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Effects of elevated temperature on *Panax quinquefolius* ecophysiology and pharmacological activity on human breast MCF-7 carcinoma cells

Gera M. Jochum
West Virginia University

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**Effects of elevated temperature on *Panax
quinquefolius* ecophysiology and pharmacological
activity on human breast MCF-7 carcinoma cells**

Gera M. Jochum

**Thesis submitted to the
Eberly College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Environmental Plant Biology**

**Richard Thomas, Ph.D., Chair
Christy Foran, Ph.D.
James McGraw, Ph.D.
Michael Miller, Ph.D.**

Department of Biology

**Morgantown, WV
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**Keywords: ginseng, ginsenosides, MCF-7 cells, *Panax quinquefolius*, photosynthesis,
secondary metabolites, temperature response**

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ABSTRACT

Effects of elevated temperature on *Panax quinquefolius* ecophysiology and pharmacological activity on human breast MCF-7 carcinoma cells

Gera M. Jochum

The purpose of this study was to determine the effects of increased temperatures associated with climate change on an understory medicinal herb, *Panax quinquefolius*. Three-year old *P. quinquefolius* were grown in a greenhouse at low (25°C day/20°C night) or elevated (30°C /25°C) temperatures for one growing season. Elevated temperatures decreased photosynthesis and accelerated leaf senescence. At the end of the growing season, biomass was detrimentally affected by increased temperatures; yet the concentration of root ginsenosides, secondary metabolites thought to be the primary pharmacological components, was higher in plants grown in the elevated temperature treatment than in plants grown in the low temperature treatment. Changes in the pharmacological activity of these extracted ginsenosides were determined by treating estrogen receptor-positive human breast cancer MCF-7 cells with known concentrations of total ginsenosides. Extracts from plants grown in the low temperature treatment were effective at lower concentrations and were overall more effective at decreasing the number of MCF-7 cells than extracts from plants in the elevated temperature treatment. This response was consistent for extracts harvested early and late in the season. Increased global air temperatures associated with climate change may have important implications for the ecology and pharmacological activity of sensitive understory herbs.

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GENERAL INTRODUCTION

Panax quinquefolius (American ginseng) is a perennial herb endemic to the eastern deciduous forests of North America (Anderson et al., 1993; Charron & Gagnon, 1991). Populations of *P. quinquefolius* are rare to uncommon and patchy throughout its distribution (Cruse-Sanders & Hamrick, 2004; McGraw, 2001). This species is a slow growing, understory shade obligate with palmately compound leaves and a large storage root (Charron & Gagnon, 1991; Lewis & Zenger, 1982; McGraw, 2001; Nantel et al., 1996; Schluter & Punja, 2000).

The highly valued roots of *P. quinquefolius* have been harvested since the 1700s and cultivated for over 100 years (Lewis, 1986; Proctor et al., 1998). Many of the harvested and cultivated roots are exported to Asia, where species of *Panax* have been used for medicinal purposes for over 2000 years (Attele et al., 1999). The exportation of *P. quinquefolius* accounts for substantial revenue; here, in West Virginia, its harvest yields approximately \$6 million per year (Bailey, 1997). The use of *Panax* species in the United States is now growing and it is common to find products containing these herbs in most grocery stores. Although it is evident that *P. quinquefolius* is economically important and gaining popularity as an herbal remedy, little is known about this plant's physiological attributes or how environmental conditions influence its pharmacological compounds or activity.

Temperature is an important environmental variable which strongly influences growth in plants (Berry & Bjorkman, 1980). Increases in greenhouse gases in the atmosphere have are expected to result in a global warming of 1 to 5°C in the northern temperate zone (Farnsworth et al., 1995). While the physiological response to global warming of canopy and tree species is well-studied, little is known about this effect on understory herbaceous species. High temperatures (30-35°C) can decrease stomatal conductance with subsequent decreases in photosynthesis and plant growth (Raven et al., 1999). The photochemical efficiency of photosystem II may also decrease, indicating plant stress at elevated temperatures (Gamon & Pearcy, 1989; Maxwell & Johnson, 2000). When plants are stressed, growth processes are often inhibited to a greater extent than photosynthetic processes, resulting in an increase in secondary metabolite production (Mooney et al., 1991). Therefore, increases in temperature due to global climate change may negatively affect growth of *P. quinquefolius* plants, yet positively affect the production of secondary metabolites.

The secondary compounds, known as ginsenosides, are thought to be the main pharmacologically active ingredients in *Panax* species (Attele et al., 1999; Oh et al., 1999; Yuan et al., 2001). Seven types of ginsenosides (Rb₁, Rb₂, Rc, Rd, Re, Rf, and Rg₁) are commonly referenced in *P. quinquefolius*, although more than thirty have been identified for all species of *Panax* (Smith et al., 1996). Studies report that the concentrations of ginsenosides vary geographically (Li et al., 1996; Mudge et al., 2001; Yuan et al., 2001) and seasonally (Li & Wardle, 2002). Previously, age was also thought to affect ginsenoside concentrations (Li & Wardle, 2002), but a recent study suggests this relationship does not hold for all populations of *P. quinquefolius* (Mudge et al., 2004). A more recent study also reported that the total ginsenoside concentration of *P. quinquefolius* has a positive linear response to light levels and duration of sunflecks (Fournier et al., 2003). Understanding how increases in temperature affect ginsenosides is an important next step in our knowledge of the variation that occurs in these compounds.

Ginsenosides exhibit stereoisomerism and structural isomerism which results in their ability to differentially target receptors, ion channels, and nuclear receptors within the membrane (Attele et al., 1999) and may be responsible for their diverse pharmacological activities. Studies report that *Panax* extracts can affect blood vessel formation (Sengupta et al., 2004), stabilize hypoglycemia (Vuksan et al., 2000), increase immunity, and enhance cognitive and physical performance (Vogler et al., 1999). *P. quinquefolius* also has anti-proliferative effects on prostate cancer cells (Murphy, 2000) and estrogen receptor-positive and -negative breast cancer cells (Murphy, 2000; Oh et al., 1999). Although studies have reported that geographical source affects the pharmacological activity of *P. quinquefolius* (Yuan et al., 2001; Yuan et al., 1998), the effects of specific environmental variables, such as temperature, on the pharmacological activity of this herb is not understood.

PROJECT OBJECTIVES

The overall objective of this project was to provide a comprehensive examination of the effect of a 5°C increase in growing temperature on *Panax quinquefolius* by studying changes in (1) ecophysiology, (2) root ginsenosides, and (3) pharmacological activity on human breast MCF-7 cancer cells.

(1) A greenhouse experiment was carried out to determine the effect of a 5°C increase in temperature on the photosynthesis and biomass production of *P. quinquefolius*. Understanding the role of temperature on seasonal patterns of biomass acquisition will contribute to the knowledge of the basic physiology of this understory species and how it will respond to a changing environment.

(2) Changes in the root ginsenosides were measured using high performance liquid chromatography in *P. quinquefolius* grown at two growth temperatures. Information about the fluctuations in these secondary metabolites is important in understanding how this herb will respond to environmental stress.

(3) Changes in the pharmacological activity of extracted ginsenosides were determined by treating estrogen receptor-positive human breast cancer MCF-7 cells with known concentrations of total ginsenosides. Combining information about changes in ginsenoside concentrations with the effect of extracts on MCF-7 cell number may help to explain why changes in the pharmacological activities of ginseng plants occur.

I hypothesized that elevated temperature will (1) negatively affect growth and photosynthesis of *P. quinquefolius*, (2) stimulate the accumulation of root ginsenosides, and (3) increase the potency of ginseng extracts in decreasing the number of MCF-7 carcinoma cells. Overall, the knowledge gained from this study will have important implications for growers, users and producers of *Panax quinquefolius* products and for ecologists interested in how global climate change may affect the physiology of understory herbaceous plants.

REFERENCES

- Amato, P., Christophe, S., & Mellon, P. L. (2002). Estrogenic activity of herbs commonly used as remedies for menopausal symptoms. *Menopause*, 9(2), 145-150.
- Anderson, R. C., Fralish, J. S., Armstrong, J. E., & Benjamin, P. K. (1993). The ecology and biology of *Panax quinquefolium* L. (Araliaceae) in Illinois. *American Midland Naturalist*, 129, 357-372.
- Attele, A. S., Wu, J. A., & Yuan, C. S. (1999). Ginseng pharmacology: multiple constituents and multiple actions. *Biochemical Pharmacology*, 58(11), 1685-1693.
- Bailey, B. (1997). *The dynamics of ginseng trade in West Virginia*. Paper presented at the Ginseng Workshop, West Virginia University, Morgantown, WV.
- Barnes, P. M., Powell-Griner, E., McFann, K., & Nahin, R. L. (2004). *Complementary and alternative medicine use among adults: United States, 2002* (No. 343). Hyattsville, Maryland: National Center for Health Statistics.
- Bassow, S. L., McConnaughay, K. D. M., & Bazzaz, F. A. (1994). The response of temperate tree seedlings grown in elevated CO₂ to extreme temperature events. *Ecological Applications*, 4(3), 593-603.
- Berry, J., & Bjorkman, O. (1980). Photosynthetic response and adaptation to temperature in higher plants. *Ann. Rev. Plant Physiol.*, 31, 491-543.
- Blumenthal, M. (2001). Ginseng sales down 27% in mainstream markets; sales follow decreasing trend for total herb category. *HerbalGram*, 52, 58-59.
- Bryant, J. P., Chapin, F. S. I., & Klein, D. R. (1983). Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos*, 40, 357-368.
- Carpenter, S. G., & Cottam, G. (1981). Growth and reproduction of American ginseng (*Panax quinquefolius*) in Wisconsin, USA. *Canadian Journal of Botany*, 60, 2692-2696.
- Charron, D., & Gagnon, D. (1991). The demography of northern populations of *Panax quinquefolium* (American ginseng). *Journal of Ecology*, 79, 431-445.
- Constable, J. V. H., Litvak, M. E., Greenberg, J. P., & Monson, R. K. (1999). Monoterpene emission from coniferous trees in response to elevated CO₂ concentration and climate warming. *Global Change Biology*, 5(3), 252-267.

