CONTAINER PRODUCTION AND POST-HARVEST HANDLING OF LOTUS

(*NELUMBO*) AND MICROPROPAGATION OF HERBACEOUS PEONY (*PAEONIA*)

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CONTAINER PRODUCTION AND POST-HARVEST HANDLING OF LOTUS (*NELUMBO*) AND MICROPROPAGATION OF HERBACEOUS PEONY (*PAEONIA*)

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Daike Tian, son of Bangzhi Xian and Renwang Tian, was born on March 3, 1968, in Longshan, Hunan, People's Republic of China. He married Yuying Sang in 2000 and has a daughter Olivia I. Tian. He graduated from Longshan First High School, Hunan in 1989. He immediately entered Hunan Normal University (HNU) in the same year and graduated with a Bachelor of Science degree in biology in June 1993. After graduation, he worked for three years as a Teaching Assistant in the Department of Biology of HNU. In August 1996, he entered the Graduate School of Chinese Academy of Sciences (CAS) in Beijing and graduated with a Master of Science in botany from Kunming Institute of Botany (KIB), CAS, in August 1999. From 1999 to 2000, he worked as a Visiting Scholar in the Botanical Gardens of Toyama in Japan. After returning to China, he worked as Research Assistant at KIB, CAS for one year and then moved to Shanghai and worked in a horticultural company. In August 2004, he entered Auburn University as a Graduate Research Assistant to pursue a Doctor of Science degree in Horticulture.

CONTAINER PRODUCTION AND POST-HARVEST HANDLING OF LOTUS (*NELUMBO*) AND MICROPROPAGATION OF HERBACEOUS PEONY (*PAEONIA*)

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Effects of container soil level, fertilization, planting time and disbudding on lotus (*Nelumbo*) growth were evaluated in container production. Container soil volume influenced EC, pH, plant growth indexes, and plant nutritional contents. The ¼ and ½ soil levels were more efficient than ¾ soil level for lotus production in containers.

Fertilization significantly increased plant growth and also influenced plant nutrient contents. Fresh underground weight, propagule number, expanded internode number, and emerging leaf number of lotus grown in 29 liter containers linearly increased with increased fertilizer rates from 0 to 8 g of Pro•Sol 20-10-20 per pot and then leveled off at 12 g rate. There was no effect on plant height and flower number by fertility treatment. Fertilization also increased N, P and K and decreased Ca content in young leaves. Soluble fertilizers (Pro•Sol 20-10-20, Miracle-Gro 24-8-16 and 15-30-15) were more efficient for

lotus growth than both urea and controlled release fertilizer (Polyon 18-6-12). Miracle-Gro 15-30-15 (higher P rate) was more beneficial for rhizome or propagule production of lotus. Miracle-Gro 24-8-16 and Pro•Sol 20-10-20 were favorable for flower production. Flowering peak of lotus was influenced by the type or composition of fertilizers. Optimum container fertilization of lotus depends on cultivar, growth stage, fertilizer type and rate, container size, soil amount and water volume.

Lotus growth was largely affected by planting time and disbudding. Lotus potted in March and April performed best, while plants planted in February and May performed worst. Flower number was generally not influenced by planting time but flowering peak was different among treatments. Disbudding increased fresh underground weight and propagule number. Positive relationships were observed among emerging leaf number, fresh underground biomass, and propagule number.

Propagules could be stored in the cooler (4 ºC, 95% RH) over 45 days. Gum acacia, sphagnum-moss and Terra-Sorb® hydrogel had no effects on moisture and viability of stored lotus propagules. Successful surface sterilization and sanitation were critical.

Optimization of tissue culture protocol for commercial production of herbaceous peony was also investigated. Young stem segments were most favorable for callus induction. Adventitious shoots did not develop from callus and were only generated on apical or axillary meristematic regions. Young nodal stems and dormant-released buds were most suitable for shoot induction. TDZ was more effective than BA for shoot induction. Gibberellic acid (GA_3) had positive effects on explant growth, shoot induction and stem elongation. Plantlets rooted in IBA-containing medium or in PGR-free medium following a short pretreatment with high-concentration IBA.

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PART I PRODUCTION AND POST-HARVEST HANDLING OF CONTAINER LOTUS (*NELUMBO*)

Chapter 1 General Introduction and Literature Review

1 General Introduction

Lotus (*Nelumbo* sp.) is a perennial aquatic plant, often confused with the genus *Lotus* in the legume family. Lotus belongs to the genus *Nelumbo* Adans. The generic name is derived from the Sinhalese *Nelum*. Dumortier (1829) and Lindley (1833) proposed that *Nelumbo* should be placed under a new family Nelumbonaceae. However, for a long time, *Nelumbo* has been treated as a member of the family Nymphaeaceae. Recently, the monotypic Nelumbonaceae is becoming widely acceptable by taxonomists based on the evidences from comparative studies in morphology, anatomy, cytology, palynology, embryology, chemotaxonomy, molecular biology, and floral development (Li, 1955; Takhtajan, 1969, 1980; Kreune and Osborn, 1999; Hu et al., 2003; Tian and Zhou, 2006). It is commonly agreed that there are two species in *Nelumbo* (Wiersema, 1997; Fu and Wiersema, 2001): *N. nucifera* Gaertn. with pink, red or white flowers, and *N. lutea* Pers. with yellow flowers. However, Huang et al. (1992) first treated the latter as a subspecies, *N. nucifera* ssp. *lutea,* which was supported by molecular analysis of *rbcL* (Les et al., 1991) and by other researchers (Borsch and Barthllott, 1994; Hayes et al 2000; Hu et al., 2003). Recently, more researchers have agreed to the classification of one species and two subspecies. *N. nucifera* ssp. *lutea* (Willd.) Pers. is distributed from eastern North

America, extending south to Columbia. It is called American lotus as well as yellow lotus, water chinquanpin, yonkapin, and yanquanpin. *N. nuciferea* spp. *nucifera* is distributed all over Asia and Oceania, from Russia to Australia. Because wild *N. nucifera* and its cultivars with rich diversity are widely distributed in China, China is considered to be a natural distribution center of *N. nucifera* (Wang and Zhang, 2004). *N. nucifera* has many diverse common names. Besides lotus and sacred lotus, it also is often called as East Indian lotus, Egyptian lotus, Indian lotus, Chinese lotus, Oriental lotus, sacred water lily (Porcher et al., 1995-2020). There are about 60 nicknames in China for *N. nucifera* (Qi, 2006).

A total of about 1500 cultivars of all types of lotus are know in the world: > 800 cvs in China (Wang and Zhang, 2004; Qiu, 2005) and additionally 120 to 200 cvs of rhizome-lotus (Ke et al., 2000; Wangjia Lotus Plantation, 2008) and more than 22 seed-lotus varieties (Zheng, 2004); 989 cultivars in Japan (655 Japanese cultivars, 328 cvs from China including all 27 cvs of edible rhizome-lotus, and 8 cvs from other countries) based on the database of the Lotus Society of Japan (2008); and ≥ 60 cvs in India. In the United States, there are about 200 cultivars, about 85% of which are imported from China and other countries. Auburn University in Alabama has in its collection about 150 cultivars. In the *Nelumbo* database of Water Gardeners International under the title 'Lotus Names' constructed by Knotts (2008), 700 named-lotus cultivars are recorded. With the development of worldwide breeding programs, it is likely that more and more new cultivars will be released.

Lotus is one of the most ancient plants. It appeared in northern hemisphere some 135 million years ago based on fossil record (Wang and Zhang, 2004) and is considered

molecular living fossil (Sanderson and Doyle, 2001). The earliest fossil record of *Nelumbo* is assigned to the Cretaceous period (Fischer, 1996). Fossils of lotus have been excavated in many countries such as China, Japan, Russia, Canada, the United States, and Argentina. In North America, there are at least 30 fossil species of the Upper Cretaceous and Tertiary age that have been assigned to *Nelumbo* (Gandolfo and Cuneo, 2005). All fossil species of *Nelumbo* came from the Northern Hemisphere until 2005 when a fossil of *N. puertae* was found in Patagonia, Argentina, which is the only and oldest fossil of *Nelumbo* in the Southern Hemisphere (Gandolfo and Cuneo, 2005). The fossil records of *Nelumbo* indicate that lotus was much more widely distributed in the past than today. However, only two species or subspecies survived through the Ice Age. Now *Nelumbo* is more restricted in its modern distributions.

There is no argument on origination of *N. lutea* which is native to North America. On origination of *N. nucifera*, there is a dispute among researchers. Based on the first Indian fossil record of *N. nucifera* from the Pleistocene epoch (1.8 to 0.01 Ma) in Kashmir and presence of *Nelumbo* in the Tertiary period in Assam, *N. nucifera* was considered to be indigenous to India (Sharma and Goel, 2000). However, the fossil age of lotus leaf excavated from Linqu, Shandong of China was much older, about 15 Ma (AOEDPI and NGPI, 1978). Based on this finding, widely distributed populations of wild lotus in Northeastern China and other abundant evidences in archaeology, history and culture in China, Ni et al. (1987), and Wang and Zhang (2004) concluded that lotus was not originally native to India but China and the common name 'East Indian Lotus' was misinterpreted. Wild lotus populations are also widely found in India and the history of Indian lotus may be traced to about 4000 years ago based on scriptural and iconographic

records (McDonald, 2004). Further study is necessary to clarify the origin of *N. nucifera*. *Nelumbo nucifera* (lotus) is confused with Egyptian white water-lily (*Nymphae lotus* L.). Because of name confusion and other historical reasons (Conard, 1901), lotus (*N. nucifera*) has been mistakenly considered to have originated in Egypt and is continuously cited wrong by some online-database sources (Bramwell, et al., 2008) and publications. *N. nucifera* was probably introduced from India to Egypt about 500 BC (Slocum, 2005) but is no longer found in the Nile region. To avoid name confusion, the common name of *N. nucifera* could be better assigned as either eastern lotus or Asian lotus, but not as Indian lotus, Chinese lotus, Eastern Indian lotus or even Egyptian lotus.

Eastern lotus, *N. nucifera*, is widely grown as an economical crop in Asian countries: China, Japan, India, Korea, Nepal, Thailand, Vietnam, Sri Lanka, Philippines and Indonesia. Lotus has an amazingly long history. For at least 6000 years sacred lotus has been known by Chinese ancestors (Wang and Zhang, 2004). In 1972, two 5000-year old lotus seeds were found in the monuments of 'Yangshao Culture' in Zhengzhou, Henan. Lotus pollen fossils more than 7000 years old were found in 1973 among cultural relics excavated from the monument of 'Hemudu Culture' in Yuyao, Zhejiang. Based on literature record, Chinese ancestors began to cultivate lotus 3000 years ago. Lotus is also deeply rooted in Chinese culture. It occurred in the earliest collection of Chinese folk songs *Shijing* • *Chenfeng* about 6th century BC. Now lotus is honored as one of the ten most famous flowers and one of the four season flowers in China. *N. nucifera* is not only loved by the Chinese, but also has a very long history in other Asian countries such as India, Japan, Korea, Thailand, Nepal, Vietnam, Sri Lanka and Egypt. Numerous documents report that *N. nucifera* is possibly related to the plant '*soma*', which was used

for traditional use and worship in early Indian prehistoric Aryan civilization originated almost 4000 years ago (McDonald, 2004). The knowledge on the role of lotus in India may be traced back to the turn of the 8th century BC (Doniger O'Flaherty, 1967) and even back to the turn of the second millennium BC (Parpola, 1995; Witzel, 1995). Japan introduced lotus (hasu in Japanese) from China prior to 500 AD (Takahashi, 1994) and then introduced rhizome (renkon in Japanese)-lotus cultivars during 1192-1333 (Ni et al., 1987) and more rhizome-lotus varieties after the Meiji Era (1911-37). *N. nucifera* has been cultivated as an ornamental and food plant in Japan for more than 1,000 years (Masuda et al., 2006). In Thailand, lotus was cultivated by the King of Thailand Kingdom 700 years ago. *Nelumbo* has strong associations with the whole Oriental culture. Sacred lotus is the National Flower of India and Vietnam. Lotus represents divinity, purity, beauty, eternity and peace. *N. nucifera* is a symbol of Indian cultural heritage deeply associated with Hindu mythology, philosophy, art, architecture, poetry and culture since the immemorial time (Sharma and Goel, 2000). In Thailand, lotus is considered to be a source of three of four necessities: life, food, medicine, and clothing (Chomachalow, 2007).

Lotus is well-known for its vegetable, medicinal and ornamental uses. The starchy rhizome of American lotus was used as food by American Indian (Watson and Dallwitz, 1992). All plant parts of *N. nucifera* are consumed throughout Asia as food or used for medicinal purposes, including rhizome, nodes, roots, seed, young shoot, leaf, stalk, petal, stamen, and pericarp (or fruit receptacle, seedpod) (Ni et al., 1987; Nguyen, 2001; Wang and Zhang, 2004). Lotus is one of the major aquatic landscape plants. It is also planted in containers or big jars as well as used as cut flowers. The uses of lotus in the fields of

environmental conservation, tourism and public science education have been developing well, especially in China and Japan. On the other hand, because lotus is one of ancient living fossil plants and is the second oldest carbon-14-dated seed (beyond Judean date palm seed 2000 years old) to ever produce a plant (Kalman, 2005), it is of particular importance in research on plant origin and evolution. *Nelumbo* also has a potential value in bionics because of its superphobic leaves and may possibly be a good source of biofuel because of its starchy rhizomes. The values of lotus are discussed in more detail in the following literature review.

2 Literature Review

2.1 Basic Biological Research on *Nelumbo*

Lotus (*Nelumbo*) is one of the most valuable plants in the world because of its amazingly diverse economical and scientific values. Lotus has never received more attention than today (Fig. 1, 2) and is becoming more attractive in scientific research in Asian countries such as China, Japan, and India, and North America. China is not only the center of germplasm resources, cultivation, production, breeding and consumption of lotus, but also the center of scientific research at least on *N. nucifera*. The diverse uses of lotus range from food and medicine to ornamental and bioremediation, biofuels and other economic opportunities. Research at Auburn University focuses on production and postharvest storage of *N. nucifera*.

The seed of Lotus can maintain viability for a very long time. American lotus seeds may remain viable for decades (Steyermark, 1963). The longevity of sacred lotus seeds is found to be extremely long under special natural conditions. Viable seeds with life span between 100 and 1300 years have been reported (Ohga, 1923; Priestley and Posthumus, 1982; Shen-Miller et al., 1995; Li et al., 2000a). Ohga lotus, also Ohga hasu, has been produced in Japan from one of three seeds about 2000 years old based on carbon-dated wood of a prehistoric boat where the seeds were found (Godwin and Willis, 1964). However, the average seed longevity of other plants under laboratory conditions usually ranges from 2 to 10 years (Pristley, 1986). The mechanism of longevity from lotus seeds remains unknown but possible explanations were given by Li et al. (2000c) who reported that high level of superoxide dismutase (SOD) activities in seeds might benefit the long

life span of lotus seeds. Huang et al. (2003) found that lotus seeds had an unusually strong heat resistance and 100% germination rate of lotus seeds was obtained after 24 hr of treatment in a 100 ºC oven. The high stability of SOD in radicles observed under high temperature is possibly related to the longevity of lotus seeds. Biological characteristics of the centuries' seeds, germination, growth and development of the seedlings, and morphology of offspring have been studied by Shen-Miller et al. (1995, 2002), Li et al. (2000c), and Nagashima (2001). Metabolic activities in germinated ancient lotus seeds have been investigated by Maeda et al. (1996).

One of the most interesting phenomena in *Nelumbo* is its gas exchange system. The adaptability of lotus to anaerobic aquatic environments has been studied by several researchers. *N. nucifera* is able to adapt to the aquatic environment through a two-way gas transport system which may carry oxygen-rich air down to the rhizome and excess air back to the atmosphere through the leaves (Mevi-Schutz and Grosse, 1988a). The thermo-osmotic gas transport existing in *N. nucifera* need not rely on a difference in humidity between two sides of a porous partition, but may be linked causally to the temperature difference and pore size (Mevi-Schutz and Grosse, 1988b). The adaxial side of the leaf of *Nelumbo* has two distinct regions in terms of gas exchange characteristics. Air enters the leaf across the expanse of the lamina, and escapes back to the atmosphere through the highly porous region at the center of the lamina (Dacey, 1987). Gas canals channel air from the leaves throughout the petioles and rhizomes. Air from a leaf flows to a rhizome through one of two petiolar canal pairs, joining with the lowermost of three canal pairs in the rhizome through a chamber in the node. The lowermost canal pair links these nodal chambers along the length of a rhizome, allowing air from a node to flow

both forward, toward a growing shoot, and backward, toward preceding leaves (Matthews and Seymour, 2006). The ventilation or gas exchange system of lotus also has been investigated by Dacey (1987), Grosse et al. (1991), Ohkuma and Tatsumi (1992), Suetomo and Tatsumi (1993), Noda and Tatsumi (1998), Tastusmi et al. (1999), Vogel (2004).

The mechanism of thermal transpiration was reported by Grosse (1996). The transpiration rate of lotus was measured by the stem heat-balance method (Takagi et al., 2006). Since there is a linear relationship between transpiration rate and the leaf-to-air vapor-pressure deficit, with no apparent maximum, high vapor-pressure deficits (3.4 kPa at maximum) don't appear to cause significant stomatal closure in lotus plants. The stomata of lotus leaves play a role as air inlets to carry oxygen-rich air to the rhizome, so their low sensitivity would help to increase air intake. The contribution of the transpiration from shaded leaves and evaporation from the water surface is considered to be minor in the seasons when the leaves are fully developed.

Other physiological characteristics of lotus have been investigated during germination and plant growth. Zuo et al. (1997) reported changes of freeze fracture ultrastructure and 27 kD polypeptide of photosynthetic membranes of lotus seeds during germination under light. Photosystem development in dark-grown lotus was investigated by Ji et al. (2001). Petiolar photosynthetic carbon assimilation (PCA) rates, elongation rates, and chlorophyll content were assessed by Al-Hamdani and Francko (1992) who reported that PCA by elongating petioles could contribute to seedling growth, even under relatively cool dark conditions near the sediment-water interphase. Liu et al. (1999) investigated adaptation of lotus roots during ontogeny. Effect of fertilizer and shade treatments on the first leaf

formation from the first node of main stem in *N. nucifera* was studied by Nagashima (1977), who found that it is treatment dependent whether the first leaf becomes a standing leaf or floating leaf. During formation of swollen rhizomes, important physiological and biochemical changes were found in rhizomes, leaf blades, and petioles (Ding et al., 1995; Xu, 2002). Differences in endogenous hormones and polyamine were found in leaves and rhizomes of both healthy and stiff lotus, and polyamine production is possibly associated with formation of stiff lotus rhizomes (Li et al., 2000a). Diaphragmatic nets in lotus rhizomes could prevent internal flooding in water depths up to 2 m, which corresponds approximately to the depths at which natural stands of the lotus grow (Blaylock and Seymour, 2000). Tatsumi et al. (1999) observed temperature gradients on the leaf surface of lotus. Physiological and biochemical changes are reported in petals of *N. nucifera* during senescence (Kong et al., 2007).

Dormancy and growth of the American lotus, *N. lutea*, was investigated by Meyer (1930). Results showed higher temperatures from 20 to 30 ºC were found to greatly accelerate growth, while below 15 ºC, growth was very limited. Meanwhile, the development of plants also was largely influenced by pH and soil types. Masuda et al. (2006) that reported high temperature and long daylength accelerated vegetable growth and short daylength rather than temperature was the main environmental factor leading to induction of dormancy in lotus plants. Phytochrome played an important role in photoperiodic response of rhizome growth (Masuda et al., 2007): rhizome enlargement occurred under an 8 to 12-hr photoperiod while rhizome elongated under a 13 to 14-hr photoperiod. The process of enlargement and elongation could be changed by a 2 hr interruption of the night with different light quality. During formation of enlarged

rhizome, the dry mass, contents of starch, soluble sugar and soluble proteins showed a significant increase. Reducing sugar increased at early stage and decreased promptly at middle stage, and then remained at a steady level until the end stage of rhizome-enlargement (Li et al., 2003). Carbohydrate metabolism of over-wintering lotus rhizome in the field has been investigated by Li et al. (2006).

Epidermic browning of lotus rhizomes often occurred during common cultural practices. The amount of brown substances changed over season, which increased continuously until late-Sept. and then decreased steadily. Iron (Fe) was dominant in brown substances and application of compost played an important role in diminishing brown phenomenon (Uchiyama et al., 1977). Resistance difference in lotus to the rhizome rot caused by *Fusarium* was investigated by Shimomura et al. (1955). The postharvest physiology of lotus rhizome and cut flower also has been much studied especially in China.

2.2 Lotus-effect and Bionic

Asian lotus (*N. nucifera*) is revered as the symbol of purity mainly based on the self-cleansing property of its leaves. This property of self-cleansing has been studied thoroughly (Lai, 2003) and is ascribed to the interaction between the surface of lotus-leaves and water, resulting in high water-repellency of the surface. Due to the impressive demonstration of these self-cleansing and high water-repellency characteristics by *N. nucifera*, this combined effect has been dubbed the “Lotus-effect” by Barthlott (1997). A surface which shows the Lotus-effect is superhydrophobic, expressed by a contact angle larger than 150º. The leaves of a lotus plant show epidermal cells on its rough surface

covered with wax crystals. The wax crystals provide a water-repellent layer, which is enhanced by the surface roughness according to the models of Wenzel (1936) and Cassie (1944).

The discovery of the Lotus-effect is of great technological interest. By transferring this effect to artificial surfaces that can be cleaned by a simple rainfall, it has been making numerous technical applications possible (Barthlott and Neinhuis, 1998). The self-cleaning property of lotus has, in recent years, stimulated much research effort worldwide, and many artificial surfaces have been synthesized with this self-cleaning property (Cheng et al., 2006; Liu et al., 2006; Guo and Liu, 2007; Lee and Kwon, 2007; Kim et al., 2008).

2.3 Ecology, Competition and Environmental Impact

Nelumbo is one of most important aquatic plants and has a critical role in ecological systems of wetlands, lakes, reservoirs and ponds. Distribution of lotus is limited by water depth where it grows. The deepest water level recorded is about 2 - 3 m for wild lotus (Unni, 1971ab, 1976; Kunii and Maeda, 1982; Wang and Zhang, 2004). The growth of lotus is affected by water level and its fluctuation. Nohara and Tsuchiya (1990) and Nohara (1991) reported that a sudden rise of 1.0 m in the lake water level after a typhoon in August caused a biomass reduction in *N. nucifera* in Japan. Fluctuation in water level and spatial difference in water depth may also be important factors determining the vegetation area and survival of *N. nucifera* and other floating-leaved plants.

Both positive and negative effects of *Nelumbo* in ecology have been widely reported. Like some of other aquatic plants, lotus has a strong ability for nutrient removal in water

and soil. Sastroutomo (1982) reported that positive correlation of total biomass of lotus with total nitrogen (N) and available phosphorus (P) in the soil, while total biomass of hydrophyte communities is negatively correlated with P. Macrophyte vegetation (including lotus) played important roles in denitrification of the nutrient dynamics of shallow sand dune lakes (Fukuhara, et al., 2003). Lotus co-cultured with tilapia or cultured alone in ponds was able to effectively remove nutrients from old pond mud. Annual nutrient losses from mud in a 1-ha pond were about 2.4 t of N and 1 t of P, among which about 300 kg of N and 43 kg of P were incorporated in lotus biomass (Yi et al., 2002). However, the ability of P removal for lotus is relatively low. Reeder (1994) reported that *N. lutea*, which covered about 23% of the wetland, was responsible for transforming approximately only 61 $kg \cdot year^{-1}$ (0.1 $g\ m^{-2} \cdot year^{-1}$) of P based on the studies of the role of autotrophs in nonpoint source phosphorus retention in a coastal wetland in Ohio. After finishing a life cycle, the leaves of *Nelumbo* demonstrated an 80% weight loss in 8 months. The percentage of N, P, and ash in decomposing leaves generally increased over time (Wylie, 1985) which made detritus as a short-term nutrient sink that concentrated nutrients for detritus-based secondary production or conserved them for later uptake by trees in wetlands.

Nelumbo is also considered to be an invasive plant or weed in some countries (Lancar and Krake, 2002) possibly based on its high productivity, fast growth, and suppression of other aquatic species during competition. The interactions of *Nelumbo* and other aquatic species have been studied by many researchers. Mastrantuono and Mancinelli (1999) reported that increasing trophy and introduction of an exotic species, *N. nucifera*, are largely responsible for the rapid qualitative reduction of the macrophyte population and

modification to non-rooted plants in shallow lakes. Some negative effects of *Nelumbo*-colonization on the invertebrate fauna indicated that further growth of this plant would cause a rapid change in the lake. Phytoplankton growth suppression by *Nelumbo* also suggested a nutrient uptake competition (Fukuhara et al., 2006). *Nelumbo* was competitively dominant in low salinity, fertile conditions, while *Potamogeton* and *Nymphaea* exhibited the greatest salinity tolerance and germination potential. Both the intensity and importance of competition of *N. lutea* were high within low-salinity, fertile conditions, but decreased as salt and nutrient stress increased (Adair, 1997). Competitive interactions between *Myriophyllum spicatum* and *N. lutea* were reported by Snow (2000) in a container experiment within a pond. *M. spicatum* dominated early but was then suppressed by *N. lutea*.

Negative effects of *Nelumbo* are also recorded in aquaculture. Burlakova and Karatayev (2007) found horizontal distribution of unionids to be limited by dense beds of invasive and noxious macrophytes (mainly *M. spicatum* and *N. lutea*). Mussel densities were significantly lower in these macrophyte beds ($P < 0.001$). In the pond with the lowest density of macrophytes (stonewort *Chara sp*.), unionids were distributed more evenly, and the average unionid biomass was the highest among all ponds studied.

2.4 Heavy-metal Assimilation and Removal

Nelumbo is not only a good bioindicator of trophic changes and ecosystem deterioration but also a good species for assimilation and removal of heavy metals. Bioaccumulation of heavy metals by *Nelumbo* has been widely investigated. Sun et al. (1987) studied absorption and accumulation of heavy metal pollutants in *N. nucifera*.

Lotus showed strong ability to absorb and accumulate all tested 6 heavy metal elements: Fe, Mn, Cu, Zn, Pb, and Cd. The contents of heavy metals were generally higher in the nodes than in the internodes while the content of Cd in the nodes is about 7 to 12 folds of that in the internodes. Cd possibly did not move after accumulation in the nodal position. The role of *N. lutea* in copper removal in wetlands was evaluated by Lung and Light (1996). The ability of *N. nucifera* in chromium removal and in bioassay was assessed by Vajpayee et al. (1999) using a few modifiable physiological responses. Plants grown in different chromium concentrations (50 to 200 μM) showed appreciable amounts of chromium accumulated in lotus tissues, maximum being in roots. Higher chromium accumulation in plant tissues resulted in a significant inhibition in chlorophyll, protein contents, and in vitro nitrate reductase activity in tested plants. Kanabkaew and Puetpaiboon (2004) reported that wastewater treatment system with lotus showed better removal efficiency than a system with *Hydrilla verticillata*. Therefore, lotus could be an alternative aquatic plant for wastewater treatment. Because of its ability of heavy metal accumulation, *N. nucifera* was used to investigate water quality of reservoirs which was possibly responsible for chronic renal failure in Sri Lankathe (Bandara et al., 2007).

