significantly different among lines, except between BLB and STK lines (Table 3). Total ginsenoside content of stock roots correlated positively and significantly with callus dry weights (r = 0.796, P = 0.001), and when both values were plotted against each other and a linear regression analysis was computed on the data (Fig. 2A), the following equation resulted:

CDW = 3.02GCSR + 17.45(Adj r² = 0.6102)

where CDW is callus dry weight (mg) and GCSR is total ginsenoside content of stock root (mg g⁻¹). Analysis of variance for linear regression was significant (P = 0.001), meaning that up to 61.02% of the variability in callus growth was accounted for by total ginsenoside content of stock plants. This could explain why explants from ML lines (which had higher ginsenoside content) had better callus induction time and higher callus biomass than those from BLB and STK lines (Table 2). Similar observations were reported in Azadirachta indica A. Juss, in which explants from stock plants with high azadirachtin-A (a secondary metabolite) showed good callus induction response and growth than explants from low azadirachtin-A stock plants (Kota et al., 2006).

Ginsenosides Rb1, Re, and Rg1 varied significantly among stock roots but Rb2, Re, and Rd did not (Table 3). Rb1 was the most abundant ginsenoside in roots (Table 3), which concurs with other reports (Assinewe et al., 2003; Lim et al., 2005; Schlag and McIntosh, 2006). Rg1 and Re ginsenosides were inversely related in roots. Roots of the ML morphotype exclusively exhibited a low Rg1/high Re profile, whereas those BLB and STK morphotypes exhibited mixed Rg1/Re profiles. However, the proportion of roots with a high Rg1/low Re profile was higher in both BLB and STK lines (83% and 66%, respectively). The inverse relationship between Rg1 and Re ginsenosides has been reported before (Schlag and McIntosh, 2006), but assessment of this relationship in roots of different morphotypes is presented here for the first time and therefore provides an important aspect of consideration in herbal formulations and root choice for targeted herbal therapeutic purposes. For instance, Rg1 has been reported as an effective neuroprotective agent (Liao et al., 2002), whereas Re has been shown to have antidiabetic and antihyperlipidemic properties (Attele et al., 2002; Cho et al., 2006; Xie et al., 2005). Therefore, roots with higher levels of Rg1 would be preferred for use in herbal formulations targeted to remedy neurological disorders, and roots with higher Re content would be preferred for use in herbal formulations geared toward remediying diabetics and lowering of high cholesterol levels.
Ginsenoside content of callus tissues. Callus tissues contained less total ginsenoside content than their stock roots (Table 3), which was expected and concurs with a previous report (Wang et al., 1999). Rg1 and Re profiles in callus tissues were similar to those of their stock roots, and, like in stock roots, Rb1 was the most abundant ginsenoside in callus tissues. Total ginsenoside content was significantly less in callus tissues from root explants of STK morphotypes compared with those from BLB or ML morphotypes; however, total ginsenoside content was not significantly different between callus tissues generated from BLB and ML morphotypes (Table 3). Total ginsenoside content of callus tissues was significantly correlated with total ginsenoside content of stock roots \( (p = 0.828, P = 0.001) \). A plot of total ginsenoside content of callus tissues against those of stock roots and linear regression analysis of the data (Fig. 2B) resulted in the following equation:

\[
\text{GCCT} = 0.175 \times \text{GCSR} + 7.75 \quad (\text{Adj} \ r^2 = 0.6665)
\]

where GCCT is total ginsenoside content of callus tissues (mg·g⁻¹) and GCSR is total ginsenoside content of stock root (mg·g⁻¹). Analysis of variance for linear regression was significant \( (P = 0.001) \), implying that total ginsenoside content of stock roots (GCSR) accounted for up to 66.65% of total ginsenoside content in callus tissues (GCCT).

Although many studies have reported growth and saponin production of in vitro tissues of ginseng, few have addressed the correlation between callus weight and ginsenoside content. In this study, that correlation was determined and found to be positive and highly significant \( (p = 0.915, P = 0.001) \). In \( P. \) ginseng, such correlation was negative (Wu and Zhong, 1999), and in \( P. \) notoginseng, there was no correlation (Zhang and Zhong, 1997). The variability in correlation between callus weight and ginsenoside content in genus \( Panax \) could be attributable to factors such as genetic differences among the species, differences in their ginsenoside profiles, and variation of their in vitro growth kinetics and nutritional requirements (Hon et al., 2003; Hong et al., 2005; Mathur et al., 2003).

Genetic variations of stock plants. The six primers used generated a total of 37 DNA fragments of which 33 (89.18%) were polymorphic (Table 1). The number of fragments per primer ranged from four (OPH-05) to nine (OPO-15). Cluster analysis showed a considerable amount of genetic variation within and among ginseng lines (Fig. 3). Four plants with the ML root morphotype clustered separately from the rest of the plants and were supported by high bootstrap values. However, two plants with the ML root morphotype clustered with plants having BLB or STK root morphotypes. There was no distinct cluster separation between plants with BLB and STK root morphotypes (Fig. 3).

Examination of the banding patterns within and among stock plants did not reveal any...
unique DNA band or banding pattern that could be associated with a particular ginsenoside profile or that would explain abundance of ginsenosides in one root morphotype over the other. However, it was noted that the stock plant whose root had the highest total ginsenosides content (ML-1, 41.14 mg g⁻¹) clustered separate from all other stock plants. On the other hand, the stock plant whose root had the lowest total ginsenoside content (STK-6, 14.31 mg g⁻¹) was an “outgroup” of its subcluster. Nevertheless, no concrete inferences could be drawn from these observations. Whereas a linkage of a particular DNA band or banding patterns to a given ginsenoside profile could exist, further analysis with more DNA markers and a larger sample size will be needed to provide more resolution to determine such a link.

Summary and Conclusions

The presented results have shown that explants from different root morphotypes (lines) of Panax quinquefolius have different callus induction response, callus growth, and ginsenoside production in vitro. Also, these results show positive and significant correlations between initial total ginsenoside content of explants and callus growth and ginsenoside content of calluses. This implies that initial ginsenoside content of explant donor plants could have a significant influence on in vitro callus growth and ginsenoside production and therefore should be considered when selecting stock plants for in vitro culture. Furthermore, the presented results show variability in ginsenoside profiles and genetics among Panax quinquefolius lines. Although no specific link between a particular DNA band or banding patterns and a given ginsenoside profile or abundance was found, this foundational research needs to be expanded to identify if unique DNA fingerprints that can be associated with desired ginsenoside profiles are present in this species. Such DNA fingerprints (if available) could be useful in selecting stock plants for use as explants for in vitro ginsenoside production or for breeding of desired cultivars of this economically important medicinal plant.

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