- Court, W. A., Hendel, J. G., & Elmi, J. (1996). Reversed-phase high-performance liquid chromatographic determination of ginsenosides of *Panax quinquefolium*. *Journal of Chromatography A*, 755, 11-17.
- Cruse-Sanders, J. M., & Hamrick, J. L. (2004). Genetic diveristy in harvested and protected populations of wild American ginseng, *Panax quinquefolius* L. (Araliaceae). *American Journal of Botany*, 91(4), 540-548.
- Duda, R. B., Zhong, Y., Navas, V., Li, M. Z. C., Toy, B. R., & Alavarez, J. G. (1999). American ginseng and breast cancer therapeutic agents synergistically inhibit MCF-7 breast cancer cell growth. *Journal of Surgical Oncology*, 72, 230-239.
- Farnsworth, E. J., Nunez-Farfan, J., Careaga, S. A., & Bazzaz, F. A. (1995). Phenology and growth of three temperate forest life forms in response to artificial soil warming. *Journal of Ecology*, 83(6), 967-977.
- Fournier, A. R., Proctor, J. T. A., Gauthier, L., Khanizadeh, S., Belanger, A., Gosselin, A., et al. (2003). Understory light and root ginsenosides in forest-grown *Panax quinquefolius*. *Phytochemistry*, 63, 777-782.
- Gamon, J. A., & Pearcy, R. W. (1989). Leaf movement, stress avoidance and photosynthesis in *Vitis californica*. *Oecologia*, 79, 475-481.
- Genty, B., Briantais, J. M., & Baker, N. R. (1989). The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta*, 990, 87-92.
- Gepstein, S. (1988). Photosynthesis. In L. Nooden & A. Leopold (Eds.), *Senescence and Aging in Plants* (pp. 85-109). San Diego, CA: Academic Press.
- Gillis, C. (1997). *Panax ginseng* pharmacology: a nitric oxide link? *Biochemical Pharmacology*, 54(1), 1-8.
- Hamilton, J. G., Zangerl, A. R., DeLucia, E. H., & Berenbaum, M. R. (2001). The carbon-nutrient balance hypothesis: its rise and fall. *Ecology Letters*, 4, 86-95.
- Herms, D. A., & Mattson, W. J. (1992). The dilemma of plants: To grow or defend. *The Quarterly Review of Biology*, 67(3), 283-335.
- Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., Linden, P. J. v. d., Dai, X., et al. (2001). *IPCC, 2001: Climate Change 2001: The Scientific Basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate*

Change. Paper presented at the Intergovernmental Panel on Climate Change, New York, NY.

- Kim, S. K., Sakamoto, I., Morimoto, K., Sakata, M., Yamasaki, K., & Tanaka, O. (1981). Seasonal variation of saponins, sucrose and monosaccharides in cultivated ginseng roots. *Planta Medica*, 42, 181-186.
- Kirschbaum, M. (2000). Forest growth and species distribution in a changing climate. *Tree Physiology*, 20, 309-322.
- Kitts, D. D., & Hu, C. (2000). Efficacy and safety of ginseng. *Public Health Nutrition*, 3(4A), 473-485.
- Konsler, T. R., Zito, S. W., Shelton, J. E., & Staba, E. J. (1990). Lime and phosphorus effects on American ginseng:II. Root and leaf ginsenoside content and their relationship. *Journal of the American Society for Horticultural Science*, 115(4), 575-580.
- Lee, J. C., Cheon, S. K., Kim, Y. T., & Jo, J. S. (1980). Studies on the effect of shading materials on the temperature, light intensity, photosynthesis and the root growth of the Korean ginseng (*Panax ginseng* C.A. Meyer). *Journal of the Korean Society for Crop Science*, 25(4), 91-98.
- Lewis, W. H. (1986). Ginseng: A medical enigma. In N. I. Etkin (Ed.), *Plants in Indigenous Medicine and Diet: Biobehavioral Approaches* (pp. 290-305). Bedford Hills, NY: Redgrave Publishing Co.
- Lewis, W. H., & Zenger, V. E. (1982). Population dynamics of the American ginseng *Panax quinquefolium* (Araliaceae). *American Journal of Botany*, 69(9), 1483-1490.
- Li, T. S. C., & Mazza, G. (1999). Correlations between leaf and soil mineral concentrations and ginsenoside contents in American ginseng. *HortScience*, 34(1), 85-87.
- Li, T. S. C., Mazza, G., Cottrell, A. C., & Gao, L. (1996). Ginsenosides in roots and leaves of American ginseng. *Journal of Agricultural Food Chemistry*, 44, 717-720.
- Li, T. S. C., & Wardle, D. (2002). Seasonal fluctuations of leaf and root weight and ginsenoside contents of 2-,3-, and 4-year-old American ginseng plants. *HortTechnology*, 12(2), 229-232.
- Litvak, M. E., Constable, J. V. H., & Monson, R. K. (2002). Supply and demand processes as controls over needle monoterpenes synthesis and concentration in Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco]. *Oecologia*, 132, 382-391.

- Liu, X., & Huang, B. (2000). Heat Stress Injury in Relation to Membrane Lipid Peroxidation in Creeping Bentgrass. *Crop Science*, 40(2), 503-510.
- Lui, J. H.-C., & Staba, E. J. (1980). The ginsenosides of various ginseng plants and selected products. *Journal of Natural Products*, 43(3), 340-346.
- Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany*, 51(345), 659-668.
- McGraw, J. B. (2001). Evidence for decline in stature of American ginseng plants from herbarium specimens. *Biological Conservation*, 98, 25-32.
- McGraw, J. B., & Furedi, M. (2005). Deer browsing and population viability of a forest understory plant. *Science*, 307(5711), 920-923.
- Mizuno, M., Yamada, J., Terai, H., Kozukue, N., Lee, Y. S., & Tsuchida, H. (1994). Differences in immunomodulating effects between wild and cultured *Panax ginseng*. *Biochemical and Biophysical Research Communications*, 200(3), 1672-1678.
- Mooney, H. A., Winner, W. E., & Pell, E. J. (1991). *Response of Plants to Multiple Stresses*. San Diego: Academic Press, Inc.
- Morison, J. I. L., & Lawlor, D. W. (1999). Interactions between increasing CO₂ concentration and temperature on plant growth. *Plant, Cell, and Environment*, 22(6), 659-682.
- Mudge, K., Lim, W., Lardner, J., & Beyfuss, R. (2004). Effects of population and age on ginsenoside content of American ginseng (*Panax quinquefolium* L.). *Acta Horticulture*, 629, 161-166.
- Mudge, K., Lim, W., Lardner, J., & Jouselin, V. (2001). *Ginsenosides as quality indicators in woods-grown American ginseng*, Department of Horticulture, Cornell University, Ithaca, NY.
- Munne-Bosch, S., & Alegre, L. (2004). Die and let live: leaf senescence contributes to plant survival under drought stress. *Functional Plant Biology*, 31, 203-216.
- Murphy, L. (2000). *Effects of America ginseng on breast cancer and prostate cancer cells*. Paper presented at the American Ginseng Production in the 21st Century, Cornell Cooperative Extension of Green County.
- Nantel, P., Gagon, D., & Nault, A. (1996). Population viability analysis of American ginseng and wild leek harvested in stochastic environments. *Conservation Biology*, 10(2), 608-621.

- Nicol, R. W., Traquair, J. A., & Bernards, M. A. (2002). Ginsenosides as host resistance factors in American ginseng (*Panax quinquefolius*). *Canadian Journal of Botany*, 80, 557-562.
- Oh, m., Choi, Y. H., Choi, S. H., Chung, H. Y., Kim, K. W., Kim, S. I., et al. (1999). Anti-proliferating effects of ginsenoside Rh2 on MCF-7 human breast cancer cells. *International Journal of Oncology*, 14, 869-875.
- Proctor, J. T. A., Louittit, D., & Jiao, J. (1998). Seasonal growth and root respiration of North American ginseng. *Journal of Ginseng Research*, 22(3), 161-167.
- Raven, P. H., Evert, R. F., & Eichhorn, S. E. (1999). *Biology of Plants sixth edition*. New York, New York: W.H. Freeman and Company Worth Publications.
- Schluter, C., & Punja, Z. K. (2000). Floral biology and seed production in cultivated North American ginseng (*Panax quinquefolius*). *Journal of the American Society for Horticultural Science*, 125(5), 567-575.
- Sengupta, S., Toh, S. A., Sellers, L. A., Skepper, J. N., Koolwijk, P., Leung, H. W., et al. (2004). Modulating angiogenesis: the yin and the yang in ginseng. *Circulation*, 110(10), 1219-1225.
- Smith, R. G., Caswell, D., Carriere, A., & Zielke, B. (1996). Variation in the ginsenoside content of American ginseng, *Panax quinquefolius* L., roots. *Canadian Journal of Botany*, 74, 1616-1620.
- Snow, M. D., Bard, R. R., Olszyk, D. M., Minster, L. M., Hager, A. N., & Tingey, D. T. (2003). Monoterpene levels in needles of Douglas fir exposed to elevated CO₂ and temperature. *Physiologia Plantarum*, 117, 352-358.
- Suits, S. A. (2003). American ginseng (*Panax quinquefolium* L.), the "other" ginseng. *Economic Botany*, 57(1), 143-144.
- Tode, T., Kikuchi, Y., Kita, T., Hirata, J., Imaizumi, E., & Nagata, I. (1993). Inhibitory effects by oral administration of ginsenoside Rh2 on the growth of human ovarian cells in nude mice. *Journal of Cancer Research and Clinical Oncology*, 120, 24-26.
- Vogler, B. K., Pittler, M. H., & Ernst, E. (1999). The efficacy of ginseng. A systematic review of randomised clinical trials. *European Journal of Clinical Pharmacology*, 55, 567-575.
- Vuksan, V., Sievenpiper, J. L., Wong, J., Xu, Z., Beljan-Zdravkovic, U., Arnason, J. T., et al. (2001). American ginseng (*Panax quinquefolius* L.) attenuates postprandial glycemia in

- a time-dependent but not dose-dependent manner in healthy individuals. *American Journal of Clinical Nutrition*, 73, 753-758.
- Vuksan, V., Stavro, M. P., Sievenpiper, J. L., Beljan-Zdravkovic, U., Leiter, L. A., Josse, R. G., et al. (2000). Similar postprandial glycemic reductions with escalation of dose and administration time of American ginseng in Type 2 diabetes. *Diabetes Care*, 23(9), 1221-1226.
- Wolfson, D. (2002). Ginseng helps regulate blood glucose. *Nutrition Science News*, 7(1), 5.
- Yuan, C.-S., Wang, X., Wu, J. A., Attele, A. S., Xie, J.-T., & Gu, M. (2001). Effects of *Panax quinquefolius* L. on brainstem neuronal activities: Comparison between Wisconsin-cultivated and Illinois-cultivated roots. *Phytomedicine*, 8(3), 178-183.
- Yuan, C. S., Wu, J. A., Lowell, T., & Gu, M. (1998). Gut and brain effects of American ginseng root on brainstem neuronal activities in rats. *American Journal of Chinese Medicine*, XXVI(1), 47-55.
- Yun, T.-K., Lee, Y.-S., & Kwon, H.-Y. (1996). Saponin contents and anticarcinogenic effects of ginseng depending on types and ages in mice. *Acta Pharmacologica Sinica*, 17(4), 293-298.
- Zito, S. W., Konsler, T. R., & Staba, E. J. (1984). *Soil and mulch effects on ginsenosides in American ginseng plants*. Paper presented at the Proceedings 4th International Ginseng Symposium, Daejeon, Korea.