Because of its ability of heavy metal assimilation, lotus planted in a polluted water body can not be used for food. Kumar et al. (2002) reported that the accumulation of Cu and Zn was very high in the rhizomes, fruiting torus, and carpels of *N. nucifera*. An average 5197 $\mu g \cdot g^{-1}$ of Cu and 3509 $\mu g \cdot g^{-1}$ of Zn (d.w.) were observed in rhizomes and 4743 $\mu g \cdot g^{-1}$ of Cu and 519 $\mu g \cdot g^{-1}$ of Zn (d.w.) in fruits, which were all higher than the prescribed limit of metal intake by humans as established by the World Health Organization. Li and Sun (2006) investigated concentration of six heavy metals (Pb, Cd,

Zn, Cu, Mn, and Cr) in different tissues of lotus harvested from a campus pond. Both Cd and Zn concentrations exceeded 228 and 43 folds of China National Food Safety Standard, respectively. The results indicated that pond water was contaminated by chemicals from the research labs.

Although lotus has the high ability of assimilation of heavy metals, the average accumulation of trace metals in *Nelumbo* is relatively little compared with other high heavy-metal-assimilation aquatic plant species. Ebrahimpour and Mushrifah (2007) investigated concentrations of heavy metals (Cd, Cu and Pb) in five aquatic plant species in Malaysia: *Lepironia articulata*, *Pandanus helicopus*, *Scirpus grossus*, *Cabomba furcata* and *N. nucifera*. Lotus contained the lowest or the second lowest concentrations of these metals. Nirmal Kumar et al. (2008) compared concentration of six heavy metals (Cd, Co, Cu, Ni, Pb and Zn) in seven species aquatic plants in Kanewal reservoir: *Eichhornia crassipes*, *E.colonum*, *Hydrilla verticillata*, *Ipomoea aquatica*, *N. nucifera*, *Typha angustata* and *Vallisneria spiralis*. The highest average concentrations of six trace elements were measured in *E. crassipes* and the lowest in *N. nucifera*. Since lotus is often dominant where it grows, the total assimilation of heavy metal may be higher than that in other species because of high biomass.

2.5 Propagation

Lotus is usually planted in ponds, rice fields, containers, and shallow lakes for different purposes. The optimal time for planting is between late March and early May depending on the local climate. Lotus can be propagated by one of the following methods: seeds, enlarged rhizomes, running rhizomes (straps), single-nodal buds, stem terminal

buds, and tissue culture. Because of permeability barrier of the extremely rigid seed coat, lotus seeds must be treated physically or chemically before sowing to favor germination. The plants from seeds can finish a full life cycle (seed-to-seed) within one year. There is no large difference in the plant growth between seed and rhizome propagation. However, some researchers reported that seedlings required at least two and sometimes probably as many as four years for floral development (Meyer, 1930). This conclusion (lack of flower in a single season) was possibly resulted from specific environmental conditions, because plant size and floral development of lotus are largely influenced by temperature, soil or medium type, container size, and nutrition availability. In fact, propagation by seed leads to plant development in a short period for a large area and saves time, labor, and large cost as compared to rhizomes. Propagation by seed also is very useful in breeding programs. In addition, propagation of lotus by seed can decrease occurrence of disease without sacrificing yield (Zhou et al., 1989). However, seeds are not encouraged for use in conservation or production of a true-to-type species or variety because of natural seedling variability. Seeds are not available for double-flower cultivars and some non-flowering rhizome lotus.

For propagation by expanded rhizomes, the farmers in Asian countries traditionally use the entire rhizome (containing the main rhizome and all branches), also called ‘parent’ rhizome, as a single propagule. In fact, both the ‘son’- (primary branches) and ‘grandson’- (secondary or third branches) rhizomes also are suitable for propagation, and the plants from this propagation method perform as good as from the parent-rhizome method (Wang and Zhang, 2004). Li (2004) compared effect of propagation methods using whole rhizome, main rhizome, secondary rhizome, top rhizome, and terminal nodal

buds. There was no significant difference in yield. Terminal nodal buds and top rhizomes can save large costs of stock plants. Peng et al. (2004) investigated effects of micro-rhizomes and regular rhizomes for use as propagules on lotus growth and yield. There was no large difference in plant height, leaf number, although the highest yield of enlarged rhizomes was observed in the propagation method using large rhizome propagules. The heeling practice of lotus using small propagules and terminal nodal buds was discussed by Wang (2007).

Propagation by division of running-stem (non-enlarged rhizomes or straps) is a method conducted during the growing season. This method can help to make up for died plants, save stock rhizomes and cost, and increase efficiency. Propagation via running stems can also prolong the flowering period of plants (Wang and Zhang, 2004). Katori et al. (2002) reported that the days to flowering were significantly shorter ($P \leq 0.01$) in the strap method than in the enlarged rhizome method. However, the rhizome strap method delayed the flowering time in each cultivar and the population flowering time was cultivar dependent. Plants generated by the rhizome strap method produced larger flowers ($P \leq 0.05$). This method may prolong the availability of rhizome propagules therefore enables a more flexible scheduling of propagation tasks.

Besides traditional propagation methods, tissue culture may potentially provide an alternative approach for lotus propagation. Tissue culture of lotus has been reported by researchers mainly in China, Japan and Thailand. Liu (1948) investigated regeneration ability of excised lotus plumules. Francko (1986a) reported that a germination of 98% in inoculated seeds was obtained. The seedlings elongated and differentiated normally in sterile liquid culture. Callus was induced from buds, cotyledons and young leaf explants

on Murashige and Skoog medium (1962), and somatic embryos were successfully induced from callus (Arunyanart and Chaitrayagun, 2005). Shoots failed to directly generate from callus induced from immature embryos, green plumule leaves and young cotyledons, but could be directly induced from plumule leaves (Ke et al., 1987a). Terminal buds were more efficient than axillary buds for shoot induction (Luo et al., 2004a). Shoots and plantlets were successfully obtained through stem tip culture (Yamamoto and Matsumoto, 1986, 1988; He and Liu, 1987). When in vitro plantlets were subcultured every 30 days the number of plants remarkably increased by 47-fold within 90 days of culture (Kakuyama and Ogawa, 1997). Acclimation of lotus plantlets before transplanting was evaluated by Sun (2005). After transplanting, about 75% of plantlets survived (Liu et al., 2002; Luo et al, 2004b). Genotype effect was investigated by Liu et al. (2002) and Pan et al. (2004). Tetraploid lotus (4n = 32) was produced through in vitro culture with colchicine treatment (Yamamoto and Matsumoto, 1990). Mutation induction of tissue cultured lotus by γ- and X-ray irradiation was investigated by Arunyanart and Soontronyatara (2002).

2.6 Cultivation and Production

American lotus is not widely planted as a crop or ornamental as is Asian lotus and it usually grows in wild areas. Asian lotus has an extremely long history in cultivation as a vegetable, medicinal, and ornamental plant in Asian countries. Recently, *N. nucifera* is becoming a potential crop in Australia (Nguyen, 2001), New Zealand (Follett et al., 2003) and the United States (Tian et al., 2006). Lotus is usually planted in a tilled pond or rice field for vegetable production. It is also planted often in bowls, containers, small ponds,

and lakes for landscape use. Cultivar selection and cultivation techniques are dependent on where lotus is planted. Large cultivars are planted in ponds and lakes for vegetable and seed production, or for landscape uses. Medium and small cultivars are usually planted in containers and water gardens for ornamental use. China is the largest country to produce and consume lotus. Currently, the planting area of rhizome lotus is about 5 to 7 million hm^2 in China. The total yield of edible rhizomes is about 6 million t.

Wild lotus can be naturally distributed at sites with water depths up to 2 to 3 m (Unni, 1971a, 1976; Kunii and Maeda, 1982; Wang and Zhang, 2004), which was supported by tank planting experiment (Nohara and Kimura, 1997) in that no petioles of *N. nucifera* elongated in 3 to 5 m depths of water. Most cultivated lotus generally can not survive in pond with water depth > 1.8 m (Zhuang et al., 2003) and is usually grown better in water < 1.5 m deep (Wang and Zhang, 2004). The survival decreased with increasing planting depth and the biomass of plants is significantly reduced from 0.5, 1.0, to 1.5 m water depth in tank experiments (Snow, 2000). Lotus planted in shallow water generated higher yield (Chen et al., 2007) and the ideal water depth was approximately 10 to 20 cm (Nguyen, 2001). However, optimum water depth is plant size dependent. Small-medium size varieties grow better in shallow water with a depth of 5 to 50 cm, so called shallow-water lotus, while large size varieties grow better in water with a depth of 50 to 100 cm, so called deep-water lotus (Zhen, 2007).

Lotus germinates or sprouts at temperatures above 13 ºC (Sou and Fujishige, 1995) and prefers warm climates. Higher temperatures from 20 to 30 ºC have been found to greatly accelerate plant growth, while below 15 ºC, growth of *N. lutea* was very limited (Meyer, 1930). Optimal temperatures are 22 to 32 ºC for lotus growth (Yang et al., 2006).

N. nucifera can endure high temperature of 41 ºC and continuous temperatures above 35 ºC for 20 days (Wang and Zhang, 2004). For lotus production in tunnel houses during cool seasons, 28 to 30 ºC soil temperatures were suitable. When soil temperature was below 18 ºC and ambient temperature was less than 20 ºC, plants almost stopped growth (Li et al., 2000b).

Lotus performs much better under full sun than in shady places. Li et al. (2000b) reported that increase of light intensity improved leaf greenness, stalk thickness, and flower number of lotus in tunnel houses for winter production. Based on results from a tank-experiment, the total biomass increased significantly with increasing light, although the survival of *N. lutea* seedlings was high in all tested light levels (Snow, 2000).

Soil is an extremely important ingredient for plant nutrition. The soil type is probably the most important factor in proper development of *Nelumbo*, and loam soil is much better than sandy soil (Meyer, 1930). Heavy garden loam containing a high percentage of clay with very small particles is best and topsoil is always good for water garden plants. A potting soil high in organic matter is not recommended because of it will float out of the containers and does not anchor the plant roots very well (Bosmans, 1994). Lotus has a wide adaptability to soil type, but, based on the studies by Wuhan East Lake Flower and Bonsai Research Institute, lake soil (pH = 6.5) is better than mountain mud (pH = 5.6) and garbage soil (pH = 7.4) (Wang and Zhang, 2004). Li and Qian (1994) compared effects of media (peat-cobble double-layer medium, yellow sand, stone powder, pebble, and soil) and nutrient solution on lotus and found that peat with cobble cover was the best for lotus growth. It has also been reported that competition of *N. lutea* with other aquatic

plants was high in low-salinity, fertile conditions, but decreased with increased salt and nutrient stress (Adair, 1997).

Lotus can tolerate a considerable pH range, from 4.5 to 9.0 (Meyer, 1930). The plant is not much affected by pH range from 5.5 to 8.0 in water. Nguyen (2001) reported that suitable electrical conductivity (EC) levels for a lotus plant at the vegetative stage of growth are between 2.8 and 3.1 $mS \cdot cm^{-1}$. However, it is questionable based on our studies (Tian, 2008), which indicated that EC should not often exceed 1.0 $mS \cdot cm^{-1}$ even for large lotus plants. A safe EC value for lotus is also dependent on plant size, growing season and temperature. *N. lutea* may be capable of limited bicarbonate assimilation at alkaline pH, and CO_2 represents the preferred dissolved carbon source in this species. Photosynthetic carbon assimilation rates in submerged shoots generated from seeds at pH 4.5 were 50-fold higher than those at pH 8.5. The pH-dependent C-assimilation curves in sections of plants grown in acidic and alkaline liquid media were statistically similar (Francko, 1986b).

2.7 Production of Vegetable Lotus

China is the largest country to produce both seeds and rhizomes of lotus (*N. nucifera*). The growing area of lotus in China is estimated at 0.3 million ha (Li et al., 2005). Based on 22.5 $t \cdot ha^{-1}$ of average yield, the capacity to produce lotus rhizome is up to 6.75 million t per year. The lotus rhizomes imported from China represent about 70% of volume of the total marketed lotus rhizomes in Japan and Korea (Li et al., 2005). Japan produced 82200 t of lotus rhizomes in 1982 on an area of 6350 ha but reduced that to 71900 t on 4900 ha in 1998 (Nguyen, 2001). Korea produces less lotus rhizomes than Japan. For lotus seed

production, 8000 to 14000 t per year were produced in China in the 1990s, 60% of which comes from Hunan and Hubei provinces (Anonymous, 2006).

Recently China has developed new technologies to increase yield of lotus, such as production in tunnel houses, off-season production using early-ripening varieties, shallow-water production, and improvement of variety quality. The yield of lotus rhizomes has increased from 500 to 750 kg·mu^{-1} (7.5 to 10.3 t·ha^{-1}) in 1980s to 1000 to 2000 kg·mu^{-1} (15 to 30 t·ha^{-1}) by traditional methods and even up to 3000 to 3500 kg·mu^{-1} (45 to 52.5 t·ha^{-1}) by new technologies for rhizome production, and from 40 to 50 kg mu^{-1} (0.6 to 0.75 t·ha^{-1}) in 1980s to 60 to 80 (100) kg·mu^{-1} [0.9 to 1.2 (1.5) t·ha^{-1}] for seed production.

2.8 Production of Ornamental Lotus

Ornamental lotuses are usually collected and limitedly produced by the lotus-related research institutions (China Lotus Research Center, Wuhan Botanical Gardens and Nanjing Zhongshan Botanical Gardens in China, the University of Tokyo in Japan, Auburn University in USA), aquatic botanical gardens (Sawara Municipal Aquatic Botanical Garden in Japan), and limited number of aquatic plant nurseries (Perry's Water Gaderns in North Carolina, Slocum Water Gardens in Florida, Springdale Water Gardens in Virginia of USA, Nanjing Yileen Water Garden in Jiangsu, Sanshui Lotus World in Guangdong, and Waterlily World in Shandong of China, Liang Lian Water Garden and Blue Lotus Water Garden in Australia). Production of ornamental lotus is at a very small scale in the world and plants are usually sold using dormant rhizomes. Lotus cut flower is mainly produced in Thailand and ranked the 3rd of cut flowers in 1994 in this country

(Sahavacharin, 1998). Mass production and sale of container lotus is just beginning in the United States (Creamer, 2008).

2.9 Planting Techniques

Many technologies have been developed for lotus production to obtain maximum yield and economical gain. Wen (1987) reported that discarding of terminal buds of main stock stem increased the total number of rhizome branches, rhizome yield and leaf number, while decreasing the length of the main rhizome. Lotus is often planted with incorporation of aquaculture to increase profits. Sang et al. (1994) compared effects of five models on the total yield of fish and lotus, a '回' (a Chinese character) style model produced the highest gain. A highly efficient cultivation technology-Tectorial Planting method was developed by Shen et al. (2001), which increases yield, advances harvest time, saves water, and improves soil quality. Lotus yield can be increased by 31% by incorporating crop residue chips in the soil (Min et al. 2006). Lime is often used in lotus cultivation to increase yield because of its advantages on soil sterilization, pH adjustment, and nutrient provision (Wang, 2007). Other new techniques, such as double-cropping lotus in spring and fall (Qing and Huang, 2005; Qing et al., 2007), and double cropping with other crops (Wang and Liang, 2001; Wei et al., 2006), have been used in China. Use of honeybees can markedly increase yield in seed production (Ke et al., 1987b).

Lotus is usually planted between late spring and early summer, and its growth ends in the fall. Yield of lotus can be predicted through foliar-age model (Zhou et al., 1994) which benefits the lotus producer to better prepare products for market. Recently, to meet the demands for an early market, out-season cultivation methods have been developed.

The availability of vegetable rhizomes could be advanced to June by a technique of growing edible lotus in the earlier season (Fu et al., 1994). Lotus flower generally blooms from June (May) to August, but the population flowering time can be extended to early October when plants are propagated by dividing growing plants in the middle of July (Deng et al., 1990). It also is feasible to prolong the flowering time of lotus through winter when plants are planted in a heated greenhouse (Li et al., 2000b). Out-of-season cultivation of lotus is discussed by Zhang (2003). Time from bud occurrence above water to opening can be controlled between 10 and 15 d by changing cultivation conditions in the greenhouse (Yang et al., 2006). If the techniques of advancing and delaying flowering are incorporated, three cycles of flowering are possible in one year (Wang and Zhang, 2004). Production of lotus cut flowers may be on a year-round schedule but water level should be adjusted to meet the requirements of flower quality based on growth stages (Chomchalow, 2004). The floating cultivation method provides a possibility to beautify a lake of more than 1.8 m water depth (Zhuang et al., 2003).

2.10 Fertilization

Lotus is a fertilizer consuming plant. The seedling can normally grow up to 4-leaf stage in pure water without fertilizer. However, if no fertilizer is added after 4-leaf stage, the seedling will die soon (Chen and Zhang, 1985). It is recommended that fertilizer applications should be split into 4-5 applications as young plants have been observed to burn quite readily (Nguyen, 2001). In the past, traditional organic fertilizer is usually the only choice for lotus production. Currently, organic fertilizer is still a major source of fertilizers for lotus production in China while chemical N-P-K fertilizers are mainly

applied in Japan (Sou and Fujishige, 1995). Song et al. (2006) investigated the effects of balanced fertilization on lotus production and suggested that the N-P-K integrated fertilizers should be applied together with B, Cu, and Fe for lotus. The optimal dose is 18-24 kg of N, 6 kg of P_2O_5, 12 kg of K_2O, 1 kg of B (sodium tetraborate), 2 kg of $CuSO_4$, and 3 kg of Fe_2SO_3 per 667 m^2. Li and Qian (1994) compared the effects of nutrient solution formulations on lotus planted in soil-alternative media and found an optimal nutrient solution with 1:0.25:0.8:0.7:0.2 of N-P-K-Ca-Mg. A single basal application of coated fertilizer not only produced almost the same yield of lotus rhizome but also saved 29% nitrogen and reduced 41% effluent nitrogen fertilizer compared with conventional fertilization method (Orimoto and Takai, 2007).

Potassium-fertilizer significantly increases yield of lotus, the highest production 1511 kg per 667 m^2 is found in soggy soil when applied at 15 kg of K-fertilizer, and 1651 kg per 667 m^2 in meadow soil when applied at 10 kg of K-fertilizer (Zhang et al., 1994). Li et al. (2002a) compared effects of K-fertilizers on production of lotus. It showed incorporated application of NPK and Fe, B fertilizers increased plant growth and yield, while K-fertilizer increased flower number, growth of leaves and rhizomes. The suitable dose of K-fertilizer is 120 to 180 $kg \cdot hm^{-2}$. Comparative effects of combinations of N, P, and B-fertilizers also were investigated by Li et al. (1998) who found the ratio of N18-K8-B1 produced the highest yield. Lotus is a Mn-loving plant, and increased Mn at the suitable levels benefited lotus growth (Li and Qian, 1994). Meanwhile, lotus is Mn-tolerant and no visible toxic symptom occurred for plants with 1340 to 3200 ppm of Mn in tissue. Qu and Zhao (1991) investigated the characteristics of nutrient absorption for

non-flower lotus and found the absorption ratio of N-P-K in rhizomes was about 2.73: 1: 5.2 during the enlargement of rhizomes.

Hicks (2005) reported effects of the major nutrients N, P, K and Ca on lotus growth. Tentative critical concentrations for 90% growth of 2.66% N, 3.9 $g \cdot kg^{-1}$ of P, and 9.97 $g \cdot kg^{-1}$ of Ca, were estimated. Toxicity concentration values of 4.25% N, 6.00 $g \cdot kg^{-1}$ of P, and 19.30 $mg \cdot kg^{-1}$ of Ca were estimated. Adequate supply ranges were 253 to 439 ppm of N, 20 to 60 ppm of P, and 82 to 195 ppm of Ca. The adequate supply range and critical concentration for K could not be determined. Leaves were considered to be the most appropriate organs for field sampling and analysis, having the greatest incidence of sensitivity to nutrient variation.

2.11 Nutrient Disorders

Except for the limited information online available, the nutrient disorders of lotus are less reported in formal publications. The following symptoms of nutrient disorders in *N. nucifera* were evidenced under the trials at the University of Western Sydney (Hawkesbury) and accurately reflected similar disorders in other plants (Nguyen, 2001).

Nitrogen (N) deficiency symptoms first appear on older leaves as an even chlorosis (yellowing) across the entire leaf blade as plant relocates nitrogen to the growing tip. Severe N deficiency will result in stunted plants. Toxic levels of nitrogen will burn the centre of the leaf blade. Lotus is very sensitive to P levels and P fertilizer must be applied with care. Plants deficient in P display a darkish green which gives way to a purplish mottling (anthocyanosis) of new leaves. The leaf will turn entirely purple under severe P stress, before an even necrosis (blackening and dehydration) of the leaf starts at the leaf

margin. Growth of plants will also be appreciably slower, though often this is not as apparent as leaf symptoms. Toxicity symptoms are indicated by deformation of new leaves which will fail to open. Potassium (K) is needed in large amounts as lotus requires K for flowering and rhizome production as well as other regulatory and metabolic functions during growth. When K is deficient, the chlorotic patches are initially seen around the entire leaf margin of older leaves. This yellowing then extends inwardly within the confines of the leaf veination before turning necrotic, from which comes necrosis, a curling of the leaf margin.

Symptoms of magnesium (Mg) deficiency initially exhibit a chlorotic mottling between leaf veins in older leaves, as the growing tip draws mobile Mg from mature leaves. Extensive deficiency will show an increased yellowing of the entire leaf blade, which could be easily mistaken as N deficiency. Deficient leaves will eventually become necrotic if unchecked. Calcium (Ca) deficiency in lotus is similar to Mg, where chlorotic mottling of older leaves becomes apparent giving way to a bronzed spotting. It differs in that leaves become stiff and brittle. The root system will also be affected, stunting between nodes, browning of the root hairs and necrosis of some of the growing tips has been observed. Iron (Fe) deficient leaves will exhibit an even chlorosis interveinially on younger leaves, leaving a dark skeleton-like appearance to the veins. Deficiency may be due to an imbalance of other nutrients.

It is also reported that high concentrations of chromium reduced chlorophyll, protein contents of *N. nucifera*, and in vitro nitrate reductase activity (Vajpayee et al., 1999). The toxicity of hexavalent chromium on *N. lutea* seedlings was investigated by Francko et al. (1993).

The nutrition deficiency of lotus can be corrected by application of the related fertilizers. However, foliar application will be ineffective due to super hydrophobicicity of lotus leaves. Solutions to nutrition toxicity of lotus have not been reported.

2.12 Weeding

Weeds compete with lotus for both space and nutrients. Control of aquatic weeds may be by physical removal during cropping and by draining off water or use of a herbicide during non-crop periods (Nguyen, 2001). Lotus may tolerate some herbicides at a very low dose. It was reported that *N. nucifera* did not show visual toxicity to the herbicide simetryn at 2.4 $\mu g \cdot L^{-1}$ (Nohara and Iwakuma, 1996). However, lotus may be very sensitive to other herbicides even at low concentrations, therefore, application of herbicide targeting on the weeds in lotus's surrounding area must be done carefully otherwise it may cause damage to untargeted lotus by herbicide drifting (Tian, 2007). Glyphosate isopropylamine (41%) can be used before planting lotus to control weeds. After 7 to 10 d of planting, one of the following herbicides, 50% Prometryn 1500 $g \cdot hm^{-2}$, 60% butachlor EC 1000 $mL \cdot hm^{-2}$, and 12.5% Oxadiazon EC 3000 $mL \cdot hm^{-2}$, can kill 80% to 90% of weeds when water level remains at 3 to 5 cm for 5 to 7 d during application (Shao et al., 2003). Control of *Eichornia crassipes* and *Typha sp*. can be achieved using simazen (3 to 6 ppm) or monuron (4 to 12 ppm) (Nguyen, 2001).

Lotus is sometimes considered to be an invasive plant or weed in some countries (Lancar and Krake, 2002). Lotus grows very fast and *N. lutea* colony can grow 15 to 30 m each year (Hall and Penfound, 1944; Rogers, 1981; Grodowitz et al., 2001). The negative influence of *Nelumbo*-colonization is largely responsible for the decrease of

both species richness and number of other aquatic plants and invertebrates in the colonized area because of large floating leaves of *Nelumbo* shading the underlying zone (Mastrantuono and Mancinelli, 1999). American lotus can be cut and removed by hand or other mechanical methods but is difficult to control physically because it can reestablish from seeds and rhizomes. Excellent herbicides to kill *N. lutea* are 2,4-D butoxyethyl ester, 2,4-D dimethylamine (DMA), and dichilobenil. Endothall dipotassium salt (K_2) and endothall dimethylalkylamine salts also have good effects for control of American lotus (Westerdahl and Getsinger, 1988). Ye et al. (1975) recommended destroying the deteriorated population of seed lotus by spraying 75 to 125 g per 667 m^2 of 72% 2,4-D Butyl ester ($C_{12}H_{14}C_{12}O_3$).

2.13 Overwintering

Rhizomes of lotus can survive through a short period of low temperatures between 0 ºC and -7 ºC, therefore nothing has to be done for winter protection in most warm regions. Cold or freeze-resistance ability of lotus is water level dependent. When lotus plants (rhizomes) are covered with suitable depth of water, they can survive through winter at -12 ºC (Wang, 2007) and even at -30 ºC (Wang and Zhang, 2004).

To avoid possible damage by freezing temperature, lotus propagules can be harvested before freezing season and stored with mud in a container at 3 to 7 ºC temperature. By this method, the survival rate from December to the next February is about 95%. A similar storage method with sand and water under 4 to 15 ºC room temperatures produces 90% survival rate (Wang and Zhang, 2004).

2.14 Pests and Diseases

Pests of lotus have been mainly reported in China, Japan, the United States, Thailand and Australia (Ni et al., 1987; Nguyen, 2001; Nguyen and Hicks, 2004; Wang and Zhang, 2004; Luo, 2006; Chomachalow, 2007). The major pests include: aphid (*Rhopalosiphum nymphaeae*) which occurs nearly every year and sucks sap from the new, young leaves, buds and stalks and reduces plant vigor; cotton worm (*Spodoptera litura*), the caterpillars of which eat lotus leaves. Lotus is ranked as the secondary favorite food of *Spodoptera litura* in 51 species crops and grasses investigated in China (Qing et al., 2004). American lotus borer (*Ostrinia penitalis*) does great damage on leaves of *N. lutea* (Sohmer and Sefton, 1978) and its biology is reported by Ainslie (1922); thrips (*Scirtothrips dorsalis*), both its larvae and imagos feed on young lotus tissues and cause severe damage (Zhang et al., 2004); *Frankliniella intonsa*, a leaf feeding pest found in Japan (Wakiya, 1988); leaf caterpillar (*Simyra conspersa*), it chews the leaves into ragged pieces; lilypad leaf beetle (*Donacia sp.*), its larvae reside and feed *N. lutea*, particularly rhizome (Schneider and Moore, 1977); caterpillars of *Heliotbis sp*. is the most important pest of lotus in Australia (Nguyen and Hicks, 2004).

Other less damaging pests are golden apple snail (*Pomacea canaliculata*), Taiwan small snail (*Radix swinhoei*), Amazonian snail (*Ampullaria gigas*), red spider mites, red swamp crawfish (*Procambarus clarkia*), two-spotted mite, and mealy bug. Adzuki bean weevil (*Callosobruchus chinensis*) was found to feed on dried and shelled lotus seeds (Furusawa, 1987).

The control of most of above mentioned pests is discussed by Ni et al. (1987), Nguyen (2001), and Wang and Zhang (2004). Feeding behavior and control of four major

lotus pests in China were reported by Luo (2006). The pests such as caterpillars may be controlled by hand at the early stage. When a heavy problem occurs, one must rely on application of pesticide. However, it is difficult to advise growers on optimal rates of application of specific chemicals as no chemicals have been registered for use with lotus and their respective withholding periods are unknown. Further, some spray oils and sprays derived from plants had phytotoxic effects on plant foliage (Nguyen, 2001).

Nelumbo nucifera is subject to few diseases. Disease is much reported in China and Japan because lotus is planted in a large scale as a major crop in these two countries. Lotus rhizome rot disease caused by *Fusarium oxysporum* sp. *nelumbicolum* (Nishikado and Watanabe, 1952; Nishizawa, 1954a) as well as possibly by *Pythium sp.* (Nishizawa, 1954b) are the most dangerous diseases causing large losses in production (Shimomura et al., 1955). It is only found in varieties planted in shallow ponds (Wang and Zhang, 2004). Other common diseases include: (1) brown strip disease, which is caused by pathogen *Alternaria nelumbii* and occurs on leaf, especially standing leaves. This disease is often seen in nursery lotus plants. When this disease happens, small yellow strips occur on leaves in the beginning and then gradually spread on the whole leaf. Under severe situations, the leaves may die (Wang and Zhang, 2004); (2) leaf spot, which causes dark patches on the pads and may eventually, if unchecked, cause leaf blades to die. Rarely is the entire plant affected. This disease can be caused by powdery mildew (*Erysiphe polygoni*), *Cercospora sp*., *Ovularia sp*. and *Cylindrocladium hawkesworthii ssp*. (Nguyen, 2001); (3) leaf dry spot disease, commonly seen on both floating and standing leaves. This disease is caused by *Phyllosticta hydrophylla sp*. Yellow spots occur on diseased plant leaves in the beginning and then grow into small to big brown patches

(Wang and Zhang, 2004); (4) lotus leaf black spot, caused by *Gloeosporium nelumbii*. The pathogen may cause purple, brown to black spots on leaves in the beginning and may eventually cause death of leaf blade (Nishikado and Watanabe, 1955); (5) lotus streak virus, rhabdovirus, is found in lotus in Japan (Yamashita et al., 1985). This virus causes black spots or streaks on rhizomes and chlorotic or ring spots on leaves; and also (6) waterlily crown rot, a serious disease caused by a species of *Phytophthora*. The fungus causes crown and stem base to black and rot and the disease can quickly spread through a pond, with early symptoms indicated by yellowing of leaves (Nguyen, 2001).

Lotus diseases and their control by chemical and physical approaches are discussed by Ni et al. (1987), Nguyen (2001), and Wang and Zhang (2004). Phytotoxicity of lotus leaves is only reported by application of some fungicides (Kanaiso and Mizuguchi, 1993).

2.15 Harvest and Postharvest

The time for rhizome harvest is July-September for early season varieties and October-March for late season varieties (Sou and Fujishige, 1995). In pond production, lotus is often harvested by a handy fork-like implement for a small-size production. Sometimes it is harvested by hand, but hand harvesting is difficult and physically strenuous. In Japan, mechanic harvesting began in 1970s and it totally replaced labor harvesting in 1990s (Sou and Fujishige, 1995). In China, although a mechanical lotus digger was developed in 2000, currently manual labor harvesting is still a major method. Lotus seeds are harvested by hand when seeds or seed pods turn black then they are processed by mechanical tools for removal of the seed coat and embryos.

Lotus can survive through winter in many regions without any other protection under

suitable water levels (Wang and Zhang, 2004). However, after harvest, enlarged edible rhizomes lose quality quickly during storage because of continued water loss, shrinkage, browning or decay (Yu et al., 2002ab; Zhan and He, 2006). Maintenance of freshness of *N. nucifera* rhizomes has been well studied in the food storage industry (Liu et al., 2000; Wang and Li, 2002; Wang and Zhang, 1994; Xu et al., 2003a; Yu et al., 2002ab). Low temperature is particularly important in lotus storage. Enlarged rhizomes used as vegetables can be stored up to 150 d at 6 to 8 ºC low temperatures with 95% to 100% RH (Anonymous, 2004), whereas, rhizomes usually have a shelf life of only 2 weeks at room temperature (Ong, 1996) and can be stored in soil for only 10 to 30 d (He et al., 1998). Lotus rhizomes keep their quality best at temperatures between 3 ºC and 7 ºC and have storage potential for up to 5 to 6 weeks. Rhizomes stored at temperatures below 3 ºC have shown evidence of surface scalding and may exhibit signs of chilling injury but this has yet to be confirmed. An acceleration of disease presence is seen at temperatures above 7 ºC, though weight loss in rhizomes is insignificant until temperatures increase above 12 ºC. Temperatures above 15 ºC break rhizome dormancy and shoots may begin growing (Nguyen, 2001).

Advances on storage and physiology of postharvest edible lotus rhizomes are discussed by Xu et al. (2003b). Lotus rhizomes can be stored in sand, plastic membrane covers, water, and soil. Browning is a big problem of stored lotus. Effect of mudpacking on appearance and structure of lotus rhizomes was investigated by Kawasaki et al. (2005). The catalytic oxidation of PPO is a main reason causing browning and senescence of lotus rhizomes during storage (Yu et al., 2002b). The relationship of enzymatic browning and its main substrates in fresh-cut lotus rhizomes were investigated by Yu et al. (2002a).

The effects of temperature, pH and inhibitors on PPO activity in fresh-cut lotus are discussed by Li et al. (2002b). Low temperature, sodium sulfite, and ascorbic acid are strong inhibitors of PPO activity, and consequently benefit storage of lotus rhizomes (Huang and Tian, 2002). Package materials also have effects on storage of lotus, PA/PE film bags (0.06, 0.08 mm) are much better than polyethylene bags (0.08 and 0.1 mm) (Wang and Li, 2002). Yuan et al. (2004) investigated effects of oxygen, temperature, and package techniques on physiological and physical characteristics (respiration, total sugar, reducing sugar, weight loss, browning, and surface appearance) during storage. Optimal usage of amylase in pretreatment of lotus rhizomes was evaluated by Zhang et al. (2005) with Response Surface Method. Lin et al. (2006) reported heterogeneity of lotus-rhizome starch granules under degradation of α-amylase. Effects of pressurized cooking methods on changes of chemical compositions and textures of lotus rhizomes were reported by Chiang and Luo (2007). Nutritional quality of electron bean-irradiated lotus seeds was studied by Bhat and Sridhar (2008).

Shipping and storage of cut lotus flowers are usually conducted in hot summer. Darkening and dropping of lotus flower petals caused by high temperatures is a serious problem. Low temperatures at 5 to 10 ºC are effective to maintain normal appearance of lotus flowers in combination with plastic film packing (Ito and Kito, 1998). Lotus flowers usually have 3 to 4 days of life span under natural conditions. High transpiration, quickly increased water loss, and quick decrease in water absorption ability cause a short vase life of cut flowers (Zhang and Liu, 2005). Application of an optimal preservative combining with low temperature 8 to 10 ºC can prolong the longevity of fresh cut flowers up to more than 9 d (Wang et al., 2003). Ethephon induces premature senescence of sacred lotus

flowers and also increases water uptake, whereas 1-MCP reduces water uptake and weight loss, delays browning and prolongs vase-life of flowers (Suanphairoch et al., 2006). Effects of chemical solutions, spraying thiosulfate before harvesting, precooling treatment, harvest time and other factors on vase-life of cut lotus flower have been investigated in Thailand (Roogtiwa, 2001; Pichayanon, 2001; Makthong, 2001).

2.16 Breeding

Flower-lotus was first cultivated in China at least 2500 years ago for ornamental purposes to meet the entertainment needs of emperors and kings (Wang and Zhang, 2004). In 1808, the first book on lotus, *Hong He Pu* (by Zhongbo Yang), described characteristics, classification and planting techniques of 33 varieties in China. Currently there are about 1500 cultivars in the world, most of which are bred in China, Japan and America. However, it remains unknown at what time the humans began to breed new cultivars of lotus.

Most of lotus varieties are bred by traditional breeding methods. Several breeding approaches were evaluated by Kong et al. (1994), which include four traditional approaches: direct selection from population, selection of terminal bud mutation, naturally crossed seeds, and artificial crossing. From a lake lotus population, a triploid plant ($2n = 3x = 24$) was found in Wuhan of China in 1983 and new varieties with small sizes were produced from it (Wang and Zhang, 2004). From vegetative plants of Chinese rhizome lotus, Kasumi et al. (2002) bred two new varieties ‘Kasumigarua’ and ‘Hayaka’ by mutation selection.

Lotus is highly compatible in hybridizing not only within *N. nucifera* but also between two species, *N. nucifera* and *N. lutea* (Huang and Xu, 1981). Huang (1983) successfully bred three varieties of bowl lotus by crossing, and the breeding of small-size lotus is discussed by Huang (1984). Kasumi and Sakuma (1998) investigated the relationships of flowering, pollination, fertilization and seed formation, and found that the high efficiency of seed formation was obtained by crossing between the pistils of flowering day and the pollens of one day after flowering. The seed formation rate was markedly decreased after pollination by pollens which have been stored for 7 d at 29 ± 3 ºC, however, there was no significant difference in seed formation between fresh pollens and those stored at -20 ± 1 ºC for 14 d (Kasumi et al., 2000), therefore the artificial pollination period can be extended by low temperature stored pollens.

Besides traditional breeding approaches, new lotus varieties are produced through treatment with physical and chemical mutagens. In 1981, 'Dian E Zhuang' was bred from seeds treated by 1000 R gamma rays and 'Dan Ding Yu Ge' with more sepals and petals was produced from the Co irradiated seeds (Wang and Zhang, 2004). The tetraploid *N. nucifera* (4n = 32) was produced by Yamamoto and Matsumoto (1990) in Japan through in vitro colchicine treatments. Recently, China has launched outer space breeding projects on crops including lotus. In 1994, 442 seeds of 13 cultivars of lotus from Jiangxi were treated in outer space and the new varieties such as 'Space No. 1', 'Space No.2', 'Space No.3', and 'No. 36' were produced (Wang and Zhang, 2004). In 2005, a local farmer in Chongqing began a five-year breeding program ($0.6 million) on space lotus with treatment of outer space conditions, and the 3000 seeds of 300 varieties would be

included (Jiang and Lu, 2005). A new breeding method through ion implantation is newly applied to lotus in China (Xie et al., 2004).

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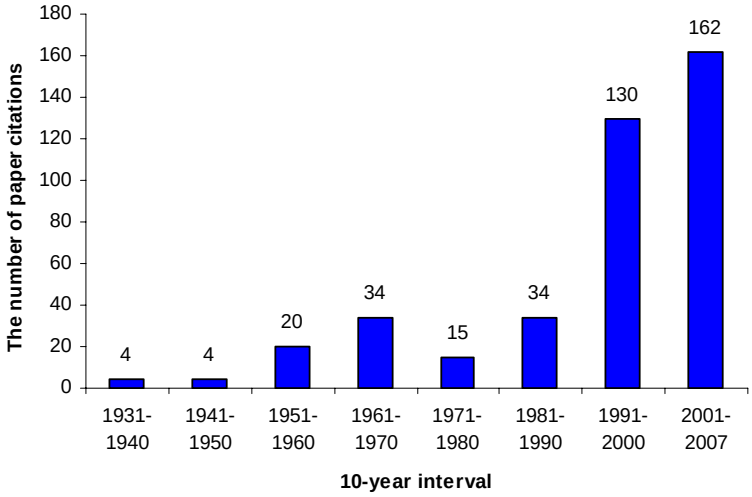

Fig. 1. Citation of papers (1931–2007) on lotus (*Nelumbo*) in CAB abstract (A bibliographic database compiled by CAB International). The result was based on searching by key word '*Nelumbo*' on March 8, 2008.

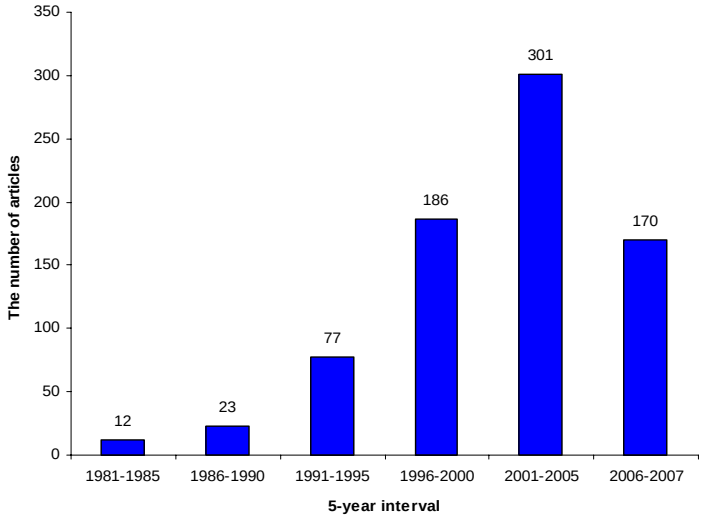

Fig. 2. The number of articles (1981–2007) on lotus (*Nelumbo*) in CNKi (China National Knowledge Infrastructure, the largest Chinese journal database). The result was based on searching by Chinese character 'Lian Ou' (lotus) in article title on March 8, 2008.

Chapter 2 Response of Lotus (*Nelumbo)* to Soil Level in Containers

Additional index words. *Nelumbo nucifera*, *Nelumbo lutea*, pH, electrical conductivity, propagule, plant growth index.

Abstract

Effect of the soil level on containerized lotus (*Nelumbo*) production has not been documented. Four lotus cultivars were investigated for growth response to container soil level in this study. Electrical conductivity, pH, plant growth indices, and plant nutritional content were influenced by soil level. Differences in some plant growth indices were significant between lotus grown in containers with ¼ soil level and higher soil levels (½ and ¾ container levels) but mostly were not different between ½ and ¾ soil level treatments. Lotus planted in ¼ soil level usually produced the greatest plant height and fresh underground weight, while the largest number of propagules often occurred in containers with ½ or ¾ soil level. The highest number of emerging leaves was observed in plants with ¼ or ½ level soil treatments, and no significant difference was found in emerging leaf number between lotus grown in ½ and ¾ level soil. Flower number generally decreased as soil level increased. The ¼ and ½ soil levels were more efficient than ¾ soil level for lotus production in containers.

Introduction

Lotus (*Nelumbo*) is an aquatic perennial plant of great economic importance especially in Asian countries. Lotus rhizomes from *N. nucifera* Gaertn. are one of the major vegetables in China, Japan and Korea. The growing area of lotus in China is estimated at 300000 ha, with production of approximately 6.75 million tons of lotus rhizomes per year based on an average yield of 22.5 ton ha^{-1} (Li et al., 2005). As an ornamental, flowering-lotus has been cultivated in pools and containers for at least 1600 to 2500 years (Wang and Zhang, 2004). Although lotus has a long history in container cultivation, only seeds and rhizomes are sold as propagules for retail purposes. Containerized plants have rarely been marketed in the horticultural industry (Creamer, 2008).

Studies on propagation and production of lotus in ponds or rice fields have been well documented (Qu and Zhao, 1991; Sou and Fujishige, 1995). Shallow water is conducive for lotus growth (Chomchalow, 2004) because temperatures in a water-soil system increase quicker in shallow water than in deeper water. However, the soil level is usually not a controllable factor in field production and lotus is free to grow to its maximum depth. In container production, lotus performance will be affected by container size, soil level and water level. Soil medium not only anchors the plants but also provides nutrition for plant growth. Little soil volume may not stabilize lotus plants well and can not provide enough space for extension of rhizomes and roots during development. Too much soil may not benefit plant growth while adding to shipping and handling costs as well as extra labor costs. Therefore, the optimal soil amount or soil level must be considered before planting lotus based on container size and plant cultivar to obtain

maximum profit. Levels of 2/3 and 3/5 soil volumes in containers for planting bowl lotus were reported by Li and Qian (1994) and Wang and Zhang (2004), respectively. However, information about effects of soil level on growth of lotus in containers is not available. The major objective of this study is to investigate how soil level influences growth indices of containerized lotus. Results will provide useful guidelines for container production of lotus in nurseries.

Materials and Methods

Three cultivars of lotus (*Nelumbo nucifera)*: 'Embolene' (medium size with pink flowers), '98 Seed' (unnamed hybrid, large size with red flowers) and 'Karizma' (medium size with white-yellow flowers), and American lotus *N. lutea* were investigated. The first experiment was conducted in 2004. Lotus rhizome-propagules of 'Embolene' and '98 Seed' were divided from stock plants with young leaves, and planted in 29 L (7.5 gal) black plastic containers (bottom 31 cm and top 37 cm diameter, 32 cm height) without holes on May 17, 2004. Each pot was planted with one propagule, usually with two internodes. Containers were filled to ½ level (1/2 depth of container, 16 cm, 13 L) and ¾ level (3/4 depth of container, 24 cm, 20 L), respectively, with natural sandy loam soil. After planting, all pots were filled with tap water (pH 7.0, EC = 0.13 mS cm^{-1}). Containers were placed under full sunlight with 25 cm spacing.

Fertilizer was applied three times once every twenty days beginning on June 9, 2004 when plants had at least several floating leaves and possibly one or two standing leaves. The last fertilizer application was applied on July 21, 2004. Soluble fertilizer Pro•Sol 20-10-20 (20% total nitrogen, 10% available phosphate, and 20% soluble potassium)

(Pro•Sol Inc, Ozark AL 36360, USA) was added as 4 g (1 tsp)/pot each time. Water solution samples were taken one hour before fertilization and 24 h after fertilization, respectively, in the late afternoon from the same pots to monitor the nutrient status. Before taking samples, pots were irrigated to nearly full container volume of water by hand to assure the same water level for all treatments. On August 23, young fully expanded leaves were sampled for nutrient analysis.

The second experiment was started on April 1, 2007. *N. lutea* (ordered from Perry's Water Gardens in NC, USA) was planted in 29 L containers filled with ¼ (8 cm, 6 L), ½ (16 cm, 13 L), and ¾ (24 cm, 21 L) level of sandy loam soil. 'Karizma' was planted in 21 L (5.5 gal) white plastic containers (bottom 26.5 cm and top 28.5 cm diameter, 36 cm height) with ¼ (9 cm, 5 L), ½ (18 cm, 10 L), and ¾ (27 cm, 15 L) level of the same type of soil. Fertilizer (Pro•Sol 20-10-20) was diluted to a 10% (w/v) solution with tap water and applied on a 20-day interval schedule: 4 g (May 10), 6 g (May 30), 8 g (June 20), 10 g (July 10), and 4 g (July 30) per pot. pH and EC were measured with Hanna instruments HI 9811, PH/EC/TDS meter on the 1st and 20th day following fertilization. The data on plant height and emerging leaf number were taken on August 20. Flower number was recorded once every 4 days until no flowers developed. Plant rhizomes were harvested on Nov. 10, 2007. Expanded rhizomes of *N. lutea* were sampled immediately following harvest on Nov. 20th and used for nutrient analysis.

The experiments used a completely randomized design with six replications. Data collected included plant height, the number of emerging leaves, flowers, propagules (normally containing 2 or 3 internodes), expanded internodes, and fresh weight of underground parts (rhizomes and roots). Plant height was measured from the bottom of

containers to the top of plant. The number of expanded internodes was calculated based on the number of swollen internodes with a diameter larger than 1.5 cm. All samples of water (for 2004 experiment only) and plant tissues were analyzed by Soil Testing Laboratory at Auburn University. Water pH was determined at 25 ºC using a Fisher Accumet Model 50 pH meter. Nutrient elements of water were determined simultaneously by Inductively Coupled Plasma (ICP) Atomic Emission Spectrometry using a Varian Vista-MPX Axial Spectrometer (Maxfield And Mindak, 1985). Plant samples were dried in forced air oven at 60 ºC (140 ºF) for 72 h to determine moisture content and dry mass. Mineral nutrients of plant tissue were analyzed by Inductively Coupled Plasma Emission Spectroscopy using a Varian Vista-MPX Axial Spectrometer (Isaac and Johnson, 1985; Plank, 1992; Odom and Kone, 1997). Total nitrogen was analyzed Elementar Vario Macro CNS Analyzer (Columbo and Giazzi, 1982). Nitrate-nitrogen was analyzed colorimetrically with a Thermo Spectronic Genesis 20 Spetrophotometer (Bremner, 1965). Data were analyzed by SAS 9.1 (SAS Institute Inc. 100 SAS Campus Drive, Cary, NC 27513-2414 USA) and mean differences were examined using Tukey (HSD) procedure at 0.05 significance level.

Results and Discussion

After addition of water to containers, soil levels decreased 2 to 6 cm in height depending on the initial soil level and container type. In the first experiment, the pH slightly increased from 7.0 to 7.27 three weeks after planting. The pH decreased after fertilization and increased as nutrients were absorbed by plants (data not shown). EC increased sharply after fertilization and was higher in the ¾ soil level treatment than in

the ½ soil level treatment for both cultivars (Fig. 1). EC decreased as plants grew and nutrients were absorbed. After the first or second fertilizer applications, absorption rates of N, P, and K were 99% to 99.6%, 93% to 96.4%, and 80.1% to 96.2%, respectively (Table 1). This suggested N was almost completely utilized by plants, and 4 g/pot of Pro•Sol 20-10-20 every 20 d might not be enough to meet the maximum growth potential of lotus. The nutrient absorption rate also increased after the second fertilizer application because plants grew faster during this period than in the period following the first application. No large differences were observed in macronutrients (N, P, K, Ca, and Mg) of young leaf tissue between the ½ and ¾ level soil treatments for both 'Embolene' and '98 Seed'. However, micronutrients in leaf tissue were generally higher in the ¾ soil level than in the ½ soil level treatment possibly because increased soil volume offered increased availability of minor nutrients (Table 2). A difference ($P < 0.05$) in B and Mn contents between two soil level treatments was found for both selections. '98 Seed' lotus grown in higher level soil (¾ level, 18 cm) showed a decrease in flower number (Table 3). Other plant growth indices were not significantly affected by soil level. However, both 'Embolene' and '98 Seed' lotus grown in containers with ¾ soil level generally produced more propagules and expanded internodes.

In the second experiment, 40 days after planting, pH increased slightly from 7.0 to 7.4-7.7 (½ to ¾ soil level) for *N. lutea* in 29 L (7.5 gal) containers (Fig. 2) but remarkably increased from 7.0 to 9.02-9.22 for 'Karizma' in 21 L (5.5 gal) containers (Fig. 4). An increase of pH in water was possibly caused by proliferation of some species of algae, nutrient changes in water-soil system or metabolism of plants. Increase in water pH in white containers also was possibly related to container's translucence since some light-

green lotus rhizomes caused by weak light were observed in 'Karizma' during harvest. pH decreased after fertilization and increased as nutrients were absorbed during the period of the first 3 applications of fertilizer. Some exceptions occurred in the earlier application period for 'Karizma', pH values generally were in the range of 6.0 to 7.0. EC increased sharply after fertilizer was applied then dropped as nutrients were absorbed by plants (Fig. 3, 5). EC was highest level in containers with the ¾ soil level because of lower water volume and higher concentration of nutrients. EC was lowest in containers with the ½ soil level because of more water and greater dilution factor.

Responses of lotus plants to the soil levels were different for *N. lutea* and 'Karizma' (Table 4). The lowest ¼ soil level treatment resulted in *N. lutea* producing the largest values in plant height (92.6 cm), standing leaf number (27.4), fresh underground weight (1495.8 g), fresh weight of total propagules (694.6 g), and the maximum diameter of internodes (3.66 cm). There were no significant differences in growth indices between ½ and ¾ soil level treatments in *N. lutea*. Although the largest fresh underground biomass was found in the ¼ soil level treatment, the largest number of propagules occurred in the treatment with the ½ soil level. Therefore, for the purpose of propagule production, containers filled with ½ level of soil would be best. It was interesting to observe that *N. lutea* failed to develop flowers in all treatments. The failure of flowering was possibly due to over fertilization. However, for the plants of *N. lutea* grown in larger containers supplied with less fertilizer, only one flower developed in four containers. Also, *N. lutea* did not perform well in Wuhan Botanical Gardens of China which has similar climate as South Alabama (personal communication). Therefore, American lotus might be more sensitive to environmental conditions. *N. lutea* performs well in ponds and shallow lakes

in the wild, but appears unsuitable for container production. Further research is necessary to investigate the reason for failure of flower bud formation of *N. lutea* in containers.

Nutrient analysis of expanded rhizome tissue showed that the ¼ soil level treatment resulted in *N. lutea* with the highest values of Fe and Cu contents but usually the lowest values of the other nutrients (Table 5). Differences between the ¼ soil level treatment and two other treatments with higher level of soil were observed in Ca, K and Mg (macronutrients), and in Al, Na and Mn (micronutrients). However, there was no difference in growth parameters between lotus in the ½ and ¾ soil level treatments. Although Fe content in expanded rhizomes of *N. lutea* sharply ranged from 90.8, 67.8, to 29.7 ppm as soil level increased from ¼, ½ to ¾ levels, the mean difference was not statistically significant because of large variation observed among the individual samples. The difference of Fe content between the highest and lowest individual values was extremely high and reached 8.3 fold. This difference was possibly caused by fertilizer toxicity due to high EC in containers with higher soil level. However, visual symptoms of Fe deficiency in leaves did not occur. Larger differences among treatments were also seen in Mn, Zn, and Cu contents. Obviously, a large sample size of lotus rhizomes would better evaluate these parameters.

'Karizma' grown in containers with ¼ soil level treatment produced the largest fresh underground weight (994.8 g/pot), as well as flower number (7 flowers/pot) (Table 4). The highest fresh weight of total propagules, and the largest number of propagules and expanded internodes were found in plants receiving ½ soil level treatment. However, there was no significant difference in all plant parameters of 'Karizma' grown in containers with ¼ and ¾ soil levels.

Natural loam soil is a good medium for planting lotus in containers. Results from both experiments suggested that soil level influenced lotus growth. Although plant height and the underground fresh weight for both *N. lutea* and 'Karizma' were the largest in containers with lowest soil level (1/4 level), the number of propagules was the lowest. Lotus grown in half level (about 16 to 18 cm) of soil produced the largest number of propagules.