CHAPTER 1

Elevated temperatures increase leaf senescence and root ginsenosides of the understory herb, *Panax quinquefolius* (Araliaceae)

ABSTRACT

The response of understory species to global warming is not well-understood but is important since these plants exhibit high sensitivity to their environmental growth conditions. *Panax quinquefolius* (American ginseng) is an understory herb endemic to the eastern deciduous forests of North America. Three-year old *P. quinquefolius* were grown in a greenhouse at 25/20°C (day/night) or 30/25°C for one growing season and analyzed each month. Plants grown in the elevated temperature treatment experienced an early onset of leaf senescence, detrimentally affecting the carbon accumulation in these plants. From May to July, *P. quinquefolius* grown in elevated temperatures decreased photosynthesis (52%), stomatal conductance (60%), and root and total biomass (33% and 28%, respectively) compared to plants grown in low temperatures. As *P. quinquefolius* prepared to over-winter, plants grown in the high temperature treatment had significantly less root biomass (53%) than plants grown in the low temperature treatment. The content of root ginsenosides was unaffected by temperature, therefore differences in root size can explain our observation that plants grown in elevated temperatures had greater concentrations of root ginsenosides (49%) than plants grown in the low temperature treatment. American ginseng is clearly sensitive to a 5°C increase in temperature, and future increases in global temperature associated with climate change may have detrimental ecological consequences for understory species of the eastern deciduous forest.

INTRODUCTION

Climate change forced by anthropogenic activities will have strong effects on many plant species. As humans continue to burn fossil fuels at an accelerated rate, greater amounts of greenhouse gases accumulate in the atmosphere, with profound implications for global climate. Mean surface air temperatures of the earth have increased 0.6°C during the last century and models project a global warming of 1.4 to 5.8°C by 2100 (Houghton et al., 2001). Many studies have focused on the potential effects of this increase in temperature on plant species, including numerous forest species (Morison & Lawlor, 1999), but few studies have examined the impacts on understory herbaceous plants (Farnsworth et al., 1995).

Many forest understory species exhibit a high sensitivity to their environmental growth conditions and, thus, are likely to be affected by increases in temperature to a greater extent than the more dominant woody species (Farnsworth et al., 1995). Elevated temperatures have been shown to reduce stomatal conductance and, subsequently, negatively impact photosynthesis and growth of many plant species (Raven et al., 1999). The photochemical efficiency of photosystem II has also been shown to decrease at elevated temperatures, indicating increased stress (Gamon & Percy, 1989; Maxwell & Johnson, 2000). When plants are stressed, growth processes are often inhibited to a greater extent than photosynthetic processes, and the carbon fixed in excess of that allocated to growth may be allocated to secondary metabolite production (Mooney et al., 1991). Several studies have examined the effects of temperature on secondary metabolite production of plants, but most of these studies have varying results. Some report that secondary metabolites increase from elevated temperatures (Litvak et al., 2002), while others report that they decrease (Snow et al., 2003). No studies have yet considered the effect of temperature on plant saponins, compounds that are the pharmacologically active ingredients in many medicinal plant species.

Panax quinquefolius L. (American ginseng) is a slow growing medicinal herb endemic to the eastern hardwood forests of North America (Anderson et al., 1993; Charron & Gagnon, 1991). Populations of this species are rare to uncommon and are decreasing in size because of a number of factors, including harvest and deer-browsing (Cruse-Sanders & Hamrick, 2004; McGraw & Furedi, 2005). *Panax quinquefolius* is an understory shade obligate with palmately compound leaves and a large storage root (Charron & Gagnon, 1991; Lewis & Zenger, 1982;

McGraw, 2001; Nantel et al., 1996; Schluter & Punja, 2000). Leaves generally emerge in late April when the tree canopy is partially to fully developed (Anderson et al., 1993; Carpenter & Cottam, 1981; Lewis & Zenger, 1982), and senescence often occurs in October, although it may occur as early as August in some populations (Anderson et al., 1993).

The pharmacologically active compounds of *P. quinquefolius* are steroidal saponins known as ginsenosides (Attele et al., 1999). These dammarene-type triterpenes are divided into two classes of compounds: those composed of two sugars, 20(S)-protopanaxadiols (including Rb₁, Rb₂, Rc, and Rd), and those with three sugars, 20(S)-protopanaxatriols (Re and Rg₁) (Fournier et al., 2003). Ginsenosides are found in all organs of the ginseng plant (Li et al., 1996) and act as antifungal (Nicol et al., 2002) and antimicrobial agents (Suits, 2003). Studies have reported that concentrations of ginsenosides vary depending on geography (Li et al., 1996; Mudge et al., 2001; Yuan et al., 2001), season (Kim et al., 1981; Li & Wardle, 2002), plant age (Li & Wardle, 2002), soil conditions (Konsler et al., 1990; Li & Mazza, 1999; Li et al., 1996), and light levels (Fournier et al., 2003). No studies have examined the potential effects of elevated temperatures on root ginsenoside concentrations of *P. quinquefolius*.

In this experiment, we conducted a greenhouse study to investigate the effects of a 5°C increase in temperature on growth and physiology of *Panax quinquefolius*. We hypothesized that elevated temperatures would negatively impact photosynthesis and biomass production of *P. quinquefolius*, but positively affect root ginsenoside concentrations. Understanding the role of elevated temperatures on seasonal patterns of biomass accumulation of *P. quinquefolius* will contribute to the understanding of how this important understory plant will respond to a changing climate.

MATERIALS AND METHODS

Plant material—Three-year old *Panax quinquefolius* (Araliaceae) roots were obtained from a woods-grown source (Shady Oaks Ginseng Co., Putnam County, West Virginia). Initial fresh weight of each root was measured to use as a covariate in the statistical analyses. In mid-March, roots were transplanted into 2 L pots containing a 1:3 mixture of sand and forest soil obtained from the West Virginia University Forest (Morgantown, West Virginia) where *P. quinquefolius* was found growing naturally. For the duration of the experiment, plants were grown in the West Virginia University Life Science Building greenhouses (Morgantown, West Virginia) under 65% shade cloth, and the light level was $216 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ averaged across the growing season. The volumetric water content, measured on five random plants from each treatment every day with a time domain reflectometry (TDR) probe (HydroSense, Campbell Scientific, Inc., Logan, UT), was maintained at 20% and no fertilizer was added to the soil.

Greenhouse treatments—*Panax quinquefolius* plants were randomly assigned to one of two temperature treatments ($n = 30$ per treatment) after they had emerged at the end of April. The temperature treatments represented the estimated mean summer temperature for understory conditions in a central hardwood forest around Morgantown, West Virginia (low temperature) and a 5°C increase associated with global warming (elevated temperature). Air temperatures in the greenhouses were monitored with a LI-1400 datalogger (Li-Cor, Lincoln, Nebraska). Mean daytime temperatures (from 7:00 to 21:00 EDT) of the low and elevated temperature treatments were $26.8 \pm 3.0^{\circ}\text{C}$ and $31.2 \pm 2.2^{\circ}\text{C}$, respectively. Mean nighttime temperatures (from 21:00 to 7:00 EDT) were $21.2 \pm 1.7^{\circ}\text{C}$ for the low temperature treatment and $26.6 \pm 1.3^{\circ}\text{C}$ for the elevated temperature treatment.

Photosynthesis and stomatal conductance—Light-saturated net photosynthesis (A_{sat}) and stomatal conductance (g_s) were measured every four weeks from May 30 until August 25 on four randomly selected *P. quinquefolius* plants from each temperature treatment using an open-flow gas exchange system (LI-Cor 6400, Li-Cor, Lincoln, Nebraska). Gas exchange measurements were made with saturating light ($\text{PPFD} = 500 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, predetermined with photosynthetic light response curves) on the largest terminal leaflet of each plant at

ambient CO₂ (360 μL · L⁻¹) and were made between 10:00 and 15:00 EDT to minimize diurnal effects. Plants grown in the high temperature treatment were photosynthetically inactive during August due to leaf senescence and were not measured.

Dark-adapted chlorophyll fluorescence was measured with a pulse-modulated fluorometer (PAM-2000, Heinz Walz GmbH, Effeltrich, Germany) prior to the July harvest on all remaining plants (n=2 in the high temperature treatment, n=16 in the low temperature treatment). Leaves were dark-adapted for ten minutes and fluorescence was measured under steady state conditions (F_s) following a saturating pulse of white light (F_m[']). The photochemical efficiency of photosystem II (F_v/F_m) was then calculated as: F_m['] - (F_s/F_m[']) (Genty et al., 1989).

Biomass production—Each plant was harvested after gas exchange measurements (n=4 per temperature per month) and separated into shoots (leaves plus sympodium) and roots (rhizome plus root). Leaf area was measured (LI 3100 Area Meter, Li-Cor, Lincoln, Nebraska). No plants from the high temperature treatment were harvested in August due to the early onset of leaf senescence. Roots from these plants were later collected in September at the same time roots from the low temperature treatment were harvested for measurements of the final over-wintering biomass. All plant tissues were dried at 60°C for 48 hrs and weighed to determine the biomass.

Root Ginsenosides—Ginsenoside concentrations from root extracts were measured from each harvested plant using reverse-phase high performance liquid chromatography (RP-HPLC). The rhizome and fibrous roots were removed from dried *P. quinquefolius* roots and the storage roots were finely ground and stored in a drying oven at 60°C until extractions were performed. Ultrasonic extraction and analytical separation procedures followed Court et al. (1996), with the following modifications. Ground root samples (50 mg) were extracted in 100% methanol and 30 μl were injected into the RP-HPLC. Samples ran for 74 min on a Waters 2690 Separations Module (Waters, Milford, Massachusetts) consisting of a Waters 996 Photodiode Array Detector adjusted to 203 nm and a C₁₈ reversed-phase column and guard column (Chrompack Standard Columns, Varian Inc, Palo Alto, California). The gradient employed the eluents (A) water, (B) acetonitrile, and (C) phosphate buffer solution at a flow-

rate of 1.15 ml/min with the following profile: 0-15 min, 0% A, 19% B, 81% C; 15-24.5 min, 0% A, 21% B, 79% C; 24.5-29 min, 0% A, 26.3% B, 73.7% C; 29-43 min, 0% A, 27% B, 73% C; 43-47 min, 0% A, 34% B, 66% C; 47-54 min, 0% A, 36% B, 64% C; 54-55 min, 0% A, 43% B, 57% C; 55-64 min, 15% A, 85% B, 0% C; and 64-74 min, 0% A, 19% B, 81% C. Ginsenosides were quantified using standard curves of known concentrations of individual ginsenosides (Rg₁, Re, Rb₁, Rc, Rb₂, and Rd from INDOFINE Chemical Co., Hillsborough, New Jersey), and the concentrations of all ginsenosides were calculated as a percentage of the dried root tissue (mg ginsenoside per mg of dried root x 100%). The concentrations of ginsenosides Rb₁, Rb₂, Rc, and Rd were added to determine the total concentration of 20(S)-protopanaxadiols (PD) and the concentrations of Re and Rg₁ were combined to give the concentrations of 20(S)-protopanaxatriols (PT). Total ginsenoside concentration was determined as the sum of all of the individual ginsenosides. The amount of ginsenosides in each root, or the ginsenoside content, was calculated as the mg of ginsenosides divided by the dried root weight (mg).

Statistical analysis—A two-way analysis of variance (ANOVA) was used to determine if growth temperature or harvest date significantly affected net photosynthetic rates, stomatal conductance, and root ginsenoside concentrations and content during the growing season (May, June, and July). Ginsenoside concentrations and content in the over-wintering root measured in September and chlorophyll fluorescence measured in July were analyzed using a one-way ANOVA with temperature as the main effect. Differences in individual ginsenosides between the pretreatment period (March and April harvests) and the growing season, or between the pretreatment period and final concentrations in September were analyzed using orthogonal contrasts following a one-way ANOVA with month as the main effect.

Biomass production was analyzed using a two-way analysis of covariance (ANCOVA) with temperature and harvest date as the main factors and the initial dry root weight as a covariate to remove any effects of differences in initial root size on biomass measurements. Biomass data from May, June, and July were used; however, data from the pretreatment harvest dates were excluded. August data were also not used since shoot senescence occurred early for plants grown in the high temperature treatment. A one-way ANCOVA with initial root weight as the covariate was used to determine if the final over-wintering root biomass

measured in September was significantly affected by temperature. All statistical analyses were performed using SAS JMP 3.2.5 (Cary, North Carolina).

RESULTS

Photosynthesis and stomatal conductance—Light-saturated net photosynthetic rates (A_{sat}) of *P. quinquefolius* grown in both temperature treatments decreased as the season progressed ($P_{\text{date}} = 0.0003$; Fig. 1.1A), but growth temperature affected the timing of this seasonal decline ($P_{\text{interaction}} = 0.0251$). In the low temperature treatment, the seasonal decline in photosynthesis of *P. quinquefolius* occurred between June and July, whereas the seasonal decline occurred between May and June in the high temperature treatment. Averaged across all measurement dates, A_{sat} of *P. quinquefolius* leaves was 52% less in plants grown in the elevated temperature treatment than plants in low temperatures ($P_{\text{temperature}} = 0.0018$). Reductions in stomatal conductance (g_s) of *P. quinquefolius* leaves also occurred as the growing season progressed ($P_{\text{date}} = 0.0264$) and as a result of elevated growth temperature ($P_{\text{temperature}} = 0.0023$; Fig. 1.1B).

Measurements of the photochemical efficiency of photosystem II (Fv/Fm) in July were low for *P. quinquefolius* leaves in both temperature treatments. At this time, Fv/Fm averaged 0.50 (± 0.14) in the low temperature treatment but was reduced by 66% in leaves in the high temperature treatment to 0.17 (± 0.03) ($P = 0.0022$). By August, the leaves and stems of *P. quinquefolius* grown in elevated temperatures were completely senesced.

Biomass production—Total biomass production of *P. quinquefolius* increased across the growing season in both temperature treatments ($P_{\text{date}} = 0.0035$), but the observed increases were 28% less for plants grown at elevated temperatures ($P_{\text{temperature}} = 0.0281$). Changes in total biomass production were reflected by changes in root biomass but not shoot biomass. Shoot biomass was not significantly affected by harvest date ($P_{\text{date}} = 0.5825$) or temperature ($P_{\text{temperature}} = 0.9106$; Fig. 1.2A). On the other hand, root biomass of *P. quinquefolius* increased across the growing season ($P_{\text{date}} = 0.0002$), but there was 33% less root mass produced in plants grown in the high temperature treatment compared to those in low temperatures ($P_{\text{temperature}} = 0.0034$; Fig. 1.2B). Thus, root to shoot ratios of *P. quinquefolius* increased throughout the growing season for all plants regardless of temperature treatment ($P_{\text{date}} = 0.0008$), but this increase was less for plants grown in elevated temperatures ($P_{\text{temperature}} =$

0.0087). Biomass production of all plant tissues depended upon the initial root weight measured at the beginning of the experiment ($P < 0.0001$).

The biomass of *P. quinquefolius* roots harvested in September after plants had senesced was affected by temperature treatments such that roots grown in elevated temperatures weighed 53% less than roots grown in low temperatures ($P = 0.0013$; Fig. 1.2B).

Root Ginsenosides—The concentrations of all individual ginsenosides from plants grown at low temperatures decreased from the pretreatment period (March and April) to the growing season (May, June, and July), except for ginsenosides Rd and Rg₁ ($P_{Rb1} = 0.0057$; $P_{Rb1} = 0.0119$; $P_{Rc} = 0.0288$; $P_{Re} = 0.0042$; Figs 1.3 and 1.4). However, the concentration of most individual ginsenosides from plants grown at elevated temperatures remained constant during this time, and only ginsenosides Rb₁ and Re decreased ($P_{Rb1} = 0.0262$; $P_{Re} = 0.0381$).

Regardless of temperature treatment, the concentrations of the two ginsenosides in greatest quantity, Rb₁ and Re, did not significantly change throughout the growing season, but the concentrations of the other ginsenosides decreased over time ($P_{Rb2} = 0.0241$; $P_{Rc} = 0.0241$; $P_{Rd} = 0.0007$; $P_{Rg1} = 0.0005$). The 20(S)-protopanaxadiols (PD), 20(S)-protopanaxatriols (PT), and total root ginsenosides grown in both elevated and low temperature treatments also decreased from May to July ($P_{PD} = 0.0092$; $P_{PT} = 0.0386$; $P_{Total} = 0.0134$; Table 1.1). During this time, all individual ginsenosides were insensitive to growth temperature, except Rb₂ which was 30% higher in roots grown in elevated temperatures compared to those in low temperatures ($P = 0.0241$).

The effect of temperature became prominent at the final measurement in September when the concentration of total root ginsenosides was 49% higher in roots grown in increased temperatures than in roots grown in low temperatures ($P = 0.0383$; Table 1.1). The concentrations of ginsenoside Re and the protopanaxatriols were also significantly higher (57% and 51% respectively) in plants grown in elevated temperatures ($P_{Re} = 0.0004$; $P_{PT} = 0.0023$). At this final measurement period, the concentration of most root ginsenosides were restored to concentrations observed in the pretreatment measurements. Only ginsenoside Rc from plants grown at low temperatures was not restored and instead decreased between the pretreatment period and September ($P = 0.0212$). The content of ginsenosides in each root, on the other

hand, was not significantly affected by temperature treatment at the final measurement time ($P \geq 0.05$ for all individual ginsenosides; Table 1.2).

Table 1.1. Total ginsenoside, protopanaxadiol (Rb₁, Rb₂, Rc, and Rd), and protopanaxatriol (Re and Rg₁) concentrations of *Panax quinquefolius* grown in the low (26.8°C day/ 21.2°C night) or elevated (31.2°C day/ 26.6°C night) temperature treatments. March (n=10) and April (n=7) values are pretreatment ginsenoside concentrations; May to July (n=3-4 per temperature treatment) values are the ginsenoside concentrations during the course of the growing season; and September (n=4 per temperature treatment) values are the over-wintering ginsenoside concentrations after shoots had senesced. Each value is the mean ($\pm$ SE).

Concentration (%)	Growth Temperature	Pretreatment		Growing Season			Over-wintering September
		March	April	May	June	July	
Total Ginsenosides	Low	2.23 (0.32)	2.08 (0.30)	1.32 (0.19)	1.40 (0.09)	0.96 (0.18)	1.50 (0.25)
	Elevated			2.12 (0.12)	1.17 (0.23)	1.22 (0.19)	2.24 (0.13)
Protopanaxadiols	Low	1.36 (0.22)	1.20 (0.17)	0.78 (0.09)	0.81 (0.02)	0.61 (0.12)	0.90 (0.23)
	Elevated			1.28 (0.09)	0.76 (0.14)	0.70 (0.09)	1.32 (0.16)
Protopanaxatriols	Low	0.92 (0.12)	0.89 (0.14)	0.54 (0.10)	0.59 (0.07)	0.35 (0.07)	0.61 (0.03)
	Elevated			0.84 (0.03)	0.41 (0.09)	0.53 (0.11)	0.92 (0.05)

Table 1.2. Content of individual and total ginsenosides for *Panax quinquefolius* roots grown in low (26.8°C day/ 21.2°C night) or elevated (31.2°C day/ 26.6°C night) temperature treatments in September. Each value is the mean ($\pm$ SE) for n=4.

Ginsenosides	Growth Temperature	Content (mg/root)
Rb1	Low	3.34 (1.45)
	Elevated	2.15 (0.44)
Rb2	Low	0.18 (0.05)
	Elevated	0.12 (0.03)
Rc	Low	0.67(0.16)
	Elevated	0.64 (0.22)
Rd	Low	0.84 (0.34)
	Elevated	0.43 (0.12)
Re	Low	2.99 (1.19)
	Elevated	2.11 (0.22)
Rg1	Low	0.35 (0.13)
	Elevated	0.17 (0.08)
Total	Low	8.36 (3.18)
	Elevated	5.61 (0.84)

Figure 1.1. Light-saturated net photosynthetic rates, A_{sat} (A) and stomatal conductance, g_s , (B) of *Panax quinquefolius* grown at pretreatment conditions, low temperatures, or elevated temperatures for one growing season. Means ($\pm$ SE) for 2-4 plants.

Figure 1.2. Shoot biomass (A) and root biomass (B) of *Panax quinquefolius* grown at pretreatment conditions, low temperatures, or elevated temperatures for one growing season. Each value is the mean ($\pm$ SE) of 3-4 plants. September root biomass is the over-wintering biomass measured after above ground senescence and each value is the mean ($\pm$ SE) of 5-9 roots.

Figure 1.3. Concentrations of protopanaxadiol ginsenosides (Rb₁, Rb₂, Rc, and Rd) from roots of *Panax quinquefolius* grown in pretreatment conditions, low temperatures, or elevated temperatures throughout one growing season.