Total availability of nutrients, especially micronutrients, increased with increase in soil amount. Soil level may influence plant growth through nutrient availability. In addition, soil level influenced plant growth indirectly by changing factors such as water level, EC, and pH. EC was remarkably affected by soil-amount related water level when the same rate of fertilizer was applied. Plants experienced different nutrient stresses due to an interaction of fertilizer rate and water level. Besides a genotypic effect, the inconsistent results between two experiments in the treatment with ½ or ¾ soil level were also possibly caused by different fertilizer stresses which were low in the preliminary experiment but high in the second experiment. Fertilization of lotus should be dependent on water level (volume) in containers for the optimal growth of plants since interactions may exist between the factors including soil level, water level, fertilizer rate, EC, pH, temperature, and plant growth. Future study is necessary to determine the relationships of these factors and the major effects on growth of lotus.

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Tables

Table 1. Influence of soil level on concentration (ppm) and nutrient absorption rate (%) of N, P, K based on water samples in 29 L containers for the first two fertilizer applications with 4 g of Pro•Sol 20-10-20 at a 20 d interval (2004).

Lotus cultivar	Soil level	N (%)		P (%)		K (%)	
		½ L	¾ L	½ L	¾ L	½ L	¾ L
Embolene	6/10/2004	21.1	40.7	4.3	6.4	29.2	42.7
	6/29/2004	0.2	0.3	0.3	0.3	5.8	6.3
	AR	99.1	99.3	93.0	95.3	80.1	85.2
	6/30/2004	27.6	40.6	8.3	9.7	34.6	49.8
	7/19/2004	0.1	0.1	0.5	0.5	5.1	5.9
	AR	99.6	99.8	94.0	94.8	85.3	88.2
98 Seed	6/10/2004	29.0	43.8	4.8	7.6	24.0	44.3
	6/29/2004	0.3	0.2	0.3	0.3	1.7	5.3
	AR	99.0	99.5	93.8	96.1	92.9	88.0
	6/30/2004	27.6	40.6	8.3	9.7	34.6	49.8
	7/19/2004	0.1	0.1	0.3	0.3	0.8	1.9
	AR	99.6	99.8	96.4	96.9	97.7	96.2

Note: L – soil level based on 29 L containers (32 cm height); AR – nutrient absorption rate (%).

Table 2. Effect of soil level in 29 L containers on nutrients of young leaves of lotus sampled 70 days after planting (2004).

Cultivar	Soil level	Macronutrients (%)					Micronutrients (ppm)						
		N	Ca	K	Mg	P	Fe	Al	B	Zn	Cu	Na	Mn
Embolene	1/2	1.87	1.30	1.15	0.38	0.17	39.2	26.2	17.7	10.5	8.7	257.0	1580.2
	3/4	1.92	1.18	1.25	0.36	0.19	50.2	33.7	35.2*	14.1	8.5	312.4	1992.6*
98 Seed	1/2	1.5	1.41	0.75	0.39	0.13	30.5	24.7	15.7	17.3	5.9	233.3	1545.8
	3/4	1.49	1.33	0.71	0.39	0.13	34.5	27.8	25.6*	13.8	6.5	222.8	2014.1*

* Significant ($P \leq 0.05$, HSD) within column for the same cultivar.

Table 3. Growth response of lotus (*Nelumbo*) to soil levels in 29 L containers (2004).

Cultivar	Soil level	Fresh weight (g)	Propagule No.	Expanded internode No.	Emerging leaf No.	Flower No.
Embolene	1/2	316.9	28.3	18.0	44.6	12.3
	3/4	333.7	31.2	22.3	41.6	12.2
98 Seed	1/2	693.4	8.6	7.4	20.2	2.0
	3/4	656.3	9.0	7.7	23.7	0.5*

* Significant ($P \leq 0.05$, HSD) within column for the same cultivar.

Table 4. Effects of soil levels on growth indices of lotus (*Nelumbo*) in 29 L containers (2007).

Cultivar	Soil level	Plant height (cm)	Standing leaf No.	Flower No.	Underground (g/pot, fw)	Propagule (g/pot, fw)	Propagule No.	Expanded internode No.	Maxim rhizome diameter (cm)
Lutea	1/4	92.6 a	27.4 a	0	1495.8 a	694.6 a	11.2 a	15.4 a	3.66 a
	1/2	55.6 b	6.4 b	0	503.6 b	424.0 b	13.8 a	17.8 a	2.92 b
	3/4	59.8 b	5.2 b	0	424.6 b	370.6 b	11.6 a	15.2 a	2.78 b
Karizma	1/4	99.8 a	59.4 b	7 a	994.8 a	480.4 a	29.2 a	7.4 a	2.04 a
	1/2	93.2 a	80.4 a	5.6 ab	851.0 ab	512.4 a	37.8 a	7.5 a	2.24 a
	3/4	85.6 a	75.6 ab	3.6 b	627.8 b	367.8 a	32.2 a	6.8 a	2.40 a

Means separation by HSD at 0.05 significance level. fw – fresh weight.

Table 5. Effects of soil levels in 29 L containers on nutrient contents in expanded rhizome tissue of *Nelumbo lutea* (2007).

Soil level	Moisture (%)	Macronutrients (%)					Micronutrients (ppm)						
		N	Ca	K	Mg	P	Fe	Al	B	Zn	Cu	Na	Mn
1/4	63.4 a	1.93 a	0.08 b	1.24 b	0.13 b	0.33 a	90.8 a	22.2 b	5.0 a	14.2 a	9.2 a	205.6 b	41.5 b
1/2	68.1 a	2.39 a	0.11 a	1.85 a	0.18 a	0.43 a	67.5 a	34.3 a	5.7 a	17.0 a	8.0 a	288.6 a	78.6 a
3/4	67.7 a	2.50 a	0.09ab	1.72 ab	0.18 a	0.39 a	29.7 a	36.4 a	5.2 a	21.9 a	9.1 a	240 ab	62.4 a
Range	61.6 - 74.5	1.66 - 3.54	0.07 - 0.13	1.07 - 2.25	0.11 - 0.21	0.29 - 0.55	18.1 - 168.4	17.0 - 43.9	3.9 - 7.5	10.9- 40.7	4.0- 14.7	152.1- 341.2	34.9- 144.8
H/L ratio	1.21	2.13	1.75	2.10	1.95	1.88	9.29	2.6	1.9	3.7	3.7	2.2	4.1

Expanded rhizomes were sampled on Nov. 10 following harvest. Means separation by HSD at 0.05 significant level within column for the same cultivar. H/L – the highest value/the lowest value.

Figures

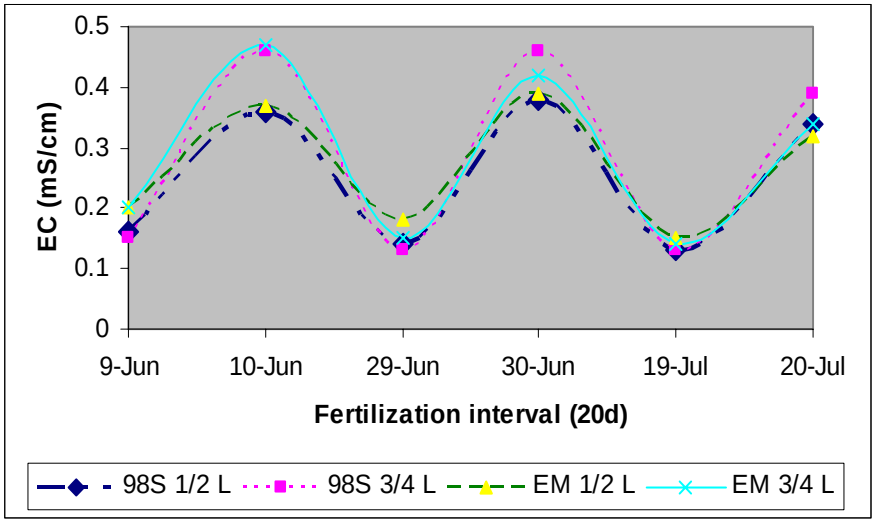

Fig. 1. Influence of soil level on water electrical conductivity in containers during fertilization of lotus (2004). Plants were supplied with 4 g of Pro•Sol 20-10-20 on June 9, June 29, and July 19 at a 20 d interval. EC was measured before each fertilizer application, 12 hrs and 20 days following fertilization, respectively. 98S – '98 Seed' lotus, EM – 'Embolene'; L – the level of soil based on 29 L (7.5 gal) containers.

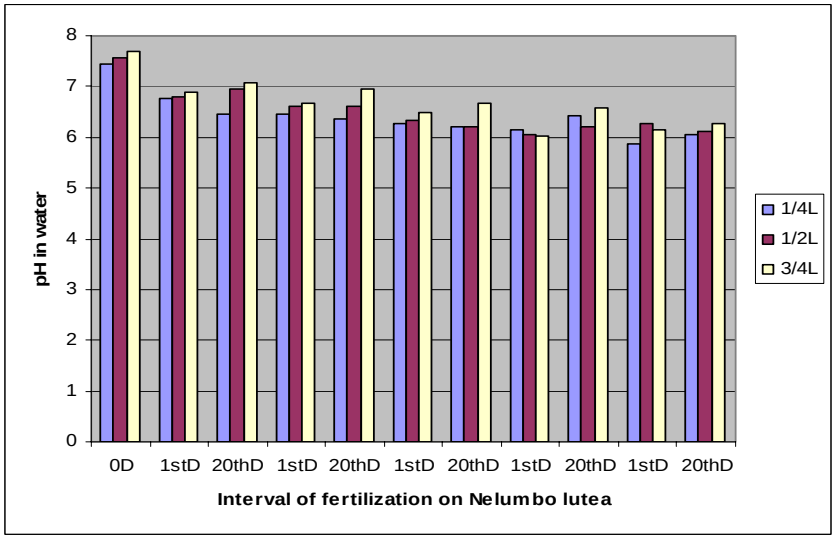

Fig. 2. Influence of container soil level on water pH during fertilization of *Nelumbo lutea* grown in 29 liter containers (2007). Fertilizer Pro•Sol 20-10-20 was applied for five applications with a sequence of 4, 6, 8, 10, and 8 g/pot from May 9 to July 30 at a 20 d interval. pH was measured on the 1st day (1stD) and 20th day (20thD) following fertilization. L – the level of soil based on 29 L (7.5 gal, 32 cm height) containers.

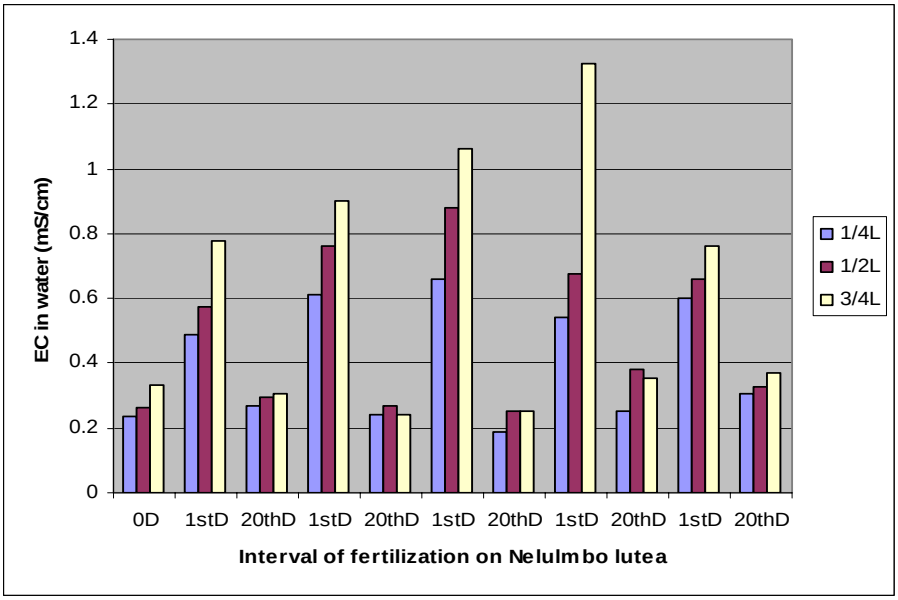

Fig. 3. Influence of container soil level on EC in water during fertilization of *Nelumbo lutea* grown in 29 liter containers (2007). Fertilizer Pro•Sol 20-10-20 was applied for five applications with a sequence of 4, 6, 8, 10, and 8 g/pot from May 9 to July 30 at a 20 d interval. EC was measured on the 1st day (1stD) and 20th day (20thD) following fertilization. L – the level of soil based on 29 L (7.5 gal) containers.

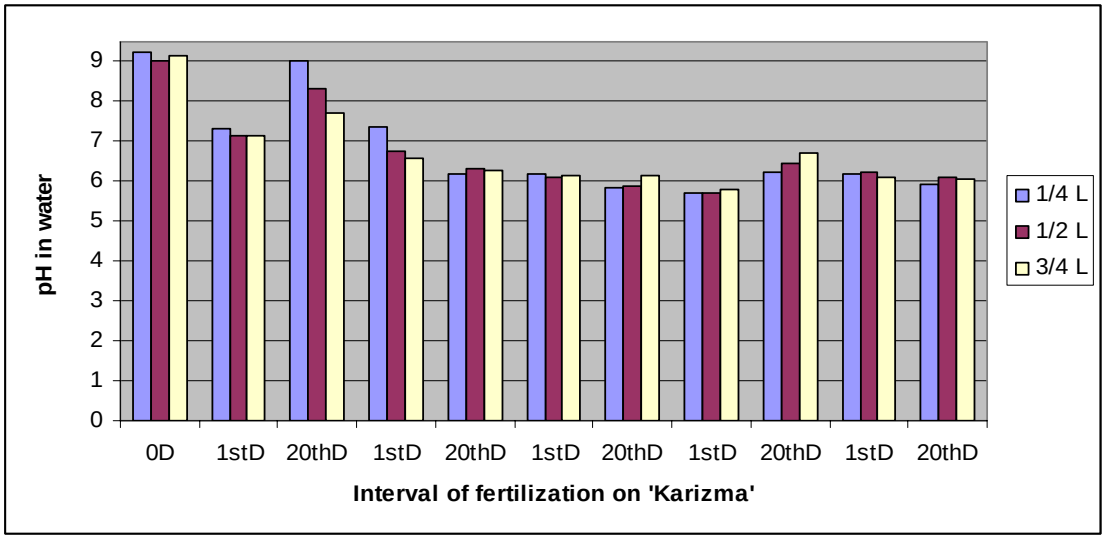

Fig. 4. Influence of container soil level on water pH during fertilization of lotus 'Karizma' grown in 21 liter containers (2007). Fertilizer Pro•Sol 20-10-20 was applied for five applications with a sequence of 4, 6, 8, 10, and 8 g/pot from May 9 to July 30 at a 20 d interval. EC was measured on the 1st day (1stD) and 20th day (20thD) following fertilization. L – the level of soil based on 29 L (7.5 gal) containers.

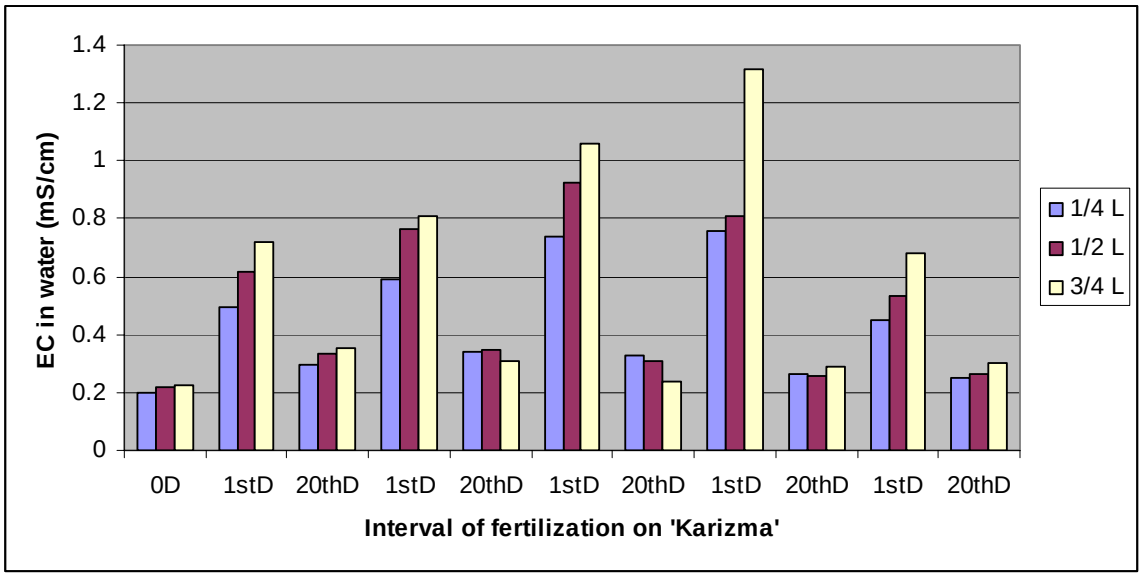

Fig. 5. Influence of container soil level on water EC during fertilization of lotus 'Karizma' grown in 21 liter containers (2007). Fertilizer Pro•Sol 20-10-20 was applied for five applications with a sequence of 4, 6, 8, 10, and 8 g/pot from May 9 to July 30 at a 20 d interval. EC was measured on the 1st day (1stD) and 20th day (20thD) following fertilization. L – the level of soil based on 29 L (7.5 gal) containers.

Chapter 3 Response of Container Lotus (*Nelumbo nucifera* Gaertn.) to Fertilization

Additional index words: fertilizer, growth index, flowering, expanded rhizome, propagule

Abstract

Two cultivars of lotus (*Nelumbo*) were used in this study to investigate the effects of fertilization on plant growth in 29 liter containers with half level of natural soil. Fertilization obviously influenced both the pH and EC values in water, and significantly increased observed plant growth and development parameters compared with the non-fertilized control. Fresh underground weight, propagule number, number of expanded internodes, and emerging leaf number linearly increased by increased fertilizer (Pro•Sol 20-10-20) rates from 0 to 8 g (0 to 2 teaspoons) per pot every 20 days and then leveled off when fertilizer reached 12 g (3 teaspoons) per pot every 20 days, while plant height and flower number changed little. Fertilization also increased the content of N, P and K and decreased the content of Ca in the young leaf tissue. However, the ratio of N:P:K in plant tissue remained relatively stable for fertilized plants compared to the control plants. The percentage and total amount of dry mass in expanded rhizomes increased with increased fertilizer rates. Young leaf tissue was suitable material to reflect the

relationships between the contents of macronutrients in tissue and the nutrient availability in the water-soil system.

Introduction

Lotus (*Nelumbo*) is an impressive perennial herb living in a soil-aquatic environment. There are two species in *Nelumbo*: *Nelumbo nucifera* Gaertn. native to Asia and *N. lutea* Pers. native to America. *N. nucifera*, called lotus, Asian lotus or sacred lotus, has an amazingly long history in the diverse cultures of the Orient. At least 6000 years ago, Asian lotus was known by Chinese ancestors and its history in cultivation can be dated to 3000 years ago (Wang and Zhang, 2004). Lotus is widely grown as an important economical crop in Asian countries. Recently, *N. nucifera* is becoming a potential crop in Australia (Nguyen, 2001), New Zealand (Follett and Douglas, 2003), and the United States (Tian et al., 2006).

Extensive studies have been conducted on the basic biology, pharmacology, horticulture, and agriculture of lotus. The practices on propagation, cultivation, field production and postharvest of edible lotus have been widely documented (Diao and Liu, 2004). Lotus is usually planted in a tilled pond or rice field for vegetable production. It also is often planted in containers, small ponds, and lakes for ornamental uses. Lotus is a fertilizer-consuming plant. Fertilization practices have significant impact on lotus growth,

therefore the optimal fertilizer types and application procedures are important to obtain maximum growth. In the past, traditional organic fertilizer has usually been the only choice for lotus production. Currently, organic fertilizer is still a major source of fertilizer in China while manufactured N-P-K fertilizers are widely used in Japan for production of vegetable lotus (Sou and Fujishige, 1995). General suggestions on fertilization of lotus are readily available. For lotus production in ponds, no or fewer side applications are necessary if the soil is fertile (Ni et al., 1987; Wang and Zhang, 2004). Otherwise, fertilizer applications should be split into 4-5 applications as young plants have been observed to burn quite readily (Nguyen, 2001). For container lotus, fertilizer should be applied frequently due to limited nutrition in container soil (Wang and Zhang, 2004). Studies on effects of fertilization on lotus plants are available but limited to vegetable lotus in pond production (Qu and Zhao, 1991; Li and Qian, 1994; Zhang et al., 1994; Li et al., 1998, 2002; Song et al., 2006; Orimoto and Takai, 2007) and in containers (Hicks, 2005). Information on fertilization of ornamental lotus in containers has not been reported. Recently, containerized ornamental lotus is becoming a potential crop in horticultural industry (Creamer, 2008). Best management practices of lotus, particularly on fertilization, are very important for nursery production of container lotus. Although Hicks (2005) reported the effects of EC, pH and fertilizer nutrition on vegetable lotus plants in containers, the results were not conclusive and only provided a basic guideline for fertilization of container lotus and did not account for the large difference in conditions between a designed experiment and an actual production system. Also, the effect of single element fertilizers in Hick's study (2005) on lotus will be different from that of incorporated fertilizers. In addition, there is no information on response of lotus

flowers to fertilization. In the following study, a soluble fertilizer was used to evaluate the influence of fertilization on container grown lotus and the interactions between fertilizer, nutrition in soil, EC, pH, and plant growth. Experimental results will provide practical information to nursery growers and the suggested fertilization procedures could be directly applied in container lotus production.

Materials and Methods

Two cultivars of *Nelumbo nucifera*: 'Yangzhou Bowl' (small size with pink flower, ordered from China) and 'UN7' (unnamed hybrid from Steve Garton in Savannah, Georgia, medium size with semi-double pink flower) were evaluated at the Department of Horticulture, Auburn University. The first study was conducted on 'Yangzhou Bowl' in 2004. Lotus rhizome-propagules were newly divided from existing container-grown stock plants which already had young leaves, and planted in 29 L (7.5 gal) of black plastic containers (31 cm bottom and 37 cm top diameter, 32 cm height) without holes on May 17, 2004. Each pot was planted with one propagule, usually with two internodes. Containers were filled to ½ container level (16 cm, 13 L) of natural sandy loam soil. After planting, all pots were filled with tap water (pH 7.0, EC = 0.13 $mS \cdot cm^{-1}$). Containers were placed in full sunlight with 25 cm of spacing between pots. Because the number of stock plants was limited in this year, the experiment included only two fertilizer level treatments without a non-fertilized control: 4 g (1 teaspoon) and 8 g (2 tsp). Soluble fertilizer Pro•Sol 20-10-20 (20% total nitrogen, 10% available phosphate, 20% soluble potassium, and minor nutrients) (Pro•Sol Inc., Ozark AL 36360, USA) was applied three times at a 20-d interval beginning from June 9, 2004 when the plants had

several floating leaves. The last application was given on July 21. Water solution samples were taken one hour before fertilization and 24 h after fertilization, respectively, in the late afternoon from the same pots to better monitor the nutrient status. Before taking samples, pots were filled with water by hand carefully to assure the same water level for all containers. On August 23, fully expanded young leaves were sampled for nutrient analysis.

The second experiment began on April 12, 2005. *Nelumbo nucifera* 'UN7' was planted in the same type of above mentioned containers with ½ container level of natural sandy loam soil. Plants were treated with 0, 4 g (1 tsp), 8 g (2 tsp), or 12 g (3 tsp) of fertilizer Pro•Sol 20-10-20 applied for each pot once every 20 d from May 23 to July 24, 2005. In total, plants were supplied with 4 applications. pH and EC were measured from water samples taken on the 2nd and 20th day after each application of fertilizer. Young leaves were collected twice on June 6 and July 15, respectively, and mature leaves were sampled on August 12 for tissue nutrient analysis. One leaf was taken from each container of the same treatments and then pooled in the same treatment. Plant height was measured on July 4 and August 4, respectively. Three samples of expanded rhizomes for each treatment were taken immediately after harvest on October 31, 2005 and used for nutrient analysis.

Experiments were arranged in a completely random design and each treatment had 6 reps for 'Yangzhou Bowl' in the first experiment and 10 reps for 'UN7' in the second experiment. Data included the number of emerging leaves, flowers, propagules (normally with 2 or 3 internodes) and expanded internodes, and the fresh weight of expanded rhizomes. Plant height was measured from the bottom of containers to the top of the

tallest leaves. The number of expanded internodes of 'Yangzhou Bowl' was calculated based on the swollen internodes with diameter larger than 1.5 cm. All samples of water and plant tissues were analyzed by the Soil Testing Laboratory of Auburn University. Water pH was determined at 25 ºC using a Fisher Accumet Model 50 pH meter. Nutrient elements of water were determined simultaneously by Inductively Coupled Plasma (ICP) Atomic Emission Spectrometry using a Varian Vista-MPX Axial Spectrometer (Maxfield And Mindak, 1985). Plant samples were dried in forced air oven at 60 ºC (140 ºF) for 72 h. Mineral nutrients of plant tissue were analyzed by Inductively Coupled Plasma Emission Spectroscopy using a Varian Vista-MPX Axial Spectrometer (Isaac and Johnson, 1985; Plank, 1992; Odom and Kone, 1997). Total nitrogen was analyzed Elementar Vario Macro CNS Analyzer (Columbo and Giazzi, 1982). Nitrate-nitrogen was analyzed colorimetrically with a Thermo Spectronic Genesis 20 Spetrophotometer (Bremner, 1965). Data were analyzed by SAS program (SAS Institute Inc., 100 SAS Campus Drive, Gary, NC, 27513-2424 USA) and mean differences were examined by Tukey (HSD) or LSD procedure at 0.05 significant level.

Results and Discussion

In the experiment on 'Yangzhou Bowl' lotus, pH and EC in water exhibited regular changes during fertilization and growth period of plants (Fig. 1, 2). The pH values of water immediately decreased following fertilizer application and then went up back close to the previous levels on the 20th day following fertilization when nutrients were absorbed by plants (Fig. 1). The decrease of pH in the treatment of 8 g fertilizer Pro•Sol 20-10-20 was obviously larger than in the treatment of 4 g and pH remained at lower

levels in the 8 g fertilizer treatment. On the contrary, EC increased sharply as fertilizer was applied and then dropped back to near the previous levels on the 20th day after fertilization (Fig. 2). EC had a larger increase after fertilization and remained at higher levels in the 8-g fertilizer treatment than in the 4 g fertilizer treatment.

The absorption rates of macronutrients (N, P and K) increased in the second application (June 30 to July 19) compared with that in the first application (June 10 to June 29) because the plants grew faster and plant size was larger in the later season (Table 1). The absorption rates were highest consistently in N followed by P and K for both treatments (4 g and 8 g). The 98.9% to 99.6% absorption rates of N in the 4 g fertilizer treatment indicated N was almost completely utilized by the plants. However, P and K in the 4 g fertilizer treatment showed 15% to 17% and 13% to 19% of residues, respectively, which suggested that 'Yangzhou Bowl' required a larger amount of N and a lower amount of P. All N, P, and K in the 8 g fertilizer treatment exhibited residuals of 12% to 15%, 18% to 29%, and 32% to 36% respectively, following a 20-day fertilization interval. Because 'Yangzhou Bowl' is a small cultivar and fewer leaves developed, application of 8-g soluble fertilizer Pro•Sol 20-10-20 at 20 d intervals appeared excessive although no visual toxicity problem occurred on leaves.

Nutrient analysis of young leaf tissue indicated higher contents of NPK and lower contents of Ca and Mg following the 8 g treatment than 4 g treatment (Table 2). The differences in macronutrients were less than 15% except Mg (38%) between the 4 g and 8 g fertilizer treatments. For micronutrients, the largest differences were respectively found in B which was 133% higher in the 8 g fertilizer treatment and in Cu which was 86% higher in the 4 g fertilizer treatment. Other micronutrients showed less difference or no

difference. Fertilization obviously promoted plant growth of 'Youngzhou Bowl' (Table 3). The treatment of 8 g fertilizer significantly increased plant fresh underground weight ($P = 0.0274$), propagule number ($P = 0.0015$), and the number of expanded internodes ($P = 0.0008$), but an increase in emerging leaf number in the 8 g fertilizer treatment was not significant ($P = 0.731$). No flowers developed for plants in either treatment.

In the experiment on 'UN7', measurement of the initial pH before fertilization on May 20 was based on water samples from containers of the non-fertilizer control (Fig. 3). This pH value could represent the initial average pH in the other treatments before the first application of fertilizer although the small differences possibly existed. For the control, pH slightly decreased from 7.6 on May 22 to 7.0 on July 4 and increased from 7.0 on July 4 to 7.3 on July 24, and decreased again from 7.3 on July 24 to 6.9 on August 12 (Fig. 3). EC continuously decreased from the initial level (0.27) on May 22 to the final value (0.14) sampled on August 12 (Fig. 4). For the plants receiving fertilizer treatments, pH generally decreased after supplying fertilizer and decreased as nutrients were absorbed by the plants (Fig. 3). The degree of decrease in pH generally increased as fertilizer rates increased. However, some exceptions were found for the pH values on May 23 in the 4 g fertilizer treatment and on June 12 in the 4 g and 8 g fertilizer treatments. These exceptions were possibly caused by a laboratory mistake or by the small sample size (only three samples in each treatment were used for analysis). EC in water for the fertilizer treatments sharply increased after fertilization and increased following fertilizer doses and then dropped down to around the initial level when nutrients were utilized by the plants during a 20 d interval of fertilization (Fig. 4). At the end of each fertilization

cycle, EC in water was generally highest in the 12 g fertilizer treatment and lowest in the control and 4 g fertilizer treatment.

The absorption of macronutrients N, P, K by plants during fertilization and plant growth showed a regular trend based on leaf tissue analysis (Fig. 5). The absolute absorption values of NPK increased with increasing fertilizer doses (Fig. 5, left). With an exception of N absorption in the 12 g fertilizer treatment, absorption values of NPK all increased and reached a peak following the 3rd application and then decreased sharply following the 4th application period. The trend well reflected the growth characteristics of lotus 'UN7' in which growth rate increased with increasing temperature and reached a growth peak between July 15 and August 5. The residue values of NPK in the water also increased as the fertilizer rates increased (Fig. 5, right). At the end of each 20 d fertilization cycle, a small amount of NPK residue remained in water following 4 g and 8 g fertilizer treatments. Following 12 g fertilizer treatment, a large amount of N residue remained in both the early and late growth seasons, while a large amount of P and K residues only occurred in the early season. These results indicated that 12 g (3 tsp) fertilizer of NPK 20-10-20 every 20 d was excessive for lotus 'UN7' in 29 L containers during some growth stages. The demand for P and K obviously increased during the growth peak of plants. The N, P, and K concentrations in the water in control were extremely low compared to fertilizer treatments, suggesting the nutrition requirement for optimal growth of plants was not met in the control.

Nutrient analysis of young leaf tissue showed that N, P, and K absorption by lotus plants consistently increased while the absorption of Ca decreased with the increasing fertilizer doses (Fig. 6). The highest contents of N, P, K and the lowest content of Ca

were observed in the young leaves showing interveinal chlorosis, possibly as a result of Fe and Ca deficiency due to antagonistic effects of N and K at high level or in excess particularly in the treatment of 12 g fertilizer. No clear relationships were observed between the fertilizer rates and the contents of other nutrients. The N content in mature leaves also increased with increased fertilizer concentrations, but the contents of K and Ca showed almost the same trend and generally decreased in a quadratic relationship (Fig. 7). The ratios of N:P:K were 8.5:1:4.5 and 10:1:5 in young leaves sampled on June 6 and July 14, respectively. The content of P in mature leaves showed a quadratic relationship for fertilizer rate decreasing first in the 4 g fertilizer treatment then sharply increasing in the 12 g fertilizer treatment. Moisture in the expanded rhizomes of 'UN7' decreased linearly with the increased fertilizer rates (Table 5). The ratio of N:P:K in expanded rhizomes was about 5:1:7 which was different from that in the young leaf tissue. The N and K contents in expanded rhizomes had the reverse change as the trends that occurred in the mature leaves (Fig. 8). There was a strong positive quadratic (nearly linear) relationship between K content and fertilizer rates (Table 5), but the relationship between N content and fertilizer rates was not clear. The P and Ca content in expanded rhizome showed a weak positive and negative quadratic relationship, respectively, with fertilizer doses (Table 5, Fig. 8). Fe content in the expanded rhizomes of 'UN7' exhibited a strong negative quadratic relationship and Mn content exhibited a strong linear relationship with fertilizer concentrations. There was a strong positive quadratic relationship between Na and the fertilizer rates.

Compared with control, fertilization obviously increased plant growth indices of lotus 'UN7' in 29 L containers. The 4 to 12 g of soluble fertilizer Pro•Sol 20-10-20 applied at

20 d intervals resulted in significant increase in plant height (1.3 to 1.6 fold), fresh underground biomass (2.4 to 3.3 fold), emerging leaf number (1.9 to 2.7 fold), flower number (2.4 to 2.7 fold), and propagule number (1.3 to 1.5 fold) (Table 4, Fig. 9, 10, 11). Significant differences were also found in emerging leaf number, propagule number, plant height and the fresh underground weight between the 4 g and 8 g fertilizer treatments but there was no difference in plant growth indices between the 8 g and 12 g fertilizer treatments. Except for flower number, a positive quadratic response was found between growth parameters and fertilizer rates (Table 5). Growth indices increased linearly from 0 to 8 g fertilizer treatments and then leveled off as the fertilizer rate reached 12 g. The most efficient rate of fertilizer for production of lotus 'UN7' in 29 L containers was 8 g of Pro•Sol 20-10-20 fertilizer applied once every 20 d.

In summary, fertilization influenced both pH and EC. The pH values ranged from 5.2 to 7.5 in 'Yangzhou Bowl' and 6.8 to 7.6 in 'UN7' during fertilization. These values were in the range of 5.5 to 8.0 in which lotus growth was not affected (Nguyen, 2001). However, EC value was a sensitive indicator of plant toxicity. Lotus young leaves often showed toxic symptoms because of either over-fertilization or a short time of fertilizer shock when EC was greater than 1.0 $mS \cdot cm^{-1}$. These results are not in agreement with the previous report that EC at 2.8 to 3.1 $mS \cdot cm^{-1}$ was optimal for lotus growth (Nguyen, 2001; Hicks, 2005). Our study supported that lotus was a fertilizer-consuming plant (Zhang et al., 1994). Fertilization significantly increased plant growth indices, while lotus in the non-fertilized control produced the lowest plant growth parameters and aged earliest. Among fertilizer treatments, fresh underground weight, propagule number, the number of expanded internodes, and emerging leaf number were significantly influenced,

while plant height and flower number usually were little changed by fertilizer treatment. Fertilization increased lotus underground fresh weight and dry mass ratio. The significant increase in fresh underground weight was through an increase in carbohydrate accumulation because enhanced photosynthesis with increased leaf area was indirectly indicated by emerging leaf number. Nutrient contents and changes in response to fertilization showed differences among young leaves, mature leaves and expanded rhizomes. In both young leaf tissue and expanded rhizome tissue, P content was always low. N content was always high in young leaf tissue and K content was high in the expanded rhizomes. The same results were seen in a previous study (Qu and Zhao, 1991). Young leaf tissue was the best material to correctly reflect the nutrient availability especially on macronutrients (N, P, and K) in water and soil system. Hicks (2005) also reported that leaves were the most appropriate organs for field sampling and analysis.

Lotus responded to fertilizer differently depending on plant size. The 8 g of fertilizer looked a little excessive for the small-size lotus ‘Yangzhou Bowl’ but produced the best result for the medium-size lotus ‘UN7’ in 29 L containers. Considering problems of fertilizer toxicity and algae competition during early growing stages of lotus and the unique characteristics of lotus growth, a modified fertility regime with a graduated application sequence of 2, 4, 8, 10, and 8 g of fertilizer at 20 d intervals may possibly be better for 'UN7' in 29 L containers. However, this fertility program should be adjusted based on plant cultivars, fertilizer types, container size, water volume in containers and monitoring of EC values. Although the best time interval of fertilization has not been clear, a 20 d interval was at least very effective for lotus production in containers. It also remains unclear if Pro•Sol 20-10-20 is one of the best fertilizers for lotus. Future studies

should be conducted on effects of fertilizer type and application frequency on lotus growth.

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Tables

Table 1. Macronutrient absorption rate (AR, %) during the first two fertilizer applications (on June 9 and 29, respectively) with Pro•Sol 20-10-20 on lotus 'Yangzhou Bowl' in 29 L containers (2004).

Time and AR	Fertilizer treatment					
	4 g/pot			8 g/pot		
	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)
6/10/2005	18.5	3.6	23.0	49.1	8.5	46.7
6/29/2005	0.2	0.6	2.9	12.1	2.5	17.0
AR (%)	98.9	83.3	87.4	75.4	70.6	63.6
6/30/2005	28.0	7.2	34.1	68.4	14.5	70.5
7/19/2005	0.1	1.1	6.5	8.1	2.6	22.8
AR (%)	99.6	84.7	80.9	88.2	82.1	67.7

Note: nutrient absorption rate is based on calculation of nutrients in water at a 20 d interval of fertilization.

Table 2. Effect of fertilization with Pro•Sol 20-10-20 on nutrient content in young leaf tissue of lotus 'Yangzhou Bowl' in 29 L containers (2004).

Fertilizer	Macronutrients (%)					Micronutrients (ppm)c						
	N	P	K	Ca	Mg	Fe	Al	B	Zn	Cu	Mn	Na
4 g	2.34	0.13	1.14	2.4	0.65	57.7	42.3	17.8	10	13.6	2156.7	416.6
8 g	2.56	0.14	1.3	2.14	0.47	64.2	44.9	41.4*	10	7.3*	2507.8	518.0

* Significant according to LSD ($P \leq 0.05$) within columns. Fertilizer (Pro•Sol 20-10-20) was applied on June 9, June 29, and July 21, respectively. Young leaves were sampled on Aug. 23 for nutrient analysis.

Table 3. Effect of fertilization with Pro•Sol 20-10-20 at a 20 d interval of application on plant growth indices of lotus 'Yangzhou Bowl' in 29 L containers (2004).

Fertilizer rate (g/pot)	Emerging leaf No.	Flower No.	Fresh underground weight (g)	Propagule No.	Expanded internode No.
4	5.9	0	248.4	6.9	7.3
8	6.3	0	337.1	10.0	10.3
P- value	0.731	-	0.0274*	0.0015*	0.0008*

* Significant according to LSD ($P \leq 0.05$) within columns. "-" indicates no flowers. Emering leaf number was recorded on Sept. 20, 2004. Plant indices of underground tissue were immediately measured following harvest on Feb. 5, 2005.

Table 4. Effect of fertilization with Pro•Sol 20-10-20 at 20 d intervals on plant growth indices of *Nelumbo* 'UN7' in 29 L containers (2005).

Fertilizer rate (g/pot)	Plant height I	Plant height II	Emerging leaf No.	Flower No.	Underground fresh weight (g)	Propagule No.
0	56.5 b	59.5 c	24.1 c	1.1 b	282.1 c	14.6 c
4	69.6 b	79.4 b	45.1 b	3.0 a	662.8 b	21.5 b
8	79.1 a	96.0 a	64.8 a	2.8 a	911.1 a	29.4 a
12	74.0 ab	94.4 a	64.3 a	3.0 a	917.1 a	29.9 a

* Significant according to HSD ($P \leq 0.05$) within columns. Plant height I and II were measured on July 4 and August 4. Emerging leaf number was counted on Aug. 12. Underground fresh weight and propagule number were measured following harvest on Oct. 31, 2005.

Table 5. Regression relationships between fertilizer rates (x, tsp/pot, 1 tsp = 4 g) of Pro•Sol 20-10-20 and plant growth indices and nutrients (y) in the expanded rhizomes of lotus 'UN7' in 29 L containers (2005).

Growth indexes	Model	P value	R^2
Plant height (cm)	$y = 58.75 + 28.25\ x - 5.375\ x^2$ (data on Aug. 4)	< 0.0001	0.813
Emerging leaf number	$y = 23.188 + 30.125\ x - 5.375\ x^2$	< 0.0001	0.721
Underground fresh weight (g/pot)	$y = 276.619 + 496.306\ x - 93.565\ x^2$	< 0.0001	0.846
Propagule number	$y = 14.206 + 10.144\ x - 1.594\ x^2$	< 0.0001	0.806
Nutrient in the expanded rhizomes			
Water	$y = 66.77 - 1.347\ x$	0.0012	0.667
K (%)	$y = 1.359 + 0.337\ x^2$	< 0.0001	0.872
Ca (%)	$y = 0.182 - 0.017\ x$	0.0139	0.47
Fe (ppm)	$y = 283.9 + 136.04 - 29.08\ x^2$	0.0007	0.8
Mn (ppm)	$y = 53.43 - 3.767\ x$	0.0207	0.43
Zn (ppm)	$y = 17.43 - 9.56\ x + 2.38\ x^2$	0.007	0.668
Na (ppm)	$y = 370.41 + 28.18\ x^2$	< 0.0001	0.683

Note: the relationships were not significant between the contents of N, P and other tested nutrients and the fertilizer rates of Pro·Sol 20-10-20.

Figures

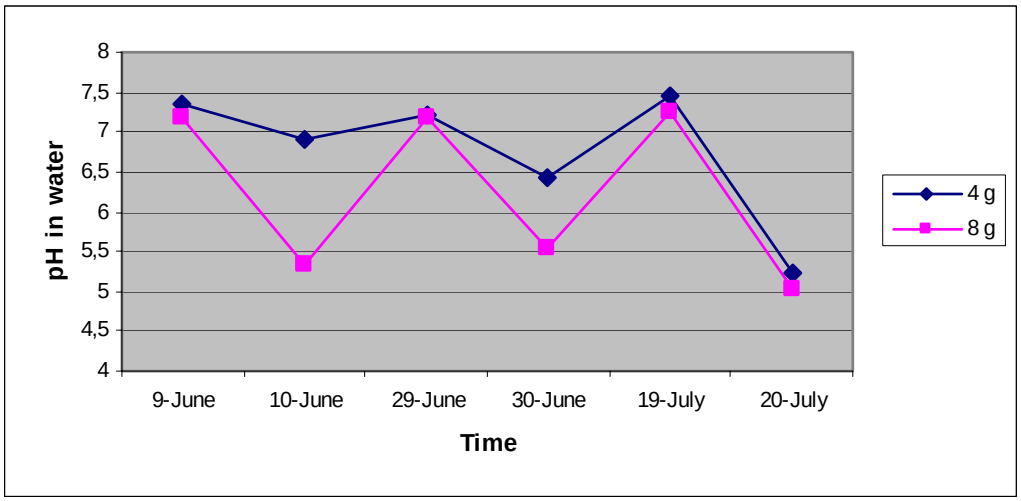

Fig. 1. Change of water pH during fertilization of lotus 'Yangzhou Bowl' in 29 L containers with 4 or 8 g of Pro•Sol 20-10-20 at 20-d intervals (2004). Fertilizer was applied on June 9, June 29, and July 19, respectively.

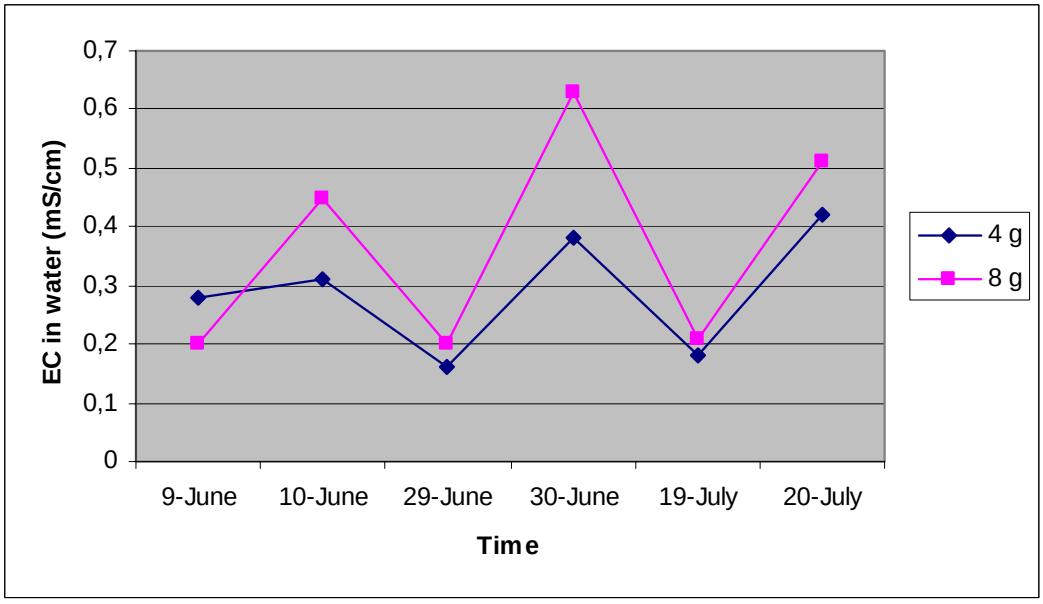

Fig. 2. Change of water EC during fertilization of lotus 'Yangzhou Bowl' in 29 L containers with 4 or 8 g of Pro•Sol 20-10-20 at 20 d intervals (2004). Fertilizer was applied on June 9, June 29, and July 19, respectively.

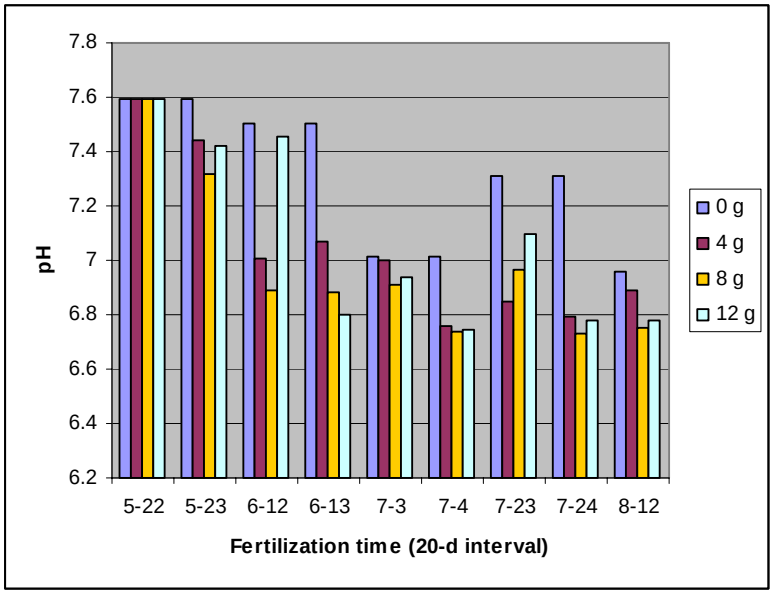

Fig. 3. Change of water pH during fertilization of lotus 'UN7' in 29 L containers (2005). Fertilizer Pro•Sol 20-10-20 was applied at 0, 4, 8, or 12 g/pot based on a 20 d interval beginning from May 23 and the last application was on July 24.

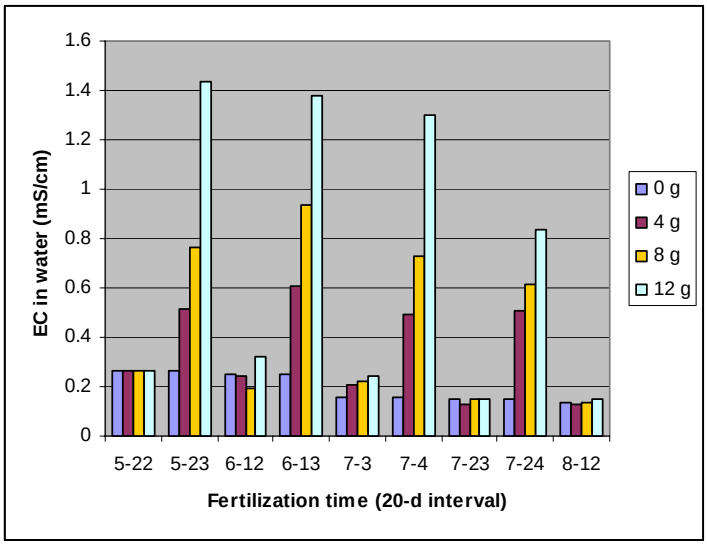

Fig. 4. Change of water EC during fertilization of lotus 'UN7' in 29 L containers (2005). Fertilizer Pro•Sol (NPK 20-10-20) was applied at 0, 4, 8, or 12 g/pot based on a 20 d interval beginning from May 23 and the last application was on July 24.

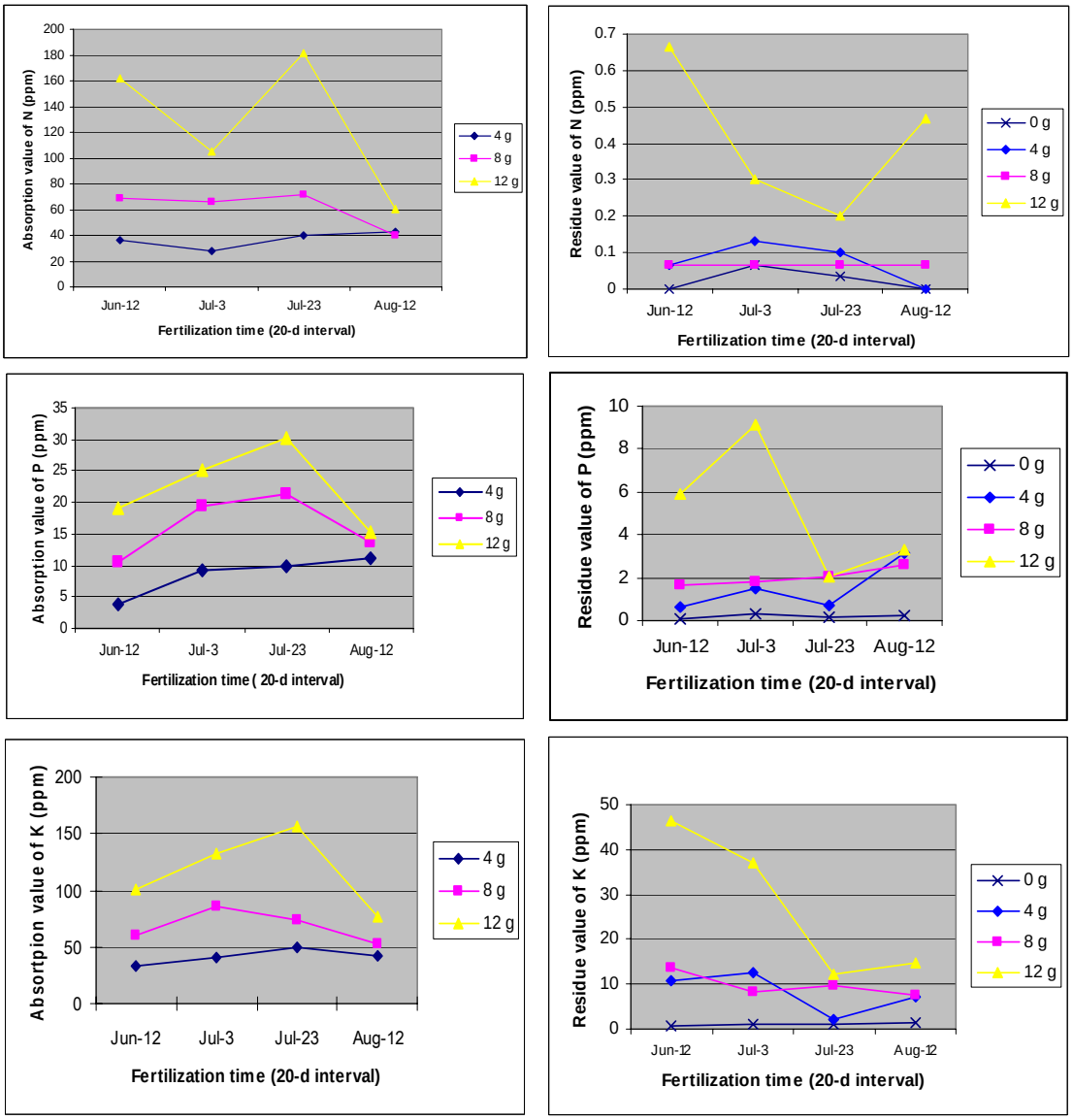

Fig. 5. Nutrient absorption values (left figures) and residuals (right figures) of N, P, and K during fertilization of lotus 'UN7' in 29 L containers (2005). Plants were supplied with soluble fertilizer Pro•Sol 20-10-20 on May 23, June 13, July 4, and July 24 with treatments of 0, 4, 8, or 12 g/pot. Nutrient absorption values were calculated by the difference of the concentration of N, P and K measured between the 24th h and the 20th day after application. Residuals were the value of nutrients in water measured on the 20th day after each time of fertilization.