Figure 1.4. Concentrations of protopanaxatriol ginsenosides (Re and Rg₁) from roots of *Panax quinquefolius* grown in pretreatment conditions, and low or elevated temperature treatments throughout one growing season.

DISCUSSION

Panax quinquefolius was highly sensitive to a 5°C increase in growth temperature. Elevated temperatures reduced net photosynthetic rates and stomatal conductance and caused premature leaf senescence. At the end of the growing season, plants grown in the high temperature treatment had significantly less root biomass but greater concentrations of total root ginsenosides than plants in the low temperature treatment. Therefore, future increases in global temperature projected by Global Circulation Models may have detrimental consequences for the growth of understory species, such as *P. quinquefolius*.

Growth temperatures strongly influence photosynthetic processes, ontogenetic development, and carbon balance of plants, and these processes may be positively or negatively impacted by increased temperatures (Morison & Lawlor, 1999). Moderate increases in temperature may positively impact the biomass production of overstory forest species (Kirschbaum, 2000). However, understory forest species are reported to be more sensitive to elevated temperatures than shrub or tree species (Farnsworth et al., 1995). Therefore, small increases in growth temperature may induce heat stress for understory herbs. Results of this experiment are symptomatic of heat stress and suggest that *P. quinquefolius* was strongly affected by a 5°C increase in temperature. Heat stress has been shown to have many negative effects on plants ranging from decreased stomatal conductance and photosynthesis to damage to chloroplast and membrane integrity (Berry & Bjorkman, 1980) and even to cell and tissue death (Liu & Huang, 2000). Extreme heat events even of short duration have been shown to be detrimental to species native to the eastern deciduous forest, especially to shade tolerant species (Bassow et al., 1994). Lee et al. (1980) reported that the photosynthetic rates of *P. ginseng* (Asian ginseng) leaves were reduced by 25% when measured at 30°C compared to 20°C. Our study, using a more moderate increase in temperature, found that light saturated photosynthetic rates of *P. quinquefolius* growing at 30°C were 52% lower than plants growing at 25°C.

The low values of photochemical efficiency of photosystem II that we observed in July indicated that *P. quinquefolius* leaves in both temperature treatments were senescing and that the senescence process had progressed farther in the shoots grown in the high temperature treatment (Liu & Huang, 2000). Photosynthetic capacity of a senescing leaf gradually declines

until the leaf dies (Gepstein, 1988). Leaf senescence is controlled by a combination of environmental factors, such as photoperiod and temperature, as well as endogenous factors including age, reproductive maturity, and plant hormones (Munne-Bosch & Alegre, 2004). Temperature strongly influences metabolic activity and plant ontology, and high temperatures can induce premature leaf senescence (Morison & Lawlor, 1999). The decreased leaf longevity in *P. quinquefolius* grown in elevated temperatures reduced time available for photosynthesis over the growing season, and consequently may be responsible for the reduced biomass accumulation in these plants.

Despite the negative effect of elevated temperatures on root biomass of *P. quinquefolius* at the end of the growing season, the concentration of total root ginsenosides was 49% higher in plants from elevated temperatures compared to plants from low temperatures. The concentration of most ginsenosides from plants grown in the low temperature treatment decreased from the pretreatment period through the growing season, while ginsenosides from plants grown in the elevated temperature treatment remained relatively constant. As the plants prepared to over-winter, the concentration of most ginsenosides were restored to pretreatment levels. Li and Wardle (2002) also studied the seasonal fluctuations in ginsenosides but reported that concentrations were highest in June and decreased through September for 3 and 4-year old *P. quinquefolius* plants.

Differences in the final over-wintering concentrations of root ginsenosides in *P. quinquefolius* grown in elevated or low temperatures can be attributed to dilution by the size of the root. The amounts of ginsenosides in roots from plants grown in both temperature treatments were not significantly different; therefore, as roots from plants grown in low temperatures increased in biomass, the concentrations of ginsenosides decreased. Likewise, greater concentrations of ginsenosides in *P. quinquefolius* grown in elevated temperatures were observed with smaller increases in root size.

The concentrations of secondary compounds in plant tissues often increase in response to stress (Mooney et al., 1991). Hypotheses such as the carbon-nutrient balance hypothesis (Bryant et al., 1983) and the growth differentiation balance hypothesis (Herms & Mattson, 1992) suggest that when plants are stressed, a trade-off occurs between carbon allocated to biomass production or to formation of defensive secondary compounds. When plants are under non-limiting conditions, these hypotheses predict that carbon is allocated to biomass

production, but when plants are stressed, carbon is allocated to secondary defense compounds and away from biomass. These hypotheses have come under some recent criticism (Hamilton et al., 2001), and reports of the response of secondary metabolites to temperature change have been inconsistent. For example, studies show that monoterpene levels in Douglas fir (*Pseudotsuga menziesii*) can either decline with a 3.5°C increase in temperature (Snow et al., 2003), increase with a 4°C increase (Litvak et al., 2002) or are not affected by the combination of elevated CO₂ (550 µmol mol⁻¹) and a 4°C increase in temperature (Constable et al., 1999). A recent review by Morison and Lawlor (1999) show that the interactive effect of elevated CO₂ and temperature increases may be negligible for secondary metabolites, as well as for biomass production. In this experiment, a 5°C increase in temperature clearly induced stress in *P. quinquefolius*, but did not stimulate production of root ginsenosides.

In summary, the carbon accumulation of *P. quinquefolius* was strongly affected by a 5°C increase in growth temperature. Leaf senescence was accelerated, and photosynthesis and stomatal conductance were reduced as the season progressed by the increase in temperature. In September, the over-wintering biomass of *P. quinquefolius* roots grown in the increased temperature treatment was half that observed in the low temperature treatment, but the content of ginsenosides was unaffected by temperature. Therefore, the concentration of total root ginsenosides was diluted by the size of the root and plants grown at elevated temperatures had greater concentrations of ginsenosides than plants grown at low temperatures. We conclude that a 5°C increase in temperature is highly detrimental to the productivity of *P. quinquefolius* and that increased temperatures associated with climate change may induce heat stress and decrease the success of other understory herbs of the eastern deciduous forest.

REFERENCES

- Anderson, R. C., Fralish, J. S., Armstrong, J. E., & Benjamin, P. K. (1993). The ecology and biology of *Panax quinquefolium* L. (Araliaceae) in Illinois. *American Midland Naturalist*, 129, 357-372.
- Attele, A. S., Wu, J. A., & Yuan, C. S. (1999). Ginseng pharmacology: multiple constituents and multiple actions. *Biochemical Pharmacology*, 58(11), 1685-1693.
- Bassow, S. L., McConnaughay, K. D. M., & Bazzaz, F. A. (1994). The response of temperate tree seedlings grown in elevated CO₂ to extreme temperature events. *Ecological Applications*, 4(3), 593-603.
- Berry, J., & Bjorkman, O. (1980). Photosynthetic response and adaptation to temperature in higher plants. *Ann. Rev. Plant Physiol.*, 31, 491-543.
- Bryant, J. P., Chapin, F. S. I., & Klein, D. R. (1983). Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos*, 40, 357-368.
- Carpenter, S. G., & Cottam, G. (1981). Growth and reproduction of American ginseng (*Panax quinquefolius*) in Wisconsin, USA. *Canadian Journal of Botany*, 60, 2692-2696.
- Charron, D., & Gagnon, D. (1991). The demography of northern populations of *Panax quinquefolium* (American ginseng). *Journal of Ecology*, 79, 431-445.
- Constable, J. V. H., Litvak, M. E., Greenberg, J. P., & Monson, R. K. (1999). Monoterpene emission from coniferous trees in response to elevated CO₂ concentration and climate warming. *Global Change Biology*, 5(3), 252-267.
- Court, W. A., Hendel, J. G., & Elmi, J. (1996). Reversed-phase high-performance liquid chromatographic determination of ginsenosides of *Panax quinquefolium*. *Journal of Chromatography A*, 755, 11-17.
- Cruse-Sanders, J. M., & Hamrick, J. L. (2004). Genetic diversity in harvested and protected populations of wild American ginseng, *Panax quinquefolius* L. (Araliaceae). *American Journal of Botany*, 91(4), 540-548.
- Farnsworth, E. J., Nunez-Farfan, J., Careaga, S. A., & Bazzaz, F. A. (1995). Phenology and growth of three temperate forest life forms in response to artificial soil warming. *Journal of Ecology*, 83(6), 967-977.

- Fournier, A. R., Proctor, J. T. A., Gauthier, L., Khanizadeh, S., Belanger, A., Gosselin, A., et al. (2003). Understory light and root ginsenosides in forest-grown *Panax quinquefolius*. *Phytochemistry*, 63, 777-782.
- Gamon, J. A., & Pearcy, R. W. (1989). Leaf movement, stress avoidance and photosynthesis in *Vitis californica*. *Oecologia*, 79, 475-481.
- Genty, B., Briantais, J. M., & Baker, N. R. (1989). The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta*, 990, 87-92.
- Gepstein, S. (1988). Photosynthesis. In L. Nooden & A. Leopold (Eds.), *Senescence and Aging in Plants* (pp. 85-109). San Diego, CA: Academic Press.
- Hamilton, J. G., Zangerl, A. R., DeLucia, E. H., & Berenbaum, M. R. (2001). The carbon-nutrient balance hypothesis: its rise and fall. *Ecology Letters*, 4, 86-95.
- Hermes, D. A., & Mattson, W. J. (1992). The dilemma of plants: To grow or defend. *The Quarterly Review of Biology*, 67(3), 283-335.
- Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., Linden, P. J. v. d., Dai, X., et al. (2001). *IPCC, 2001: Climate Change 2001: The Scientific Basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change*. Paper presented at the Intergovernmental Panel on Climate Change, New York, NY.
- Kim, S. K., Sakamoto, I., Morimoto, K., Sakata, M., Yamasaki, K., & Tanaka, O. (1981). Seasonal variation of saponins, sucrose and monosaccharides in cultivated ginseng roots. *Planta Medica*, 42, 181-186.
- Kirschbaum, M. (2000). Forest growth and species distribution in a changing climate. *Tree Physiology*, 20, 309-322.
- Konsler, T. R., Zito, S. W., Shelton, J. E., & Staba, E. J. (1990). Lime and phosphorus effects on American ginseng: II. Root and leaf ginsenoside content and their relationship. *Journal of the American Society for Horticultural Science*, 115(4), 575-580.
- Lee, J. C., Cheon, S. K., Kim, Y. T., & Jo, J. S. (1980). Studies on the effect of shading materials on the temperature, light intensity, photosynthesis and the root growth of the Korean ginseng (*Panax ginseng* C.A. Meyer). *Journal of the Korean Society for Crop Science*, 25(4), 91-98.