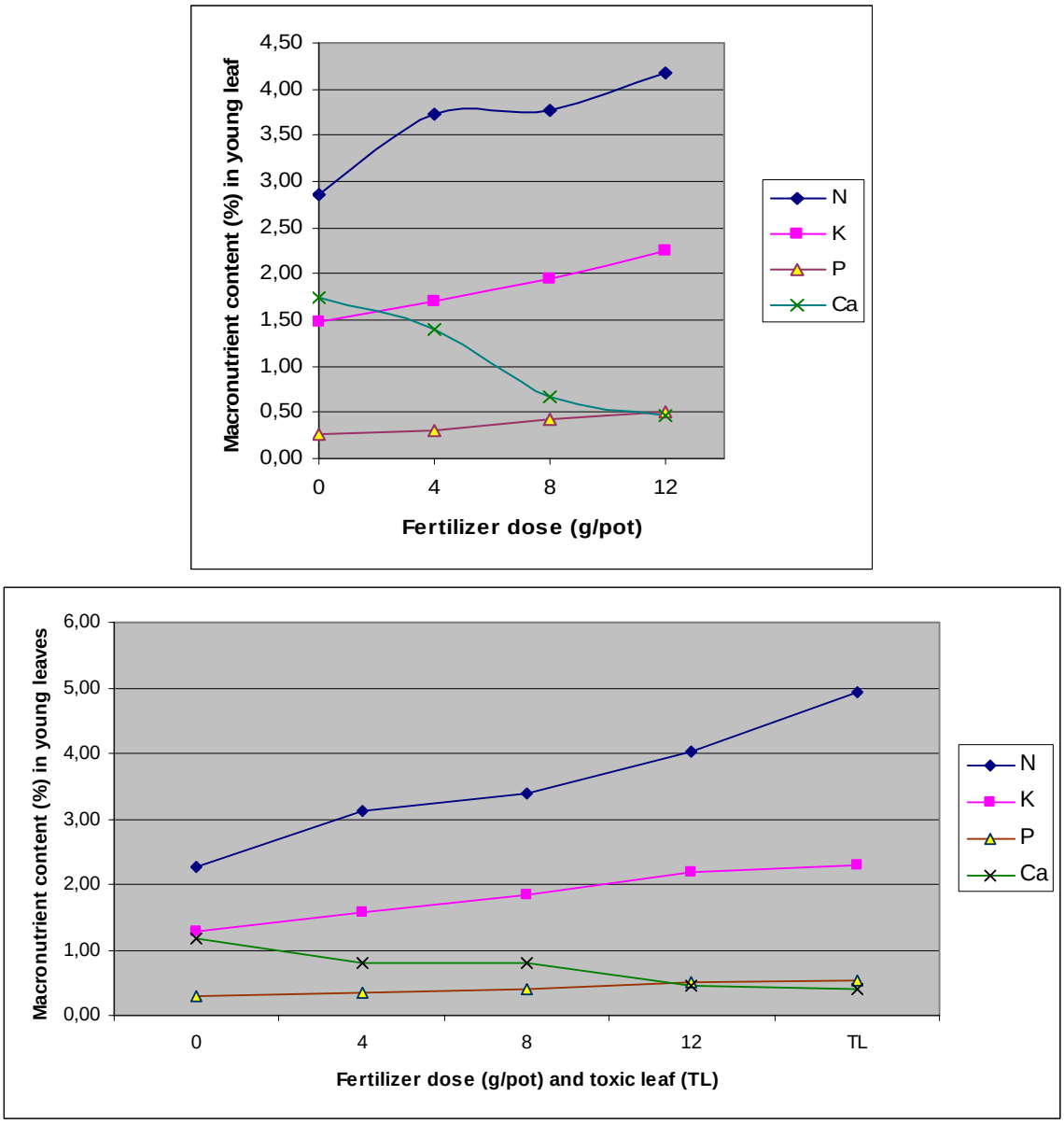

Fig. 6. Effect of fertilization on macronutrient (N, K, P and Ca) content in young leaf tissue of lotus 'UN7' in 29 L containers (2005). Plants were supplied with soluble fertilizer Pro•Sol 20-10-20 on May 23, June 13, July 4, and July 24 with treatments of 0, 4, 8, or 12 g/pot. Young leaves were sampled on June 6 (above) and July 14 (below), respectively. 'TL' – the fertilizer toxic leaf with obvious interveinal chlorosis in the treatment of 12 g fertilizer.

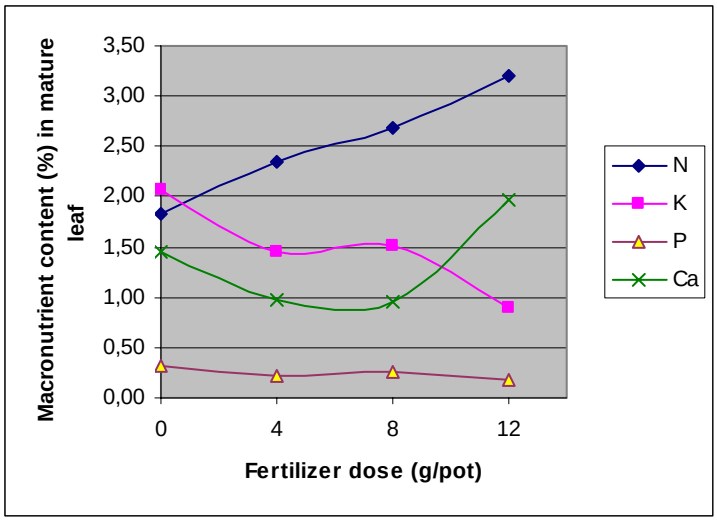

Fig. 7. Effect of fertilization on macronutrient (N, K, P, and Ca) content in mature leaves of lotus 'UN7' in 29 L containers (2005). Plants were supplied with soluble fertilizer Pro•Sol 20-10-20 on May 23, June 13, July 4, and July 24 with treatments of 0, 4, 8, or 12 g/pot. Mature leaves were sampled on Aug. 12, 2005.

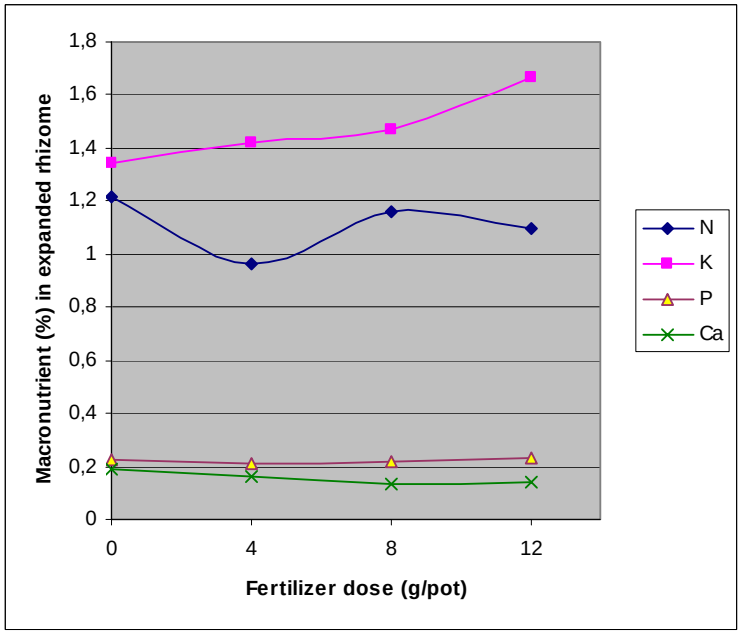

Fig. 8. Effect of fertilization on macronutrient (N, K, P, and Ca) content in expanded rhizomes of lotus 'UN7' in 29 L containers (2005). Plants were supplied with soluble fertilizer Pro•Sol 20-10-20 on May 23, June 13, July 4, and July 24 with treatments of 0, 4, 8, or 12 g/pot. Rhizome samples were taken on Oct. 31, 2005 following harvest.

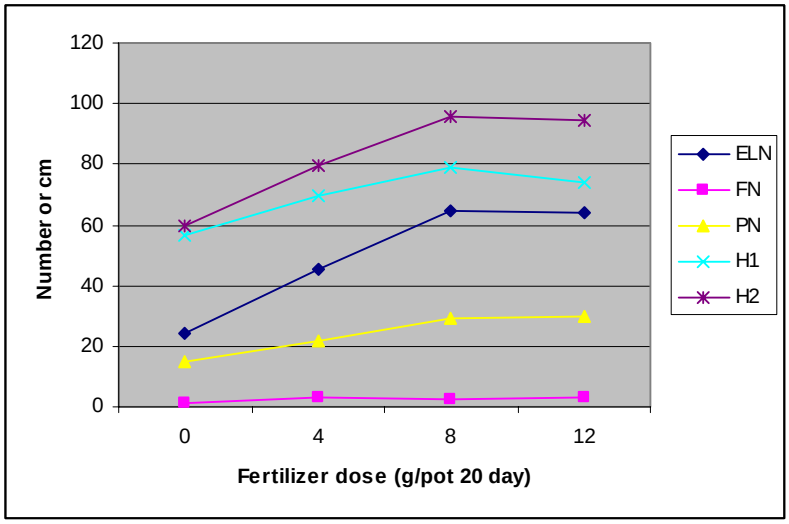

Fig. 9. Effect of fertilization with Pro•Sol (NPK 20-10-20) on growth indices of lotus 'UN7' in 29 L containers (2005). Plants were treated by fertilization with soluble fertilizer Pro•Sol 20-10-20 at 0, 4, 8, or 12 g/pot every 20 d. ELN – emerging leaf number, FN – flower number, PN – propagule number, H1 and H2 – plant height I and II (cm) were measured on July 4 and August 4, respectively.

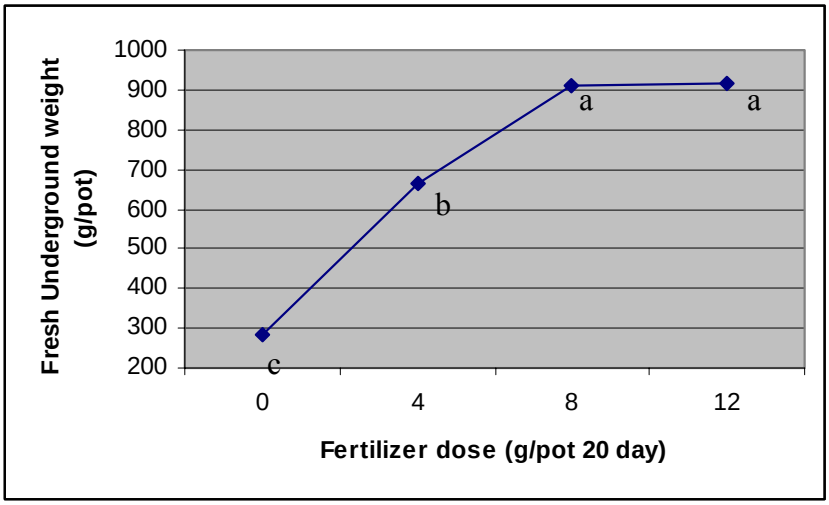

Fig. 10. Effect of fertilization with Pro•Sol 20-10-20 on fresh underground weight of lotus 'UN7' in 29 L containers (2005). Plants were treated by fertilization with soluble fertilizer Pro•Sol (NPK 20-10-20) at 0, 4, 8, or 12 g/pot every 20 d. Means were separated by Tukey (HSD, $\alpha = 0.05$).

Fig. 11. Effect of fertilization on growth of underground part of lotus 'UN7' in 29 L containers (2005). Plants were treated by fertilization with soluble fertilizer Pro•Sol 20-10-20 at 0, 4, 8, or 12 g/pot every 20 d.

Chapter 4 Response of Container Lotus (*Nelumbo nucifera* Gaertn.) to Five Types of Fertilizers

Additional index words: fertilization, controlled release fertilizer, growth index, flowering, expanded rhizome

Abstract

Effects of five fertilizers on growth of *N. nucifera* 'Embolene' were investigated in 29 liter container production in this study. Fertilization increased all evaluated growth indices: plant height, emerging leaf number, fresh underground biomass, and propagule number. Large differences in nutrient content of expanded rhizomes were found between the non-fertilized control and fertilization treatments. Flowering peak of lotus was influenced by the type or composition of fertilizers. Based on applications of equal rates of N, soluble fertilizers (Pro•Sol 20-10-20 and Miracle-Gro 24-8-16, 15-30-15) were more effective for increasing lotus growth indices than both nitrogen fertilizer (urea) and controlled release fertilizer (Polyon 18-6-12). High phosphorus ratio fertilizer (Miracle-Gro 15-30-15) led to the largest growth parameters except for flower number. Miracle-Gro 15-30-15 was more beneficial for rhizome or propagule production. Miracle-Gro 24-8-16 and Pro•Sol 20-10-20 produced the highest flower numbers, therefore these two fertilizers would be a better selection for lotus flower production. Controlled release

fertilizer Polyon 18-6-12 did not provide enough nutrition for requirements of plant growth especially in the early season with lower temperatures. However, Polyon 18-6-12 prolonged leaf life for about two weeks compared to other fertilizers. With exception of flower number, there were no differences in plant growth indices and in nutrients of expanded rhizomes between treatments of high-frequency-low-rate and low-frequency-high-rate of soluble fertilizer Pro•Sol 20-10-20. Plant height, emerging leaf number, fresh underground biomass, and propagule number were quadratically or linearly related to each other. However, no relationship was detected between flower number and other plant growth parameters.

Introduction

Lotus (*Nelumbo nucifera* Gaertn.) is an aquatic perennial herbaceous plant that has been a valuable crop for centuries in China (Wang and Zhang, 2004). Lotus rhizomes are one of the major vegetables in Asian countries. Recently, lotus is becoming a potential crop in Australia (Nguyen, 2001), New Zealand (Follett et al., 2003), and the United States (Tian et al., 2006). Lotus is a fertilizer-consuming plant and fertilization obviously increases yield and profit (Zhang et al., 1994). Various types of organic manure has provided the major source of nutrition for lotus production in the past, and is still often used in China (Sou and Fujishige, 1995). Effects of fertilization on lotus growth have been much studied mainly in production of vegetable lotus in ponds and fields (Qu and Zhao, 1991; Zhang, 1994; Li et al., 1998; Li et al., 2002; Song et al., 2006; Orimoto and Takai, 2007). Information on fertilization of ornamental lotus in containers is sparse (Hicks, 2005). Based on the difference in biological characteristics among

selections of vegetable and ornamental lotus, responses of lotus plants to fertilization can possibly be different. It is assumed that type and dose of fertilizers and application procedures should be genotype dependent. In lotus 'Embolene', our previous studies showed that 8 g (2 tsp) of soluble fertilizer Pro•Sol 20-10-20 applied at 20-day intervals in 29 L (7.5 gal) containers was optimal for plant growth and 12 g (3 tsp) of fertilizer at same interval resulted in toxic symptoms on lotus leaves, especially in the earlier growth stage. Since there are many diverse commercial fertilizers available for crops, it is not clear if Pro•Sol is necessarily the best choice for lotus growth and production. Effects of fertilization frequency on lotus performance also remain unknown. The major goal of this study was to evaluate effects of fertilizer type and application frequency on plant growth parameters and nutrition of rhizome tissue of lotus.

Materials and Methods

A medium-sized ornamental lotus, *Nelumbo nucifera* 'Embolene', was used in this study in the Department of Horticulture at Auburn University, Alabama USA to evaluate response of plant to fertilization with five types of fertilizers. 'Embolene' rhizomes were divided from stock plants with young leaves already emerged and planted in 29 L (7.5 gal) black plastic containers (31 cm bottom and 37 cm top in diameter, 32 cm in height) without holes on April 14, 2006. Each pot was planted with one propagule with 2 internodes. The experiment utilized a completely randomized block design with 7 treatments and 10 blocks. Each treatment included 10 plants (containers). All containers were filled to ½ container level (16 cm or 13 L, with natural sandy loam soil and immediately filled to ¾ container level with municipal tap water (pH = 7.0, EC = 0.13

$mS \cdot cm^{-1}$) after planting. Fertilization began on June 2 at a 10 or 20 d interval (Table 1). The last application was on August 11, 2006. Except for the control group, plants were treated by fertilization, respectively, using five types of fertilizers: urea, Pro•Sol 20-10-20 (20% total N, 10% available P, 20% soluble K, and minor nutrients) (Pro•Sol Inc., Ozark AL 36360, USA); Miracle-Gro 24-8-16 and Miracle-Gro 15-30-15 (Scotts Miracle-Gro Products, Inc., Marysville, OH, USA), and controlled release fertilizer Polyon 18-6-12 (8 to 9 month at 27 ºC or 80 ºF, Pursell Technology Inc., Sylacauga, AL, USA). In total, there were seven treatments (Table 1): (A) control without fertilization; (B) Urea at 2.2 g/10 d; (C) Pro•Sol 20-10-20 at 5 g/10 d; (D) Pro•Sol 20-10-20 at 10 g/20 d but 5 g/pot for the initial application; (E) Miracle-Gro 24-8-16 at 4.2 g/10 d; (F) Miracle-Gro 15-30-15 at 6.7 g/10 d; and (G) controlled release fertilizer Polyon 18-6-12 at 11.2 g/10 d (applied double rate of equivalent N compared with other treatments considering slow release factor). Urea and controlled release Polyon fertilizer were weighed and applied directly. For soluble fertilizers, freshly prepared solution (10%, w/v) was applied using a graduated cylinder. Fertilizer dose was calculated based on the same amount of N. Data included plant height, emerging leaf number, flower number, fresh underground weight, and the number of marketable and total propagules. A propagule was defined as a rhizome segment consisting of 2 to 3 internodes with shoots. Marketable propagule was defined as a rhizome segment consisting of 2 to 3 expanded or larger internode with shoots. Secondary propagules were usually small, without expanded internodes and were not suitable for sale. The total propagule number was added by the numbers of marketable and secondary propagules. Marketable propagule rate was calculated by dividing the marketable propagule number by total propagule number. Flowering time

and flower number were recorded once every 2 to 3 d. Plant height was measured on the 90th, 105th, 120th and 135th day after planting. Underground parts were harvested on Nov. 23, 2006. The data on fresh biomass and propagule number were taken immediately following harvest. Expanded internodes (4 samples/treatment) were sent to the Soil Testing Laboratory at Auburn University for nutritional analysis. Water samples were filtered with a 0.45 μm filter before analysis. Nutrient elements of water were determined simultaneously by Inductively Coupled Plasma (ICP) Atomic Emission Spectrometry using a Varian Vista-MPX Axial Spectrometer (Maxfield And Mindak, 1985). Plant samples were dried in forced air oven at 60 ºC (140 ºF) for 72 h to determine moisture content and dry mass. Mineral nutrients of plant tissue were analyzed by Inductively Coupled Plasma Emission Spectroscopy using a Varian Vista-MPX Axial Spectrometer (Isaac and Johnson, 1985; Plank, 1992; Odom and Kone, 1997). Total nitrogen was analyzed Elementar Vario Macro CNS Analyzer (Columbo and Giazzi, 1982). Nitrate-nitrogen was analyzed colorimetrically with a Thermo Spectronic Genesis 20 Spetrophotometer (Bremner, 1965). The means of variables were separated by LSD procedure at the 0.05 significance level using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Relationships among plant growth parameters were determined by regression analysis under the SAS environment.

Results and Discussion

In this study, fertilization obviously improved performance of lotus plants. All plant growth indices evaluated for *N. nucifera* ‘Embolene’ increased following applications of five types of fertilizers as compared to control receiving no supplemental fertilization

(Table. 2, Fig. 1, 2, 4, 5, 6). Plant height reached maximum earliest on the 105th day after planting in control group but latest on the 135th day in the treatment with controlled release Polyon fertilizer. Plant height was significantly increased by fertilization compared with control (Table 2, Fig. 1). In fertilizer treatments, plants receiving urea produced the least in plant height. No differences were observed in plant height among plants receiving treatments with other soluble fertilizers. Emerging leaf number of 'Embolene' was also affected by fertilization (Table 2, Fig. 2). The largest number of emerging leaves, 96 leaves/pot, was found in plants treated by fertilization with Miracle-Gro 15-30-15 while only 7 leaves/pot developed in control. No differences in emerging leaf number were observed in plants treated with Pro•Sol 20-10-20, Miracle-Gro 24-8-16 or controlled release fertilizer.

Flowering characteristics were largely influenced by fertilization. At least one major flowering peak was found for each treatment (Fig. 3). For the control treatment, the first flowering peak was the greatest number of flowers for the year and flower number decreased sharply after this peak. No flowers developed later than August 10. In fertilized treatments, the first flowering peak occurred between June 10 and June 20 but the highest flowering peak occurred between June 30 and August 30. The highest flowering peak of plants occurred differently in plants among fertilizer treatments. The order of the time period for the highest flowering peak was around June 30 to July 9 (3.2 flowers/pot) for urea, July 20 to 29 (2.5 flowers/pot) for Miracle-Gro 15-30-15, Aug. 5 to 15 (3.3 flowers/pot) for Miracle-Gro 24-8-16, Aug. 10 to 19 (2.7 flower/pot) for Pro•Sol 20-10-20 at 5 g/10 d intervals, Aug. 10 to 19 (2.8 flowers/pot) for Pro•Sol 20-10-20 at 10 g/20 d intervals, and Aug. 20 to 29 (2.3 flowers/pot) for controlled release Polyon. In the control,

Pro•Sol 20-10-20 at 5 g/10 d intervals and Miracle-Gro 24-8-16, a flowering slump occurred between July 20 to 29. Flowering time was extended about 10 days in the treatment of controlled release Polyon fertilizer. Mean flower number was also increased by fertilization (Table 2, Fig. 4). The lowest flower number (3 flowers/pot) was observed in the control and the highest flower number (19 flowers/pot) was obtained in the treatment of Miracle-Gro 24-8-16 with 4.2 g/10 d. In the fertilizer treatments, plants treated with controlled release Polyon fertilizer produced the least flower number, 11.4 flowers/pot, followed by 13 flowers/pot for plants treated with Miracle-Gro 15-30-15.

There were large differences in both biomass and propagule number of lotus receiving different fertilizer treatments (Table 2, Fig. 5, 6). Plants treated with Miracle-Gro 15-30-15 produced the highest biomass (1090 g/pot) followed by 973.7 g/pot for plants fertilized with Miracle-Gro 24-8-16. The highest number of marketable propagules (55.1 per pot), total propagules (74 per pot), and marketable propagule rate (74.2%) were also found in plants treated with Miracle-Gro 15-30-15. Plants fertilized with urea produced the least in fresh underground weight, propagule number, and the ratio of marketable propagules of 'Embolene' compared with plants receiving other fertilizer treatments. Regression analysis showed strong positive quadratic or linear relationships among growth parameters except for flower number of plants in the fertilizer treatments, thus one plant growth index can be predicated by another based on regression model (Table 3).

Fertilization not only influenced plant growth but also influenced moisture and nutrient content in rhizomes of 'Embolene' (Table 4). Moisture content of expanded rhizomes was reduced by fertilization. The highest moisture (83.6%) was found in plants in control followed by plants treated with controlled release Polyon which contributed to the highest

moisture (78.64%) in rhizomes among fertilizer treatments. There was no difference in moisture among rhizomes receiving other treatments. Nutrition analysis showed that the highest values were found in plants in control for most macro- or micro-elements (Table 4). Compared with control, a decrease occurred in the contents of P, Ca, Mg, Fe, Mn, Zn and Al in plants with fertilizer treatments. Plants treated with Urea produced the highest N and Na but the least P, K, B, Cu and Al in expanded rhizomes. No differences were found in Ca, Mg, Fe, Zn, and Al among rhizomes receiving fertilizers, excluding urea. There was no difference in nutrients for rhizomes receiving 5 g/10 d and 10 g/20 d of Pro•Sol 20-10-20. Except for the urea treatment, K content in rhizomes was the highest followed by N content, and P content was the lowest. However, the ratio of N-P-K in rhizomes was influenced by fertilizer types (Table 4). N-fertilizer (urea) increased N accumulation but significantly decreased absorption of P and K in expanded rhizomes.

The above results confirmed that lotus is a fertilizer-consuming plant (Zhang et al., 1994). Lotus 'Embolene' in the control group produced the lowest parameters of plant growth and aged earliest, while fertilization increased plant growth indices 2 to 14 fold (Table 2). Therefore, for lotus production in containers, fertilization is particularly critical to obtain maximum growth. Based on application of an equal rate of N, soluble fertilizers were more effective for lotus growth than urea and controlled release fertilizer Polyon 18-6-12. Fertilizers with higher P and K ratio to N were beneficial for development of rhizomes or propagules with respect to increases in plant height and emerging leaf number. High P ratio fertilizer Miracle-Gro 15-30-15 produced the highest in growth parameters other than flower number. Therefore, this type fertilizer was more efficient for rhizome or propagule production. Controlled release fertilizer Polyon 18-6-12 did not

provide enough nutrition to meet optimum requirements of plant growth, especially in the early season, because of lower temperatures. Early in the season plants receiving Polyon fertilizer were almost the same size as plants in the control group. However, leaf life was extended in the Polyon fertilizer treated plants for about two weeks compared with other fertilizers. Further studies are necessary to investigate if better effects could be obtained by improving fertilization methods using controlled release Polyon or using other types of controlled release fertilizers with varying nutrient release curves. With exception of flower number, there were no large differences in plant growth indices and nutrient content in expanded rhizomes among treatments of high-frequency-low-concentration and low-frequency-high-concentration soluble fertilizer Pro•Sol 20-10-20. Therefore, an application with 10 g/20 d of this fertilizer was preferable to decrease labor cost compared with a 5 g/10 d.

Moisture of expanded rhizomes in plants with fertilization treatments decreased compared to plants in the control treatment. Concentrations of macronutrients P, Ca, Mg and micronutrients Fe, Mn, Zn, and Al in rhizomes of plants with fertilization treatments were also obviously lower than those in plants in the control treatment. This indicated accumulation of these nutrients in expanded rhizomes was inhibited by fertilization. Both increase and decrease in content of other nutrients in expanded rhizomes occurred in plants with fertilizer treatments. These phenomena suggested that the increased dry mass in expanded rhizomes in fertilizer treatments could not be explained by nutrient levels alone (Table 4). It was reported that the yield of rhizomes, starch, and carbohydrate in expanded rhizomes were significantly increased by application of K fertilizer in vegetable lotus (Zhang et al., 1994), therefore, the increased dry rhizome mass rate of

‘Embolene’ in the fertilizer treatments could be caused by accumulation of carbohydrates through photosynthesis. Further study is necessary to determine major factors responsible for an increase in both amount and percentage of dry mass in expanded rhizomes. Compared with the fertilizer treatments, a relatively high N content and the highest contents of P and K in expanded rhizomes were found in the control. Thus, the contents of N, P and K in rhizome tissue did not proportionally relate to the total availability of nutrients in the water. However, the contents of N, P and K in rhizomes were associated with fertilizer sources: urea led to highest N content, Miracle-Gro 15-30-15 led to highest P content, and Pro•Sol 20-10-20 led to highest K in expanded rhizomes. Except for plant treated with urea, K content was always the highest followed by N content. P content was the lowest in expanded rhizomes (Table 4). This result agreed with the previous report in non-flowering vegetable lotus (Qu and Zhao, 1991).

In conclusion, fertilization significantly increased parameters of lotus ‘Embolene’ but plants responded to the fertilizers differently. A correct choice of fertilizer type is important in lotus production. There are diverse types of fertilizers available for various crops but no lotus-specific fertilizer has been developed. Future studies should evaluate effects of other fertilizers and fertilizer specific ratios of nutrients for specific production purposes such as seed, rhizome or flower production and time of flowering. Research should concentrate on aquatic plant fertilizers and shorter duration controlled release fertilizers, in lotus container production.

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Tables

Table 1. Description on fertilization treatments on lotus 'Embolene' in 29 L container production.

Treatment		N% in fertilizer	Fertilizer dose per pot and interval of application	Total applications
Code	Fertilizer or control			
A	Control	0	0	0
B	Urea	46%	2.2 g/10 d	7
C	Pro•Sol 20-10-20	20%	5 g/10 d	7
D	Pro•Sol 20-10-20	20%	10 g/20 d (5 g for initial time)	7
E	Miracle-Gro 24-8-16	24%	4.2 g/10 d	4
F	Miracle-Gro 15-30-15	15%	6.7 g/10 d	7
G*	Controlled release Polyon 18-6-12	18%	11.2 g/10 d	7

Note: G* – the rate of fertilizer was doubled because of a temperature based slow release rate of 8 to 9 months at 27 ºC (80 ºF). The rate of other fertilizers was calculated based on the same total rate of N. For fertilizer treatments, plants were supplied with fertilizer from June 2 to Aug. 11, 2006 at a 10 or 20 d interval.

Table 2. Response of lotus 'Embolene' to different fertilizers in 29 L container production.

Treatment	Plant height (cm)	Emerging leaf No.	Flower No.	Fresh underground weight (g)	Marketable propagule No.	Total propagule No.	Marketable propagule rate (%)	Days to maxim height
A	41.9 d	6.9 d	3.0 c	104.6 f	10.0 f	17.5 e	57.1 c	105
B	75.1 c	63.3 c	16.6 a	563.5 e	22.1 e	47.7 d	46.3 d	120
C	97.1 a	81.0 b	17.2 a	855.1 c	33.5 c	52.8 cd	63.4 bc	120
D	92.1 a	72.8 b	12.4 b	859.5 c	36.6 bc	51.8 cd	70.7 ab	120
E	94.7 a	78.8 b	19.0 a	973.7 b	39.7 b	56.7 c	70.0 ab	120
F	96.9 a	96.0 a	12.0 b	1090.0 a	55.1 a	74.3 a	74.2 a	120
G	81.0 b	77.8 b	11.4 b	749.4 d	37.4 bc	64.7 b	57.8 c	135

Treatments: A – control; B – Urea at 2.2 g/10 d; C – Pro•Sol 20-10-20 at 5 g/10 d; D – Pro•Sol 20-10-20 at 10 g/20 d; E – Miracle-Gro 24-8-16 at 4.2 g/10 d; F – Miracle-Gro 15-30-15 at 6.7 g/10 d; G – Controlled release Polyon 18-6-12 (8-9 month) at 11.2 g/10 d. For fertilization treatments, plants were supplied with fertilizer from June 2 to Aug. 11, 2006 at a 10 or 20 d interval. Means were separated by LSD at a 0.05 significance level.

Table 3. Regression relationships among plant growth indices of lotus 'Embolene' using the pooled data of fertilizer treatments in 29 L container production.

Plant index	Model	P value	R^2
Plant height (x) * Emerging leaf No. (y)	$y = -36.95 + 0.03534\ x^2 - 2.31*10^{-4}\ x^3$	< 0.0001	0.715
Plant height (x) * Fresh biomass (y)	$y = 22164 - 79.057\ x + 9.457\ x^2 - 0.0367\ x^3$	< 0.0001	0.652
Plant height (x) * propagule No. (y)	$y = 47.59 + 1.27*10^{-5}\ x^3$	0.0295	0.082
Emerging leaf No. (x) * Fresh biomass (y)	$y = 406.21 + 5.618\ x$	< 0.0001	0.335
Emerging leaf No. (x) * propagule No. (y)	$y = 40.0 + 3.2*10^{-4}\ x^2$	< 0.0001	0.558
Fresh biomass (x) * propagule No. (y)	$y = 40.04 + 2.4*10^{-5}\ x^2$	< 0.0001	0.397
Flower No. (x) * other parameters (y)	No relationship	> 0.05	-

Note: The relationships among plant growth indices were evaluated by regression analysis using SAS 9.1. The unit of plant growth index: cm/pot for plant height and g/pot for fresh biomass.

Table 4. Effect of fertilizers on nutrition in expanded rhizomes of lotus 'Embolene' harvested from a 29 L container production.

Treatment	H_2O (%)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	N-P-K ratio
A	83.6 a	1.24 c	0.82 a	3.66 a	0.34 a	0.5 a	1.46-1-4.46
B	75.16 cd	1.79 a	0.4 d	1.63 c	0.28 b	0.33 b	4.48-1-4.08
C	75.54 cd	0.96 d	0.66 b	3.7 a	0.23 c	0.24 c	1.45-1-5.61
D	73.58 cd	1.05 d	0.67 b	3.73 a	0.22 c	0.25 c	1.57-1-5.57
E	74.07 cd	1.17 cd	0.53 c	3.01 b	0.24 c	0.24 c	2.21-1-5.68
F	75.79 bc	1.31 c	0.79 a	3.28 b	0.22 c	0.27 c	1.66-1-4.15
G	78.64 b	1.6 b	0.49 c	3.24 b	0.22 c	0.27 c	3.27-1-6.61
	Fe (ppm)	Mn (ppm)	B (ppm)	Zn (ppm)	Cu (ppm)	Al (ppm)	Na (ppm)
A	558.53 a	283.42 a	11.55 cd	27.5 a	11.88 ab	163.38 ab	717.27 bc
B	316.16 b	171.89 c	8.19 de	21.53 b	7.46 bc	92.95 c	1629.54 a
C	155.48 b	209.91 bc	15.57 ab	18.89 bc	16.97 a	118.16 bc	527.85 d
D	165.83 b	217.09 bc	15.79 a	17.02 c	13.79 ab	145.13 bc	599.84 cd
E	161.73 b	168.08 c	12.38 cd	17.52 c	10.59 ab	127.63 bc	786.75 b
F	221.53 b	229.7 b	13.13 bc	17.07 c	9.48 bc	132.52 bc	685.06 bc
G	178.88 b	186.5 bc	10.04 de	16.94 c	8.27 bc	139.37 bc	603.38 cd

Treatments: A – control; B – Urea at 2.2 g/10 d; C – Pro•Sol 20-10-20 at 5 g/10 d; D – Pro•Sol 20-10-20 at 10 g/20 d; E – Miracle-Gro 24-8-16 at 4.2 g/10 d; F – Miracle-Gro 15-30-15 at 6.7 g/10 d; G – Controlled release Polyon 18-6-12 (8-9 month) at 11.2 g/10 d. For fertilization treatments, plants were supplied with fertilizer from June 2 to Aug. 11, 2006 at a 10 or 20 d interval. Means were separated by LSD at a 0.05 significance level.

Figures

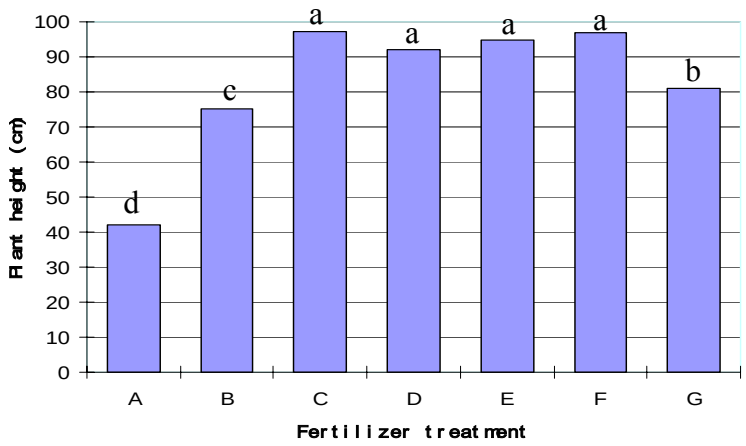

Fig. 1. Effect of fertilizers on plant height of lotus 'Embolene' in 29 L containers. Treatments: A – control; B – Urea at 2.2 g/10 d; C – Pro•Sol 20-10-20 at 5 g/10 d; D – Pro•Sol 20-10-20 at 10 g/20 d; E – Miracle-Gro 24-8-16 at 4.2 g/10 d; F – Miracle-Gro 15-30-15 at 6.7 g/10 d; G – Controlled release Polyon 18-6-12 (8-9 month) at 11.2 g/10 d. For fertilization treatments, plants were supplied with fertilizer from June 2 to Aug. 11, 2006 at a 10 or 20 d interval. Bars with the same letter were not significantly different by LSD ($\alpha = 0.05$).

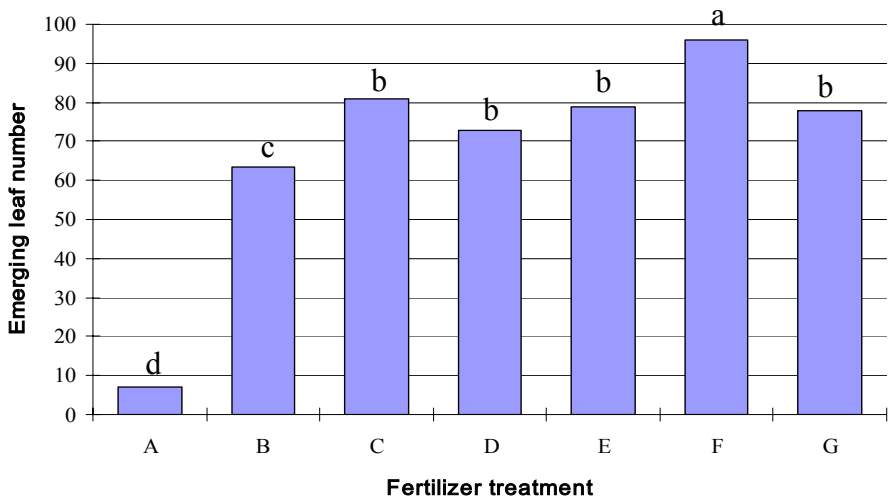

Fig. 2. Effect of fertilizers on emerging leaf number of lotus 'Embolene' in 29 L containers. Treatments: A – control; B – Urea at 2.2 g/10 d; C – Pro•Sol 20-10-20 at 5 g/10 d; D – Pro•Sol 20-10-20 at 10 g/20 d; E – Miracle-Gro 24-8-16 at 4.2 g/10 d; F – Miracle-Gro 15-30-15 at 6.7 g/10 d; G – Controlled release Polyon 18-6-12 (8-9 month) at 11.2 g/10 d. For fertilization treatments, plants were supplied with fertilizer from June 2 to Aug. 11, 2006 at a 10 or 20 d interval. Bars with the same letter were not significantly different by LSD ($\alpha = 0.05$).

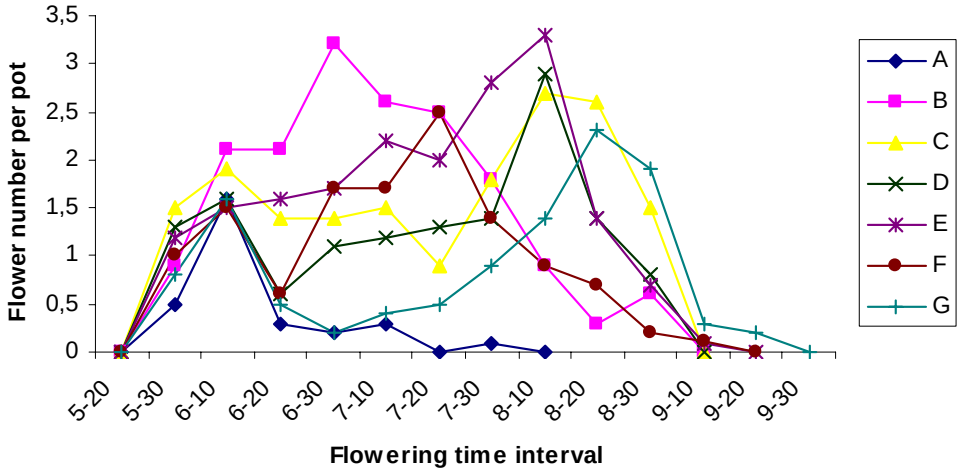

Fig. 3. Effect of fertilizers on flowering of lotus 'Embolene' in 29 L containers. Treatments: A – control; B – Urea at 2.2 g/10 d; C – Pro•Sol 20-10-20 at 5 g/10 d; D – Pro•Sol 20-10-20 at 10 g/20 d; E – Miracle-Gro 24-8-16 at 4.2 g/10 d; F – Miracle-Gro 15-30-15 at 6.7 g/10 d; G – Controlled release Polyon 18-6-12 (8-9 month) at 11.2 g/10 d. For fertilization treatments, plants were supplied with fertilizer from June 2 to Aug. 11, 2006 at a 10 or 20 d interval. Calculation of flowering number was based on 10 d periods, namely 10-19, 20-29, and 30-9 of a month, respectively.

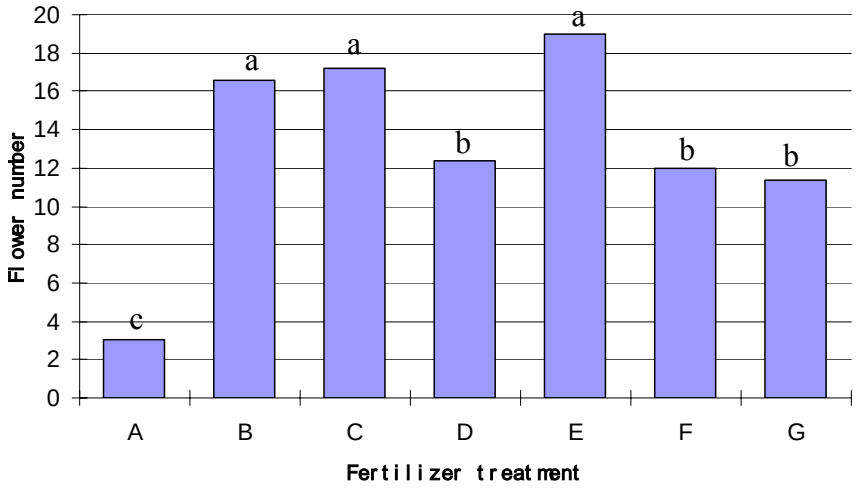

Fig. 4. Effect of fertilizers on flower number of lotus 'Embolene' in 29 L containers. Treatments: A – control; B – Urea at 2.2 g/10 d; C – Pro•Sol 20-10-20 at 5 g/10 d; D – Pro•Sol 20-10-20 at 10 g/20 d; E – Miracle-Gro 24-8-16 at 4.2 g/10 d; F – Miracle-Gro 15-30-15 at 6.7 g/10 d; G – Controlled release Polyon 18-6-12 (8-9 month) at 11.2 g/10 d. For fertilization treatments, plants were supplied with fertilizer from June 2 to Aug. 11, 2006 at a 10 or 20 d interval. Bars with the same letter were not significantly different by LSD ($\alpha = 0.05$).

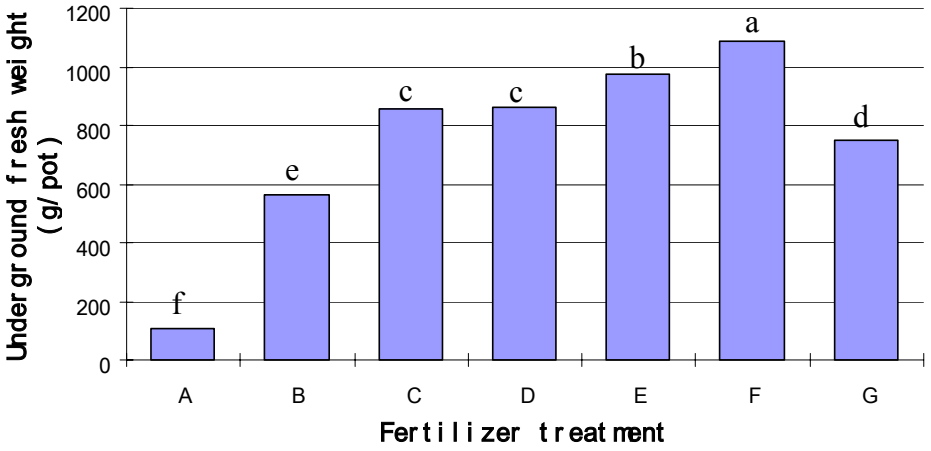

Fig. 5. Effect of fertilizers on underground fresh weight of lotus 'Embolene' in 29 L containers. Treatments: A – control; B – Urea at 2.2 g/10 d; C – Pro•Sol 20-10-20 at 5 g/10 d; D – Pro•Sol 20-10-20 at 10 g/20 d; E – Miracle-Gro 24-8-16 at 4.2 g/10 d; F – Miracle-Gro 15-30-15 at 6.7 g/10 d; G – Controlled release Polyon 18-6-12 (8-9 month) at 11.2 g/10 d. For fertilization treatments, plants were supplied with fertilizer from June 2 to Aug. 11, 2006 at a 10 or 20 d interval. Bars with the same letter were not significantly different by LSD ($\alpha = 0.05$).

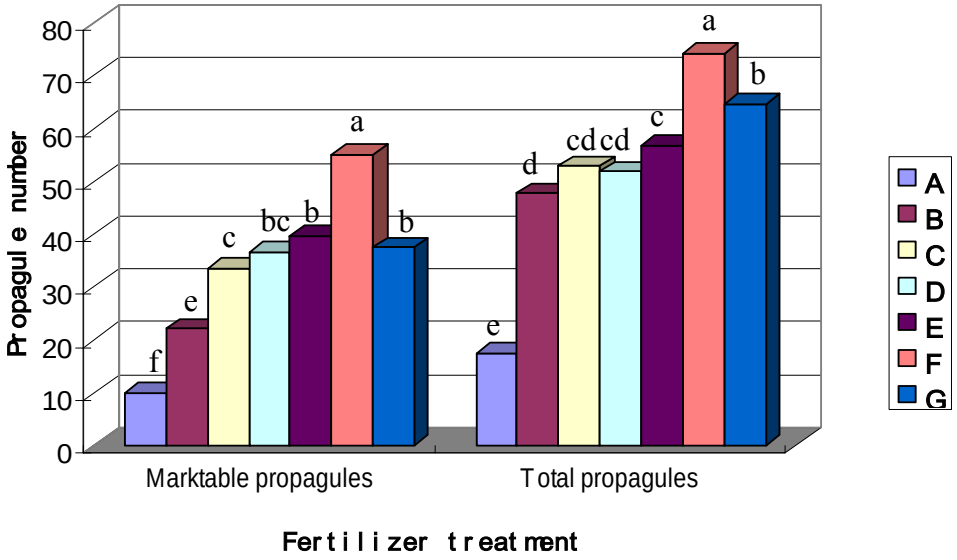

Fig. 6. Effect of fertilizers on marketable and total propagule number of lotus 'Embolene' in 29 L containers. Treatments: A – control; B – Urea at 2.2 g/10 d; C – Pro•Sol 20-10-20 at 5 g/10 d; D – Pro•Sol 20-10-20 at 10 g/20 d; E – Miracle-Gro 24-8-16 at 4.2 g/10 d; F – Miracle-Gro 15-30-15 at 6.7 g/10 d; G – Controlled release Polyon 18-6-12 (8-9 month) at 11.2 g/10 d. For fertilization treatments, plants were supplied with fertilizer from June 2 to Aug. 11, 2006 at a 10 or 20 d interval. Bars with the same letter were not significantly different by LSD ($\alpha = 0.05$).

Chapter 5 Response of Lotus (*Nelumbo nucifera* Gaertn.) to Planting Time and Disbudding

Additional index words: growth index, flower number, expanded rhizome, plant height

Abstract

Effects of planting time and disbudding on plant performance were evaluated for an ornamental lotus**,** *Nelumbo nucifera* 'Embolene'. Plant growth indices were not greatly affected by planting time with a time gap less than one month when lotus was planted in March but were significantly influenced by a larger time gap more than one month in planting time when lotus was planted between February 25 and May 25. Plants potted and placed outside in March and April performed best and lotus planted in the greenhouse in February and planted outside in February and May performed worst. Flower number was not largely influenced by the planting time but flowering characteristics, especially the flowering peaks, were obviously different among treatments. Disbudding had no impact on plant height but significantly increased fresh underground weight and the number of propagules, and non-significantly increased the number of emerging leaves (α = 0.05, HSD). Strong positive linear or quadratic relationships were detected among emerging leaf number, fresh underground biomass, and propagule number.

Introduction

Lotus (*Nelumbo nucifera* Gaertn.), also called Asian lotus, is an aquatic perennial herbaceous plant. *N. nucifera* has an extremely long history in cultivation as a vegetable, medicinal and ornamental plant in Eastern countries (Wang and Zhang, 2004). Lotus rhizome is one of the major vegetables in Asia. Recently, lotus has become a potential crop in Australia (Nguyen, 2001), New Zealand (Follett and Douglas, 2003), and the United States (Tian et al., 2006). Lotus is usually planted in a tilled pond or rice field for vegetable production. It also is often planted in containers, small ponds, and lakes for ornamental uses. Planting time is often between late spring and early summer (Sou and Fujishige, 1995) with plant growth ending in the fall under natural conditions. Growth and yield of lotus may be influenced by diverse factors such as genotype (Zhou et al., 2004), media (Meyer, 1930; Li and Qian, 1994; Wang and Zhang, 2004), water depth (Snow, 2000; Nguyen, 2001; Wang and Zhang, 2004), light (Li et al., 2000; Snow, 2000), temperature (Meyer, 1930), planting time, propagation methods (Katori et al., 2002; Wang and Zhang, 2004), planting technique (Wen, 1987; Sang et al., 1994; Shen et al., 2001; Min et al., 2006), fertilization (Li and Qian, 1994; Zhang et al., 1994; Sou and Fujishige, 1995; Hicks, 2005; Song et al., 2006; Orimoto and Takai, 2007), and other environmental factors (Nguyen, 2001; Hicks, 2005).

Cultivar selection and cultivation techniques are dependent on the environmental setting of the lotus plant. Lotus can be propagated by seeds, rhizome divisions with viable growing points, and tissue culture. Effects of propagation methods and planting techniques on yield of lotus have been much reported. Propagation by division of running stems (non-enlarged rhizomes or straps) during the growing season can not only save

stock rhizomes and cost, increase efficiency, and prolong flowering period of plants, but may replace plants which did not survive early in the year (Wang and Zhang, 2004). Days to flowering are significantly shorter in the strap propagation method than in the enlarged rhizome propagation method where flowering time is delayed (Katori et al., 2002). Plants generated via the rhizome strap method also produce larger flowers.

Off-season cultivation methods have been developed to meet the demands of the market. Availability of vegetable rhizomes could be advanced to June by a technique of growing edible lotus earlier in the season (Fu et al., 1994). Flowering-lotus generally blooms from June (May) to August, but population flowering time can be extended to early October when lotus is propagated by dividing growing plants in July (Deng et al., 1990). It is feasible to prolong flowering time of lotus through the winter when plants are planted in a heated greenhouse (Li et al., 2000). If the technologies of advancing and delaying flowering are incorporated, three cycles of population flowering are possible in one year (Wang and Zhang, 2004). Reports indicate that production of lotus cut flowers may be on a year-round schedule (Chomchalow, 2004). However, little information is available on effects of planting time on overall lotus performance. The productive organs of lotus plants are nutrient sinks and therefore disbudding of lotus would possibly increase plant yield of lotus rhizomes and other plant growth indices. The major goal of this study was to investigate effects of planting time and disbudding on lotus growth and development in containers.

Materials and Methods

A medium-sized ornamental lotus, *N. nucifera* 'Embolene', was used to evaluate response to planting time and disbudding. Experiments were conducted in 2005 and 2007, respectively. In experiment I, 4 ºC-cooler-stored lotus propagules (2 to 3 internodes with shoots) were planted outdoors in 29 L (7.5 gal) black plastic containers (31 cm bottom and 37 cm top in diameter, 32 cm in height) without holes on Mar. 1, Mar. 16, and Mar. 31, 2005, respectively. Twenty propagules were planted each time and each pot contained one propagule. All containers were filled to 2/3 container level with natural sandy loam soil and immediately filled to ¾ container level (18 cm, 21 L) with municipal tap water (pH 7.0, EC = 0.13 $mS \cdot cm^{-1}$) after planting. Fertilization started when several floating leaves developed. Each plant was fed 8 g (2 tsp) of Pro.Sol 20-10-20 (Pro•Sol Inc., Ozark AL 36360, USA) at 20 d intervals. Fertilizer was applied 4 times and each treatment received the same amount of fertilizer. During the flowering period, flower buds were discarded once every 3 d for one half of plants from each planting date, while the other half was not pruned with flower buds and fruits allowed to mature. Plant height and emerging leaf number were measured on Aug. 20, 2005 when plants reached maximum height. Underground parts were harvested on Jan. 1, 2006.

The second experiment was conducted in 2007. The same type of containers were used but only filled to half container level (16 cm, 13 L) of the same type of soil in experiment I. Cooler stored propagules of lotus 'Embolene' were planted on Feb. 25, Mar. 25, Apr. 25, and May 25, respectively. There were two treatments for the plants planted on Feb. 25: greenhouse and outdoors. Plants planted on all other dates were placed outdoors. Each treatment contained 6 plants (pots). Soluble fertilizer Pro•Sol 20-10-20 was applied

beginning on Apr. 20 for plants planted on Feb. 25; May 10 for plants planted on March 25; May 20 for plants planted on Apr. 25; and June 15 for plants planted on May 25, respectively, based on the equal dose and sequence with 4, 8, 12, 12, 8, and 4 g/pot at a 20 d interval. The blooming time and flower number were recorded once every one or two days. Data on plant height and emerging leaf number were taken on June 20, July 10, Aug. 2, Aug. 20 and Sept. 12, respectively. Underground parts were harvested on Nov. 23, 2007.

Data on fresh biomass, the number of marketable propagules (2 to 3 expanded internodes with shoots), secondary propagules (2 to 3 non-expanded internodes with shoots), total propagules (including both marketable and secondary propagules), and the number of expanded internodes (≥1 cm diameter) were immediately taken following harvest. The expanded internodes (4 samples/treatment) were sent to the Soil Testing Laboratory at Auburn University for nutritional analysis. Means of variables were separated by Tukey (HSD) or LSD procedure at the 0.05 significance level using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). The relationships among plant growth parameters were determined by regression analysis with SAS 9.1 using pooled data from all outdoor treatments.

Results and Discussion

In experiment I, plant growth indices of 'Embolene' were little affected by planting time with a gap between 15 and 30 d but much influenced by disbudding treatment (Table 1, Fig. 1, 2, 3). Planting time and disbudding treatments did not affect plant height (87.1 to 92.6 cm, Table 1). Significant difference (LSD, $P \leq 0.05$) in emerging leaf

number was only observed between plants planted on Mar. 1 in non-disbudding control and other treatments (Table 1, Fig. 1). Emerging leaf number generally increased as a result of disbudding among planting dates. Fresh underground biomass significantly increased (HSD, $P \leq 0.05$) by disbudding (Table 1, Fig. 2). A significant increase in total propagule number was observed in disbudded plants planted on Mar. 1 and Mar. 16 compared with non-disbudded control. Plants planted on Mar. 31 had a larger number of total propagules than plants planted earlier (Table 1, Fig 3).