- Lewis, W. H., & Zenger, V. E. (1982). Population dynamics of the American ginseng *Panax quinquefolium* (Araliaceae). *American Journal of Botany*, 69(9), 1483-1490.
- Li, T. S. C., & Mazza, G. (1999). Correlations between leaf and soil mineral concentrations and ginsenoside contents in American ginseng. *HortScience*, 34(1), 85-87.
- Li, T. S. C., Mazza, G., Cottrell, A. C., & Gao, L. (1996). Ginsenosides in roots and leaves of American ginseng. *Journal of Agricultural Food Chemistry*, 44, 717-720.
- Li, T. S. C., & Wardle, D. (2002). Seasonal fluctuations of leaf and root weight and ginsenoside contents of 2-, 3-, and 4-year-old American ginseng plants. *HortTechnology*, 12(2), 229-232.
- Litvak, M. E., Constable, J. V. H., & Monson, R. K. (2002). Supply and demand processes as controls over needle monoterpenes synthesis and concentration in Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco]. *Oecologia*, 132, 382-391.
- Liu, X., & Huang, B. (2000). Heat Stress Injury in Relation to Membrane Lipid Peroxidation in Creeping Bentgrass. *Crop Science*, 40(2), 503-510.
- Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany*, 51(345), 659-668.
- McGraw, J. B. (2001). Evidence for decline in stature of American ginseng plants from herbarium specimens. *Biological Conservation*, 98, 25-32.
- McGraw, J. B., & Furedi, M. (2005). Deer browsing and population viability of a forest understory plant. *Science*, 307(5711), 920-923.
- Mooney, H. A., Winner, W. E., & Pell, E. J. (1991). *Response of Plants to Multiple Stresses*. San Diego: Academic Press, Inc.
- Morison, J. I. L., & Lawlor, D. W. (1999). Interactions between increasing CO₂ concentration and temperature on plant growth. *Plant, Cell, and Environment*, 22(6), 659-682.
- Mudge, K., Lim, W., Lardner, J., & Jousselein, V. (2001). *Ginsenosides as quality indicators in woods-grown American ginseng*, Department of Horticulture, Cornell University, Ithaca, NY.
- Munne-Bosch, S., & Alegre, L. (2004). Die and let live: leaf senescence contributes to plant survival under drought stress. *Functional Plant Biology*, 31, 203-216.

- Nantel, P., Gagon, D., & Nault, A. (1996). Population viability analysis of American ginseng and wild leek harvested in stochastic environments. *Conservation Biology*, 10(2), 608-621.
- Nicol, R. W., Traquair, J. A., & Bernards, M. A. (2002). Ginsenosides as host resistance factors in American ginseng (*Panax quinquefolius*). *Canadian Journal of Botany*, 80, 557-562.
- Raven, P. H., Evert, R. F., & Eichhorn, S. E. (1999). *Biology of Plants sixth edition*. New York, New York: W.H. Freeman and Company Worth Publications.
- Schluter, C., & Punja, Z. K. (2000). Floral biology and seed production in cultivated North American ginseng (*Panax quinquefolius*). *Journal of the American Society for Horticultural Science*, 125(5), 567-575.
- Snow, M. D., Bard, R. R., Olszyk, D. M., Minster, L. M., Hager, A. N., & Tingey, D. T. (2003). Monoterpene levels in needles of Douglas fir exposed to elevated CO₂ and temperature. *Physiologia Plantarum*, 117, 352-358.
- Suits, S. A. (2003). American ginseng (*Panax quinquefolium* L.), the "other" ginseng. *Economic Botany*, 57(1), 143-144.
- Yuan, C.-S., Wang, X., Wu, J. A., Attele, A. S., Xie, J.-T., & Gu, M. (2001). Effects of *Panax quinquefolius* L. on brainstem neuronal activities: Comparison between Wisconsin-cultivated and Illinois-cultivated roots. *Phytomedicine*, 8(3), 178-183.

CHAPTER 2

Growth temperature of *Panax quinquefolius* L. (Araliaceae) affects the pharmacological activity of root extracts on MCF-7 human breast cancer cells

ABSTRACT

Panax quinquefolius (American ginseng) is used as a medicinal herb worldwide. The primary active ingredients are steroidal saponins called ginsenosides. While studies report that ginsenoside concentrations and pharmacological activities differ between sources, the potential effect of different growth temperatures is unknown. Our objectives were to determine the effects of growth temperature and harvest date on root ginsenoside concentrations and on the pharmacological activity of root extracts on MCF-7 human breast cancer cells. Three-year old *P. quinquefolius* plants were grown in a greenhouse at low (26.8 day/21.2°C night) or high (31.2/26.6°C) temperatures from April until September. Extracts were prepared from dried roots using ultrasonic extraction, and ginsenosides were analyzed using HPLC. Extracts at known total ginsenoside concentrations were applied to MCF-7 cells for 48 hours and the number of cells was assayed using MTS tetrazolium compound (Owen's reagent). *P. quinquefolius* root extracts caused a dose-dependent decrease in the number of MCF-7 cells, regardless of temperature treatment or harvest date. Extracts from plants grown in low temperatures reduced MCF-7 cell numbers at lower concentrations than root extracts from plants grown at elevated temperatures. Although the total concentration of major ginsenosides was higher in *P. quinquefolius* roots grown in high temperatures than in roots grown in low temperatures, the relative proportion of individual ginsenosides was not significantly affected by temperature. All results are consistent for extracts of *P. quinquefolius* harvested early and late in the growing season. We conclude that there may be other active ingredients in addition to ginsenosides Rb₁, Rb₂, Rc, Rd, Re, and Rg₁ in *P. quinquefolius* that may be responsible for reducing the number of MCF-7 carcinoma cells.

INTRODUCTION

The use of complementary and alternative medicines (CAM) is growing in the United States. A recent survey estimates that over one-third of American adults use CAM therapies, and natural products are among the most frequently used of these therapies (Barnes et al., 2004). Ginseng is reported as one of the top ten selling herbal supplements in the United States, and in 2001, the sale of herbal dietary supplements containing ginseng totaled approximately \$56 million (Blumenthal, 2001). In spite of this, herbal supplements are not regulated by the Food and Drug Administration and so the safety, efficacy, and quality of ginseng products is unregulated (Gillis, 1997). In fact, many products even fail to identify which species of ginseng (Asian, *Panax ginseng*, or American, *Panax quinquefolius*) is processed, making the study of efficacy difficult (Kitts & Hu, 2000).

There are many purported medicinal uses of ginseng. From a recent review of clinical studies evaluating the effectiveness of ginseng, there are reports that it can be used to enhance physical performance, cognitive function, and immunostimulatory activity (Vogler et al., 1999). Studies also report that extract from ginseng berries has a regulatory effect for treating blood glucose (Wolfson, 2002) and may be used to treat patients with type II diabetes (Vuksan et al., 2001). There is growing evidence for ginseng's antioxidant activities (Kitts & Hu, 2000), and it is effective in treating some types of cancers, including lung (Yun et al., 1996) and ovarian cancers (Tode et al., 1993). Ginseng has also been reported to inhibit proliferation of prostate cancer cells (Murphy, 2000) and estrogen receptor-positive and -negative breast cancer cells (Duda et al., 1999; Murphy, 2000; Oh et al., 1999). Contradictory reports for the reputed medicinal uses of ginseng do exist (Vogler et al., 1999), including reports of estrogenic activity in estrogen receptor-positive breast cancer cells (Amato et al., 2002). Lack of agreement for the pharmacological activities of ginseng may be attributed, at least in part, to differences in the species of ginseng used and, ultimately, to differences in the effective compounds.

The main pharmacologically active ingredients in ginseng are steroidal saponins known as ginsenosides (Attele et al., 1999; Oh et al., 1999). These compounds exhibit stereoisomerism and structural isomerism which may result in their ability to differentially target receptors, ion channels, and nuclear receptors within the membrane (Attele et al., 1999)

and may be responsible for ginseng's reported diverse pharmacological activities. Though more than thirty ginsenosides have been identified (Smith et al., 1996), only six (Rb₁, Rb₂, Rc, Rd, Re, and Rg₁) are typically referenced for American ginseng (Li et al., 1996). How different cultivation and extraction procedures may contribute to variation in these specific compounds are not well-understood. Studies report that the concentrations of ginsenosides may vary with harvest date (Li & Wardle, 2002), nutrient availability (Konsler et al., 1990; Zito et al., 1984), and growth light (Fournier et al., 2003). Extraction and processing techniques can also cause differences in the concentrations of ginsenosides detected (Lui & Staba, 1980; Yun et al., 1996). Further, the geographic source of ginseng has been reported to influence not only the ginsenoside concentrations (Li et al., 1996; Mudge et al., 2001; Yuan et al., 2001), but also the pharmacological activity (Yuan et al., 2001; Yuan et al., 1998). Reports of differences in medicinal activity between wild versus cultivated ginseng also exist (Mizuno et al., 1994).

As the demand for ginseng grows, it is important to understand the factors which influence its medicinal activity. Linking information about variations in ginsenosides with pharmacological activity is an important step. In this study, we determined how growth temperature and the time of harvest (May versus September) of *Panax quinquefolius* influenced concentrations of the main root ginsenosides and the effect of root extracts on the number of estrogen-receptor positive human breast MCF-7 cancer cells *in vitro*.

MATERIALS AND METHODS

American ginseng treatments—Three-year old *Panax quinquefolius* L. (Araliaceae) roots were obtained from a woods-grown source (Shady Oaks Ginseng Co., Putnam County, WV) and were transplanted in mid-March of 2002 into 2 L pots containing a 1:3 mixture of sand and forest soil obtained from the West Virginia University Forest (Morgantown, WV) where American ginseng was found growing naturally. For the duration of the experiment, plants were grown under 65% shade cloth and maintained at 20% volumetric water content with no fertilizer added. American ginseng plants were randomly assigned to one of two temperature treatments (n=15 per treatment) after they had emerged at the end of April. Mean daytime temperatures (from 7:00 to 21:00 EDT) were 26.8 ± 3.0 °C for the low temperature treatment and 31.2 ± 2.2 °C for the high temperature treatment. Mean nighttime temperatures (from 21:00 to 7:00 EDT) of the low and high temperature treatments were 21.2 ± 1.7 °C and 26.6 ± 1.3 °C, respectively.

Ginsenoside extraction and analysis—Plants were harvested early in the growing season and after leaf senescence (May and September, respectively). The rhizome and root fibers were removed from root tissues, the storage roots were dried in a drying oven at 60°C for 48 hrs, and the tissue was finely ground and stored at 60°C until extracted. Ultrasonic extraction and analytical separation procedures followed Court et al. (1996), with the following modifications. Ground root samples (100 mg) were extracted in 100% methanol. After the residue was concentrated *in vacuo*, it was diluted in 5 ml of methanol, divided into 2 new vials each containing 2.5 ml, and the extracts were again concentrated *in vacuo*. One of the vials containing the extract was analyzed for ginsenosides by dissolving it in 0.5 ml of acetonitrile containing the internal standard (*m*-cresol) and using reversed-phase high performance liquid chromatography (RP-HPLC). The second vial was used for the cell proliferation assays. RP-HPLC analysis was performed using 30 µl injections on a Waters 2690 Separations Module (Waters, Milford, MA) consisting of a Waters 996 Photodiode Array Detector adjusted to 203nm and a C₁₈ reversed-phase column and guard column (Chrompack Standard Columns, Varian Inc, Palo Alto, CA) for a total elution time of 74 min. The gradient employed the eluents (A) water, (B) acetonitrile, and (C) phosphate buffer solution at a flow-rate of 1.15

ml/min with the following profile: 0-15 min, 0% A, 19% B, 81% C; 15-24.5 min, 0% A, 21% B, 79% C; 24.5-29 min, 0% A, 26.3% B, 73.7% C; 29-43 min, 0% A, 27% B, 73% C; 43-47 min, 0% A, 34% B, 66% C; 47-54 min, 0% A, 36% B, 64% C; 54-55 min, 0% A, 43% B, 57% C; 55-64 min, 15% A, 85% B, 0% C; and 64-74 min, 0% A, 19% B, 81% C. Ginsenosides were quantified using standard curves of known concentrations of individual ginsenosides (Rg₁, Re, Rb₁, Rc, Rb₂, and Rd from INDOFINE Chemical Co., Hillsborough, NJ) and the concentrations of all ginsenosides were calculated (mg total ginsenosides per mg of dried root). Individual ginsenosides were summed to give the total ginsenoside concentration and results are means of 3-4 individual roots.

Cell culture—Human breast cancer MCF-7 cells (American Type Culture Collection, Manassas, VA) were maintained in phenol red-free minimum essential medium (GIBCO MEM alpha, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, Utah), 0.05% gentamycin (Invitrogen), and 0.05% penicillin-streptomycin (Mediatech, Herndon, VA). Cells were incubated at 37°C and 5% CO₂ and the medium was changed every 2-3 days. At ~70% confluence, cells were passed by trypinization. All assays were done using estrogen free conditions, i.e. MEM supplemented with 5% charcoal-dextran FBS (CD-FBS, Hyclone). Estrogen free conditions allowed assessment of the inhibitory effects of ginseng extracts on MCF-7 proliferation (Duda et al., 1999; Murphy, 2000; Oh et al., 1999), as well as potential growth-stimulatory effects due to estrogenic activity (Amato et al., 2002).

Cell assays—MCF-7 cells were seeded at an initial density of 10⁴ cells/well in 96-well microtiter plates. Twenty-four hours later, the media was changed to 5% CD-FBS. Cells were allowed 48 hours for steroidal withdraw, and cells were treated with 100 µl/well of indicated concentrations of total ginsenosides. Extracts were combined for each sample group (April Pretreatment, May High Temperature, May Low Temperature, Sept High Temperature, Sept Low Temperature) and diluted in 5% CD-FBS medium for the adjusted concentrations of total ginsenosides. The control group contained 5% CD-FBS medium and no extract. After 48 hours of treatment, the media was removed, each well was washed once with 100 ul of phosphate buffer solution (PBS, BioWhittaker, Walkersville, MD) and replaced with 100 ul of 5% MTS tetrazolium compound (CellTiter 96 AQueous One Solution, Promega, Madison, WI). The plates were incubated for 3 hours, shaken for 4 min, and read on a Tecan Microplate Reader (GENios TECAN, Research Triangle Park, NC) at 492 nm. These methods were repeated so there was a total of two assays for the experiment in order to determine the reproducibility

of the results. Absorbance at 492 nm and cell numbers are directly proportional (Promega, Madison, WI), and results are expressed as the mean absorbance $\pm$ SE.

Statistical analysis—Within each harvest date, a three-way analysis of variance (ANOVA) was used to determine if temperature, treatment concentration, or assay significantly affected the number of MCF-7 cells independently or in combination. Within each growth temperature, we used Dunnett's test to determine which treatments were significantly different from the control. One-way ANOVAs were used to determine if temperature significantly affected the total ginsenoside concentration or the relative proportion of individual ginsenosides. All statistics were completed using JMP 3.2.5 (SAS, Cary, NC).

RESULTS AND DISCUSSION

Panax quinquefolius extract harvested during the pretreatment in April decreased the number of MCF-7 cells in a dose-dependent manner (Figure 2.1). A reduction in the number of MCF-7 cells was first evident with 0.25 mg/ml of total ginsenosides and was maximally reduced by 0.5 mg/ml of total ginsenosides. Therefore, all assays comparing growth temperatures and harvest dates were done encompassing these concentrations over the range of 0 to 0.75 mg/ml of total ginsenosides.

A dose-dependent decrease in the number of MCF-7 cells with *P. quinquefolius* root extracts from both harvest dates and temperature treatments was observed ($P_{\text{concentration}} < 0.0001$ for all; Figure 2.2). Although Amato et al. (2002) reported that ginseng extracts have a stimulatory effect on MCF-7 cell proliferation, a dose-dependent decrease in the number of MCF-7 cells has also been observed by Duda et al. (1999) and Murphy (2000) using standardized American ginseng powder, and by Oh et al. (1999) using standardized ginsenoside Rh₂ from *Panax ginseng*. Only Duda et al. (1999) provided enough information to allow us to compare our effective dosages, and the extracts in our experiment reduced the number of MCF-7 cells at considerably lower doses (0.04 mg of total ginsenosides per 10⁴ cells) than the standardized American ginseng powder used by Duda et al. (0.1-0.2 mg of ginsenosides per 10⁴ cells). This difference may be the result of many factors, including inherent differences in the plant material used to make the extract or differences in the processing or extraction procedures. Processing and extraction methods can greatly influence the recovery of ginsenosides in a sample, at least in part, due to the thermal instability of some ginsenosides (Court et al., 1996).

The dose-dependent reduction of the number of MCF-7 cells depended on the growth temperature of *P. quinquefolius* ($P_{\text{interaction}} < 0.0001$), and root extracts from plants grown at low temperatures reduced the number of cells at lower total ginsenoside concentrations than extracts from plants grown at high temperatures, regardless of harvest date (Figure 2.2). For May extracts, a significant decrease in the number of MCF-7 cells from the control was first evident at 0.125 mg/ml of total ginsenosides from roots grown at low growth temperatures, whereas a significant reduction in cell number was first observed at 0.3 mg/ml for extracts from roots grown at high temperatures. Extracts from *P. quinquefolius* harvested in September appeared to reduce cell number at lower concentrations: at 0.075 mg/ml for extracts from plants grown at low temperatures and at 0.125 mg/ml for extracts from high temperatures. The point at which the cell number remained consistently low, reducing the number of cells at least 75% compared to the control, occurred at 0.25 mg/ml of total ginsenosides for May Low

Temperature extracts, at 0.3 mg/ml for Sept. Low Temperature extracts, and at 0.4 mg/ml for May and Sept. High Temperature extracts. Results were reproducible and were not affected by assay ($P_{\text{May}} = 0.5363$ and $P_{\text{Sept}} = 0.5607$).

Differences in the medicinal activities of ginseng extracts may be explained by variations in the pharmacologically active compounds. A recent study has reported that ginseng's ability to have two opposing effects on blood vessel formation was correlated with the compositional ratios of ginsenosides Rg₁ and Rb₁ (Sengupta et al., 2004). With more of Rg₁, the growth of blood-vessels was stimulated, while more of Rb₁ inhibited angiogenesis. However, differences in the main ginsenosides, Rg₁, Re, Rb₁, Rc, Rb₂, and Rd, in this experiment cannot account for the differences in the ability of *P. quinquefolius* extracts to decrease the number of MCF-7 cells. Although the total ginsenoside concentration was higher in *P. quinquefolius* roots grown at high temperatures compared to those grown at low temperatures, 61% for plants harvested in May ($P = 0.0233$) and 49% in roots harvested in September ($P = 0.0383$), the relative proportions of individual ginsenosides were not significantly affected by temperature treatment ($P > 0.05$ for all ginsenosides; Figure 2.3). Therefore, there must be compounds in root extracts of *P. quinquefolius* other than the six ginsenosides quantified in this experiment that are responsible for decreasing the number of MCF-7 cancer cells.

Our data are inconsistent with Murphy (2000), who identified ginsenoside Rc as the main ginsenoside from *P. quinquefolius* extract that is responsible for the inhibition of MCF-7 cancer cells. In this experiment, ginsenoside Rc was not significantly affected by temperature treatment and thus is not responsible for the decrease in the number of MCF-7 cells. Yun et al. (1996), on the other hand, were unable to identify the ingredient in *Panax ginseng* extracts that was responsible for decreasing the incidence of lung adenoma in mice,; and, like us, concluded that pharmacologically active compounds other than the main ginsenosides, Rb₁, Rb₂, Rc, Rd, Re, and Rg₁, must be present in ginseng extracts.

Despite the popularity and economic importance of ginseng, little is known about how environmental growth conditions may influence its pharmacological activity. In this study, a small difference in growth temperature ($\sim 5^{\circ}\text{C}$) elicited significant changes in the ability of *P. quinquefolius* extract to reduce the number of MCF-7 cells in culture, regardless of harvest date. Geographical growing location has been reported to cause differences in the ability of

Panax quinquefolius to modulate brainstem neuronal activity *in vitro*: the effect of American-grown *P. quinquefolius* was stronger compared to that grown in China (Yuan et al., 1998), and on a smaller geographical scale, *P. quinquefolius* cultivated in Illinois was more effective than that cultivated in Wisconsin (Yuan et al., 2001). In addition, Mizuno et al. (1994) reported that extract from wild *Panax ginseng* exhibited mitogenic activity to lymphocytes but extract from cultivated *P. ginseng* did not. Because it is clear that pharmacological activity is influenced by growth conditions, a large amount of variability in the potency of ginseng products is likely. Therefore, regulation and standardization of marketed ginseng is recommended.

Figure 2.1. MTS assay of the number of MCF-7 cells after 2 days of exposure to *Panax quinquefolius* extracts with total ginsenosides at 0 (control), 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2 mg/ml from plants harvested during the pretreatment in April. Bars are the mean $\pm$ SE ($n = 7-15$), and bars marked with an asterik (*) are significantly different from the control.

Figure 2.2. MTS assay of the number MCF-7 cells after 2 days of exposure to *Panax quinquefolius* extracts with total ginsenosides at 0 (control; gray bar), 0.075, 0.125, 0.25, 0.3, 0.4, 0.5, and 0.75 mg/mL from plants at low (closed bars) or high (open bars) growth temperatures harvested in May or September. Bars are the mean $\pm$ SE (n = 10-12). Bars marked with an asterik (*) are significantly different from the control.

Figure 2.3. Proportion of individual ginsenosides in roots of *Panax quinquefolius* grown in low or high temperature treatments in May and September. The total concentration of ginsenosides for each treatment (mg total ginsenosides/ mg dried root) is given below the figure and is the mean of three to four roots.

REFERENCES

- Amato, P., Christophe, S., & Mellon, P. L. (2002). Estrogenic activity of herbs commonly used as remedies for menopausal symptoms. *Menopause*, 9(2), 145-150.
- Attele, A. S., Wu, J. A., & Yuan, C. S. (1999). Ginseng pharmacology: multiple constituents and multiple actions. *Biochemical Pharmacology*, 58(11), 1685-1693.
- Barnes, P. M., Powell-Griner, E., McFann, K., & Nahin, R. L. (2004). *Complementary and alternative medicine use among adults: United States, 2002* (No. 343). Hyattsville, Maryland: National Center for Health Statistics.
- Blumenthal, M. (2001). Ginseng sales down 27% in mainstream markets; sales follow decreasing trend for total herb category. *HerbalGram*, 52, 58-59.
- Court, W. A., Hendel, J. G., & Elmi, J. (1996). Reversed-phase high-performance liquid chromatographic determination of ginsenosides of *Panax quinquefolium*. *Journal of Chromatography A*, 755, 11-17.
- Duda, R. B., Zhong, Y., Navas, V., Li, M. Z. C., Toy, B. R., & Alavarez, J. G. (1999). American ginseng and breast cancer therapeutic agents synergistically inhibit MCF-7 breast cancer cell growth. *Journal of Surgical Oncology*, 72, 230-239.
- Fournier, A. R., Proctor, J. T. A., Gauthier, L., Khanizadeh, S., Belanger, A., Gosselin, A., et al. (2003). Understory light and root ginsenosides in forest-grown *Panax quinquefolius*. *Phytochemistry*, 63, 777-782.
- Gillis, C. (1997). *Panax ginseng* pharmacology: a nitric oxide link? *Biochemical Pharmacology*, 54(1), 1-8.
- Kitts, D. D., & Hu, C. (2000). Efficacy and safety of ginseng. *Public Health Nutrition*, 3(4A), 473-485.
- Konsler, T. R., Zito, S. W., Shelton, J. E., & Staba, E. J. (1990). Lime and phosphorus effects on American ginseng:II. Root and leaf ginsenoside content and their relationship. *Journal of the American Society for Horticultural Science*, 115(4), 575-580.
- Li, T. S. C., Mazza, G., Cottrell, A. C., & Gao, L. (1996). Ginsenosides in roots and leaves of American ginseng. *Journal of Agricultural Food Chemistry*, 44, 717-720.

- Li, T. S. C., & Wardle, D. (2002). Seasonal fluctuations of leaf and root weight and ginsenoside contents of 2-,3-, and 4-year-old American ginseng plants. *HortTechnology*, 12(2), 229-232.
- Lui, J. H.-C., & Staba, E. J. (1980). The ginsenosides of various ginseng plants and selected products. *Journal of Natural Products*, 43(3), 340-346.
- Mizuno, M., Yamada, J., Terai, H., Kozukue, N., Lee, Y. S., & Tsuchida, H. (1994). Differences in immunomodulating effects between wild and cultured *Panax ginseng*. *Biochemical and Biophysical Research Communications*, 200(3), 1672-1678.
- Mudge, K., Lim, W., Lardner, J., & Jousselin, V. (2001). *Ginsenosides as quality indicators in woods-grown American ginseng*, Department of Horticulture, Cornell University, Ithaca, NY.
- Murphy, L. (2000). *Effects of America ginseng on breast cancer and prostate cancer cells*. Paper presented at the American Ginseng Production in the 21st Century, Cornell Cooperative Extension of Green County.
- Oh, m., Choi, Y. H., Choi, S. H., Chung, H. Y., Kim, K. W., Kim, S. I., et al. (1999). Anti-proliferating effects of ginsenoside Rh2 on MCF-7 human breast cancer cells. *International Journal of Oncology*, 14, 869-875.
- Sengupta, S., Toh, S. A., Sellers, L. A., Skepper, J. N., Koolwijk, P., Leung, H. W., et al. (2004). Modulating angiogenesis: the yin and the yang in ginseng. *Circulation*, 110(10), 1219-1225.
- Smith, R. G., Caswell, D., Carriere, A., & Zielke, B. (1996). Variation in the ginsenoside content of American ginseng, *Panax quinquefolius* L., roots. *Canadian Journal of Botany*, 74, 1616-1620.
- Tode, T., Kikuchi, Y., Kita, T., Hirata, J., Imaizumi, E., & Nagata, I. (1993). Inhibitory effects by oral administration of ginsenoside Rh2 on the growth of human ovarian cells in nude mice. *Journal of Cancer Research and Clinical Oncology*, 120, 24-26.
- Vogler, B. K., Pittler, M. H., & Ernst, E. (1999). The efficacy of ginseng. A systematic review of randomised clinical trials. *European Journal of Clinical Pharmacology*, 55, 567-575.
- Vuksan, V., Sievenpiper, J. L., Wong, J., Xu, Z., Beljan-Zdravkovic, U., Arnason, J. T., et al. (2001). American ginseng (*Panax quinquefolius* L.) attenuates postprandial glycemia in a time-dependent but not dose-dependent manner in healthy individuals. *American Journal of Clinical Nutrition*, 73, 753-758.

- Wolfson, D. (2002). Ginseng helps regulate blood glucose. *Nutrition Science News*, 7(1), 5.
- Yuan, C.-S., Wang, X., Wu, J. A., Attele, A. S., Xie, J.-T., & Gu, M. (2001). Effects of *Panax quinquefolius* L. on brainstem neuronal activities: Comparison between Wisconsin-cultivated and Illinois-cultivated roots. *Phytomedicine*, 8(3), 178-183.
- Yuan, C. S., Wu, J. A., Lowell, T., & Gu, M. (1998). Gut and brain effects of American ginseng root on brainstem neuronal activities in rats. *American Journal of Chinese Medicine*, XXVI(1), 47-55.
- Yun, T.-K., Lee, Y.-S., & Kwon, H.-Y. (1996). Saponin contents and anticarcinogenic effects of ginseng depending on types and ages in mice. *Acta Pharmacologica Sinica*, 17(4), 293-298.
- Zito, S. W., Konsler, T. R., & Staba, E. J. (1984). *Soil and mulch effects on ginsenosides in American ginseng plants*. Paper presented at the Proceedings 4th International Ginseng Symposium, Daejeon, Korea.

GENERAL DISCUSSION

This study provides a comprehensive understanding on the effects of elevated temperatures associated with climate change on *Panax quinquefolius*. Increased temperatures induced heat stress and reduced the efficacy of *P. quinquefolius* extract to decrease the number of estrogen receptor-positive MCF-7 cancer cells.

Photosynthesis and biomass production was lower in *P. quinquefolius* grown in elevated temperatures than in plants grown in low temperatures, as hypothesized. Throughout the growing season, stomatal conductance was reduced by the increased temperatures, subsequently decreasing photosynthetic rates in plants from the high temperature treatment. The photochemical efficiency of photosystem II, measured in July, was significantly lower in leaves from the elevated temperature treatment than those in the elevated temperature treatment, indicating that these plants were senescing prematurely (Liu & Huang, 2000). The accelerated leaf senescence reduced time available for plants to photosynthesize and acquire carbon. Therefore, in September, the over-wintering biomass of *P. quinquefolius* roots grown in the increased temperature treatment was half that observed in the low temperature treatment. Reductions in photosynthesis, stomatal conductance, and leaf longevity are general symptoms of heat stress (Berry & Bjorkman, 1980). Although large temperature extremes are generally associated with heat stress (Kirschbaum, 2000), our results suggest that even small increases in growth temperature may induce heat stress for understory species such as *P. quinquefolius*.

At the end of the growing season, the concentration of root ginsenosides was higher in *P. quinquefolius* grown in elevated temperatures than in low temperatures. However, the ginsenoside content, or overall amount of ginsenosides in each root, was not affected by growth temperature. Differences in the final over-wintering concentrations of root ginsenosides in *P. quinquefolius* grown in elevated or low temperatures can thus be attributed to dilution by the size of the root. We did not support our hypothesis that these secondary metabolites would increase in elevated temperatures.

The hypothesis that elevated temperatures would increase the ability of *P. quinquefolius* extracts to decrease the number of MCF-7 cancer cells was not supported. In fact, the opposite was observed. Extracts from plants grown at low temperatures decreased the number of MCF-7 cells at lower concentrations and were overall more potent than extracts

from plants grown at elevated temperatures. The ability of *P. quinquefolius* extract to decrease the number of MCF-7 carcinoma cells could not be explained by the observed differences in the total ginsenoside concentrations since temperature did not cause any significant changes in the relative proportion of individual ginsenosides. Therefore, compounds other than the six ginsenosides measured here (Rb₁, Rb₂, Rc, Rd, Re, Rg₁) must be affected by increased temperatures and are responsible for decreasing the number of MCF-7 cancer cells.

In conclusion, elevated temperatures projected by Global Circulation Models negatively affected the ecophysiology and efficacy of *P. quinquefolius*. This research indicates that increased global air temperatures associated with climate change may have detrimental effects on the ecology of sensitive understory herbs of the eastern deciduous forests, such as *P. quinquefolius*. Carbon acquisition of *P. quinquefolius* was strongly affected by elevated temperatures. Another important implication of this research is that growth conditions may influence the pharmacological activity of medicinal plants and thus should be considered for the safety, efficacy, and quality of natural products, such as those containing *P. quinquefolius*.

REFERENCES

- Berry, J., & Bjorkman, O. (1980). Photosynthetic response and adaptation to temperature in higher plants. *Ann. Rev. Plant Physiol.*, 31, 491-543.
- Kirschbaum, M. (2000). Forest growth and species distribution in a changing climate. *Tree Physiology*, 20, 309-322.
- Liu, X., & Huang, B. (2000). Heat Stress Injury in Relation to Membrane Lipid Peroxidation in Creeping Bentgrass. *Crop Science*, 40(2), 503-510.