In the second experiment, plants responded to environmental conditions in the greenhouse and outdoors differently. Because of higher temperatures plants in the greenhouse grew faster in the early stage and reached a peak plant height, flowered earlier but had the lowest values in plant height and emerging leaf number measured on Sept. 12 (Table 2, Fig. 4, 5, 6, 7, 8). Plants in the greenhouse also entered dormancy and ceased growth earlier (data not shown). Flowering characteristics were obviously influenced by plant growth environment (greenhouse vs. outdoors) and planting time. Days to the first flower were 76, 90, 71, 45, and 60, for plants planted on Feb. 25 in the greenhouse, on Feb. 25, Mar. 25, Apr. 25, and May 25 outdoors, respectively. Plants in all treatments had at least one or two major flowering peaks and 0 to 2 small minor flowering peaks determined on 10 d intervals (Fig 6). Except for plants planted on May 25, the first flowering peak occurred between May 10 and June 20 and this peak was always the largest flowering peak. During Aug. 10 to 20 a flowering peak occurred in all outdoor treatments. An obvious decrease in flowering occurred between June 30 and July 20 except for plants planted on May 25. The occurrence of the major flowering peak was associated with planting time, and the time order was May 20 to 30 for the greenhouse

treatment (planted on Feb. 25); May 30 to June 10, June 10 to 20, and June 10 to 20 for the outdoor plants planted on Feb. 25, Mar. 25, and Apr. 25, respectively. The flowering peak of plants planted on May 25 occurred latest between Aug. 10 and Aug. 20. The days to the largest flower peak were 90, 95, 85, 55, and 85 days, respectively. No flowers developed after Aug. 20 for plants in the greenhouse and after Sept. 20 for all plants planted outdoors. Plants grown outside showed similar growth patterns, which were reflected in plant height and emerging leaf number: plants initially grew slowly and later grew fast, linearly between June and August for plants planted on Feb. 25, Mar. 25 and Apr. 25 and between June and August for plants planted on May 25. Plant growth leveled off after late August as plants gradually entered dormancy. Leaves of plants planted on Feb. 25 died back earliest and plants planted on May 25 died back latest. An estimated 10-day difference in the time of leaf death was found between the earliest planting and the latest planting treatments. No visible difference in the time of leaf death was found between the treatments of plants planted on Mar. 25 and Apr. 25. All leaves on plants died in October for outside growing plants.

Plant growth indices were largely influenced by treatments in the greenhouse or outdoors as well as planting time. Plants planted in the greenhouse had the lowest values in most growth indices while plants planted outdoors on Mar. 25 and Apr. 25 had the highest values in all evaluated plant growth indices (Table 2, Fig. 7, 8). Significant differences were observed in plant height, emerging leaf number, fresh underground biomass, marketable propagule number, total propagule number, expanded internode number, and the average maximum diameter of rhizomes between the plants planted in the greenhouse and outside. But, total flower numbers and the number of secondary

propagules were similar. Outdoor lotus plants planted on Mar. 25 and Apr. 25 performed best, and no significant differences in observed growth parameters were detected between these treatments. Performance of plants was similar in plants planted on Feb. 25 and May. 25.

Regression analysis of major plant indices showed a strong positive linear or quadratic relationship between emerging leaf number and fresh underground weight ($P < 0.0001$, $R^2 = 0.685$), plant height and fresh underground weight ($P < 0.0001$, $R^2 = 0.662$), emerging leaf number and propagule number ($P < 0.0001$, $R^2 = 0.53$), fresh underground weight and total propagule number ($P < 0.0001$, $R^2 = 0.496$), fresh underground weight and commercial propagule number ($P < 0.0001$, $R^2 = 0.689$), fresh underground weight and expanded internode number ($P < 0.0001$, $R^2 = 0.569$), and between fresh underground weight and maximum rhizome diameter ($P < 0.0001$, $R^2 = 0.78$). Little- or non-significant relationship occurred between emerging leaf number and plant height ($P < 0.0111$, $R^2 = 0.209$), and between emerging leaf number and flower number ($P < 0.0452$, $R^2 = 0.136$) (Table 3). No correlation was determined between flower number and other plant growth indices.

Results in this study indicated that lotus plant growth was mainly regulated by temperature which was in agreement with previous reports by Li et al. (2000) and Zhang (2003). In earlier growth stages (before June), plants grew slow and fewer leaves developed because of lower temperatures. Plant growth rate increased when temperatures became higher from June to August and then growth gradually decreased and entered senescence and dormancy after August. Emerging leaves began to die in early September and totally desiccated around mid-October. A similar situation was reported by Peng et al.

(2004) in field production of vegetable lotus. Lotus performed better in an environment with flexible temperatures. Continuous high temperatures (32 to 40 ºC) above the optimal range (22 to 32 ºC) and small difference of day-night temperature in the greenhouse inhibited plant growth and advanced plant maturation and senescence. Fresh underground weight in the greenhouse treatment was only 25% to 39% of that in the outdoor treatments. This suggested that a possible increase of night time respiration at higher temperatures inhibited enlargement of rhizomes while flexible day/night temperatures were more beneficial for accumulation of carbohydrates which were responsible for expansion of lotus rhizomes. Therefore, the greenhouse system with somewhat constant day/night temperatures was not optimal for production of lotus as propagules or vegetables.

Selection of the best planting time is especially critical for a nursery to maximize yields and profits. This study indicated that March and April are the best seasons for lotus container production in Auburn area of Alabama. However, an optimal planting time should be case dependent considering differences of genotype (early or late season cultivars), local climate and production system (container or field, greenhouse or field, regular or offseason production). Under natural conditions, it is not necessary to plant lotus early because of a limitation in temperatures. On the other hand, because of a short life cycle constrained by temperature and photoperiod, lotus should not be planted too late. Disbudding was a useful practice to increase yield of underground weight, propagule number and emerging leaf number. Large mean differences were evident in emerging leaf number, fresh underground weight, and total or marketable propagule number which were positively correlated with each other. These plant parameters were very effective for

evaluation of effect of planting time and disbudding on lotus plants. Report on lotus production in the greenhouse is limited. Since there are large differences in the conditions between the greenhouse and outdoors, further studies are necessary to evaluate the effects of air circulation, moisture, temperature, photoperiod and light intensity on lotus production.

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Tables

Table 1. Response of lotus 'Embolene' to planting time and disbudding in 29 L containers in 2005.

Planting Time	Disbudding	Height (cm)	Emerging leaf number*	Underground biomass (g)	Total propagule number
Mar. 1	No	88.7 a	40.7 b	450.8 d	32.3 b
	Yes	87.1 a	51.1 a	559.4 bc	40.8 a
Mar. 16	No	87.9 a	44.9 ab	506.9 cd	33.4 b
	Yes	92.6 a	50.3 a	648.4 a	40.4 a
Mar. 31	No	87.5 a	47.8 ab	516.3 cd	42.6 a
	Yes	87.1 a	50.9 a	596.3 ab	45.5 a

Means were separated by HSD at the 0.05 significant level except means of emerging leaf number* separated by LSD ($\alpha = 0.05$) based on data collected on Aug. 20, 2005 for plant height and emerging leaf number, and on Jan, 1, 2006 for underground biomass and total propagule number.

Table 2. Response of lotus 'Embolene' to planting time in 29 L containers in 2007.

Planting Time	Height (cm)	Emerging leaf No.	Flower No.*	Fresh underground biomass (g)	Fresh weight of propagules (g)	Marketable propagule No.	Secondary propagule No.*	Total propagule No.	Expanded Internode No.	Maximum rhizome diameter (mm)
Feb. 25*	63.2 c	38.8 c	11.7ab	294.8 d	199.6 d	20.8 b	19.6 ab	40.4 c	17.6 b	1.7 b
Feb. 25	92.3 b	73.2 ab	14.7ab	715.7 c	514.8 c	26.7 ab	22.5 ab	49.2 bc	27.8 a	2.6 a
Mar. 25	100.7ab	90.5 a	15.8 a	1133.8 a	762.0 ab	33.8 a	27.3 a	61.0 a	31.3 a	2.8 a
Apr. 25	102.3 a	75.8 ab	15.0 a	1119.3 ab	802.0 a	32.7 a	24.5 ab	57.2 ab	34.0 a	2.9 a
May. 25	98.7 ab	43.3 c	10.3 b	755.17 c	595.5 c	23.7 b	17.7 b	41.3 c	30.2 a	2.6 a

Plants potted on Feb. 25* were placed in the greenhouse, plants potted on Feb. 25, Mar. 25, Apr. 25, or May 25 were placed outdoors. Means were separated by HSD at 0.05 significant level, but for flower number and secondary propagule number, LSD ($\alpha = 0.05$) was used when mean difference couldn't be detected by HSD. Means within a column not followed by same letter are significantly different ($P \leq 0.05$).

Table 3. Relationships of major plant growth indices of lotus 'Embolene' grown in 29 L containers in 2007.

Plant indexes	Model	P value	R^2
Emerging leaf No. (x) – Height (y, cm)	$y = 81.48 + 0.0021\ x^2$	0.0111	0.209
Emerging leaf No. (x) – Underground weight (y, g)	$y = -816.9 + 17.72\ x$	< 0.0001	0.685
Emerging leaf No. (x) – Flower No. (y)	$y = 9.68 + 0.059\ x$	0.0452	0.136
Emerging leaf No. (x) – Total propagule No. (y)	$y = 28.51 + 0.331\ x$	< 0.0001	0.530
Underground weight (y, g) – Height (x, cm)	$y = 475.52 + 0.069\ x^2$	< 0.0001	0.496
Underground weight (x, g) – Total propagule No. (y)	$y = 39.16 + 1.389\times10^{-8}\ x^3$	< 0.0001	0.662
Underground weight (x, g) – Marketable propagule No. (y)	$y = 19.5 + 1.064\times10^{-5}\ x^2$	< 0.0001	0.689
Underground weight (x, g) – Expanded internode No. (y)	$y = 13.95 + 0.018\ x$	< 0.0001	0.569
Underground weight (x, g) – Maximum rhizome diameter (y, mm)	$y = 0.76 + 0.0039\ x + 1.89\times10^{-6}\ x^2$	< 0.0001	0.780

Relationships among major plant growth indices were determined by regression analysis using pooled data for outdoor treatments in which plants were potted on Feb. 25, Mar. 25, Apr. 25, or May 25 and placed outdoors.

Figures

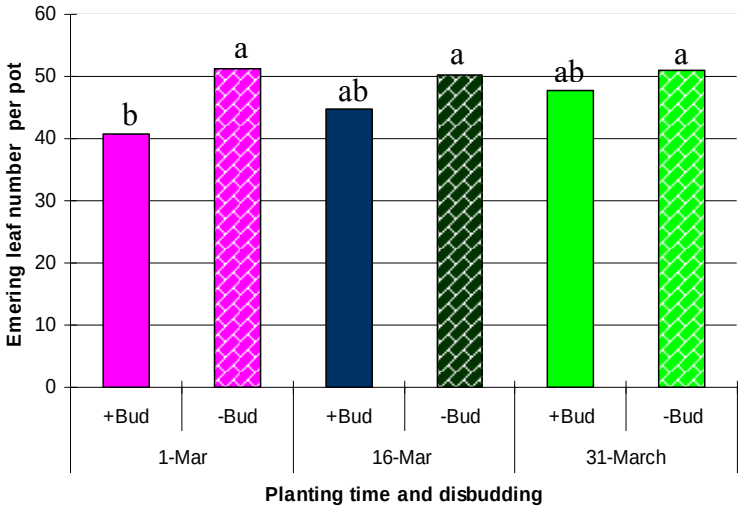

Fig. 1. Effect of planting time and disbudding on emerging leaf number of lotus 'Embolene' in 29 L containers in 2005. Note: '+Bud': keeping buds; '-bud': disbudding. Means were separated by LSD ($\alpha = 0.05$).

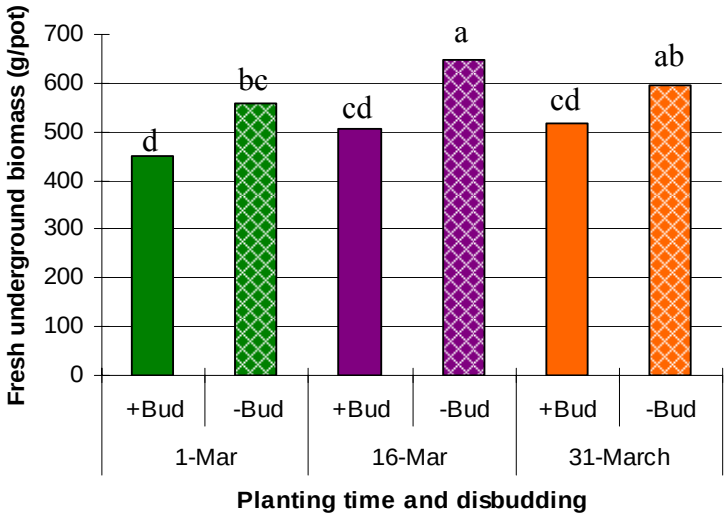

Fig. 2. Effect of planting time and disbudding on fresh underground biomass of lotus 'Embolene' in 29 L containers in 2005. Note: '+Bud': keeping buds; '-bud': disbudding. Means were separated by HSD ($\alpha = 0.05$).

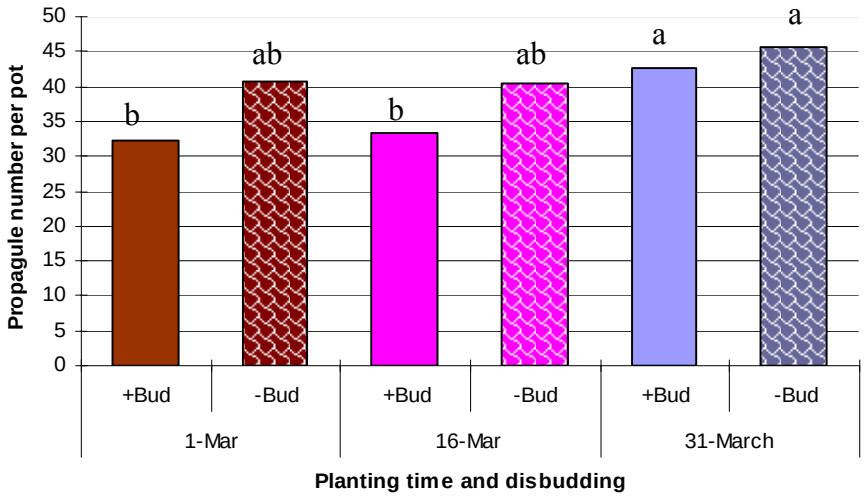

Fig. 3. Effect of planting time and disbudding on propagule number of lotus 'Embolene' in 29 L containers in 2005. Note: '+Bud': keeping buds; '-bud': disbudding. Means were separated by HSD ($\alpha = 0.05$).

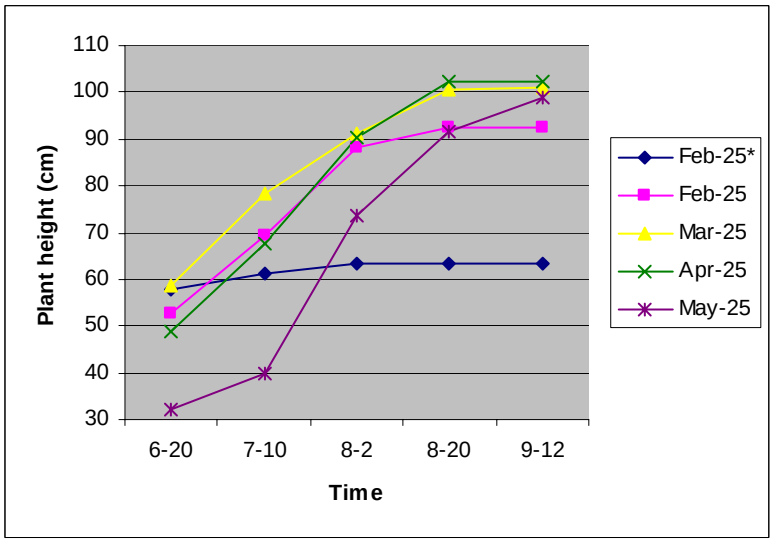

Fig. 4. Effect of planting time on plant height of lotus 'Embolene' in 29 L containers. Plants were planted on Feb. 25* (greenhouse), Feb. 5, Mar. 25, Apr. 25, and May 25 outside.

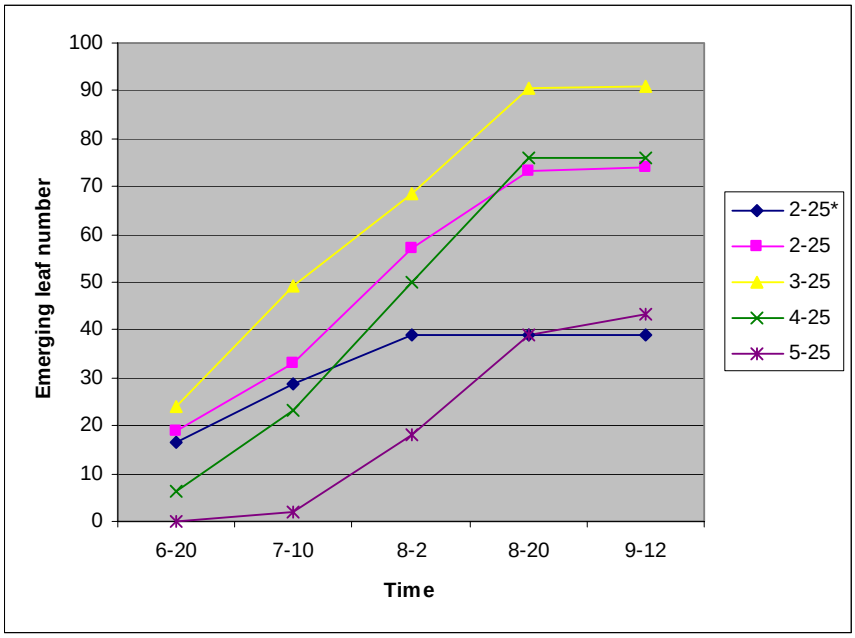

Fig. 5. Effect of planting time on emerging leaf number of lotus 'Embolene' in 29 L containers. Plants were planted on Feb. 25* (greenhouse), Feb. 25, Mar. 25, Apr. 25, and May 25 outside.

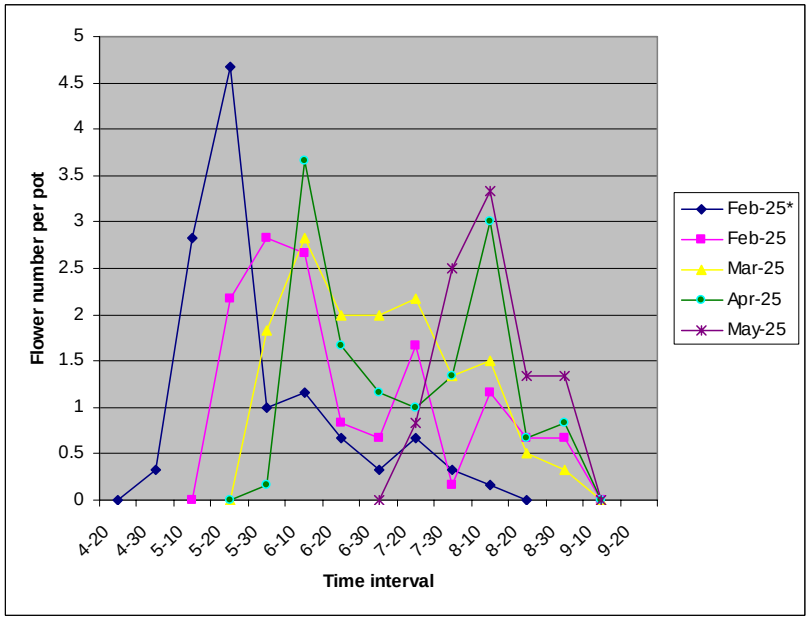

Fig. 6. Effect of planting time on flowering of lotus 'Embolene' in 29 L containers in 2007. Plants were planted on Feb. 25* (greenhouse), Feb. 25, Mar. 25, Apr. 25, and May 25 outside. Calculation of flower number was based on a 10 d interval.

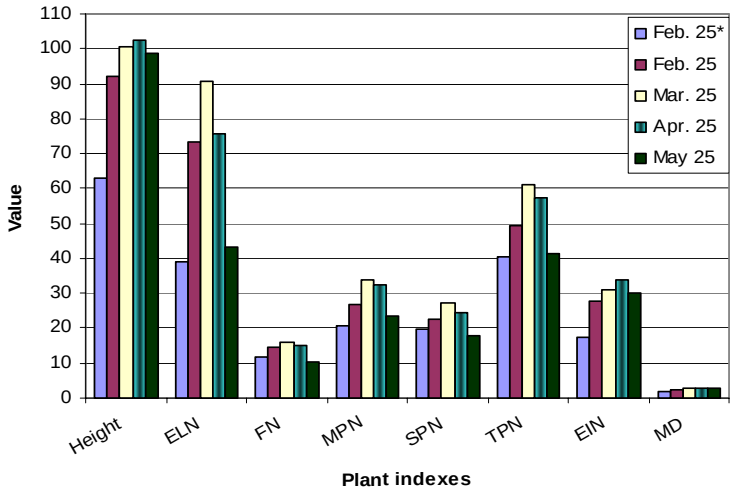

Fig. 7. Effect of planting time on plant growth indices of lotus 'Embolene' in 29 L containers in 2007. Plants were planted on Feb. 25* (greenhouse), Feb. 25, Mar. 25, Apr. 25, and May 25 outside. Height – plant height (cm); ELN – emerging leaf number; FN – flower number; MPN – marketable propagule number; SPN – secondary propagule number; TPN – total propagule number; EIN – expanded internode number; MD – maximum diameter (mm) of rhizomes.

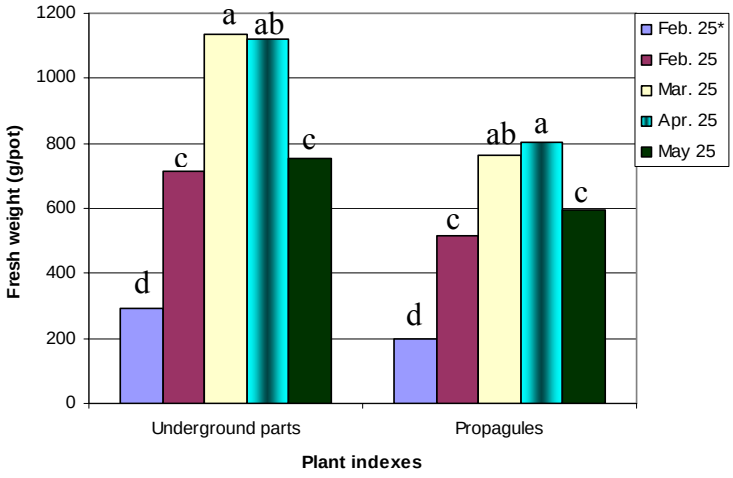

Fig. 8. Effect of planting time on fresh weight of underground part and propagules of lotus 'Embolene' in 29 L containers in 2007. Plants were planted on Feb. 25* (greenhouse), Feb. 25, Mar. 25, Apr. 25, and May 25 outside.

Chapter 6 Postharvest Longevity and Viability of Cooler-stored Lotus Propagules

Abstract

Storage of enlarged lotus rhizomes used as vegetables has been extensively studied, but little is known about the viability of lotus propagules (rhizomes with shoots) during storage. In this study, an ornamental lotus, *Nelumbo nucifera* 'Embolene', was used to evaluate the effects of gum acacia, sphagnum moss and Terra-Sorb® hydrogel on the physiology and postharvest longevity or viability of stored lotus propagules. After 45 days of storage in the cooler at 5C (42F) and 95% RH, there were no decayed propagules, and 94% of total propagules maintained their viability after planting. Water retention and shelf-life of rhizomes were similar among all treatments during storage. However, more water loss occurred with treatments of higher concentrations of gum acacia. After harvest, large differences in total sugar were found among individual treated samples, whereas starch content remained almost unchanged. Strong quadratic relationships were observed between total sugar and storage time for all treatments but there was no significant effect of treatment on total carbohydrate change in lotus propagules after 45 days of cooler storage.

Index words: *Nelumbo nucifera*; storage; carbohydrate; aquatic plants; gum acacia; hydrogel.

Species used in this study: Lotus (*Nelumbo nucifera* Gaertn.)

Chemical used in this study: ZeroTol™ (Hydrogen Peroxide), Terra-Sorb® Hydrogel (Potassium Polyacrylamide Acrylate Copolymer), Gum acacia (a complex mixture of saccharides and glycoproteins).

Significance to the Nursery Industry

Ornamental lotus, *Nelumbo* nucifera 'Embolene', was used to evaluate the effects of gum acacia, sphagnum peat moss and hydrogel on the longevity and quality of cooler-stored lotus propagules. Nurseries often deal daily with potentially hundreds of plants with varying cultural requirements. Such diverse crops need intensive scheduling to meet specific production requirements. If some plants can offer more flexibility in their scheduling through the implementation of simple effective postharvest storage, shipping and handling techniques, it is a great help to the growers. Lotus propagules in this study retained strong viability after 45 days of storage in the cooler at 5C (42F) and 95% RH. Surface sterilization by soaking propagules in 1% ZeroTol™, or another surface sterilant, followed by low temperature storage is required to maintain the viability of lotus rhizomes during storage.

Introduction

Lotus (*Nelumbo*, Nelumbonaceae) is a well-known perennial aquatic plant with edible, ornamental, medicinal and ecological uses. Only two species exist within genus

Nelumbo, *N. lutea* Willd. native to North America and *N. nucifera* Gaertn. native to China. However, more than 1,000 cultivars have been introduced from these two species. Sacred lotus (*N. nucifera*) has been a popular crop in China for more than 6,000 years (19) and is one of the most famous traditional flowers in this country. Lotus is grown and consumed throughout Asia, with all parts of the plant (including seed, rhizome, leaf, stalk, petal, anther, pericarp, and fruit receptacle) used as food or for medicinal purposes (9, 10). Besides typical ornamental use, flowers are also used in religious ceremonies (9). Lotus seeds are among the oldest in the world and have been noted to maintain the viability more than a thousand years (13, 15). In contrast, rhizomes survive poorly in long-term storage because of continued water loss, shrinkage, browning and decay (21, 22). Maintenance of freshness of edible lotus rhizomes has been well studied in the food storage industry (8, 18, 19, 20, 21). Enlarged rhizomes used as vegetables can be stored up to 150 days at 6-8C (43-46F) with 95-100% RH (2), whereas, rhizomes usually have a shelf life of only 2 weeks at room temperature (11) and can be stored in soil for only 10-30 days (5). Lotus rhizomes for propagation remain viable through winter in containers and ponds without additional protection in Southern China (17). However, post-harvested propagules are quite difficult to be stored for extended durations and little research is available on longevity of liner propagules for shipping and production purposes.

Lotus propagules (usually rhizomes with shoots) are mainly used for propagation to maintain the homogenous genotypes. Shelf-life extension of propagules during shipping and planting season will benefit both lotus producers and consumers. Although low temperature storage is the most practical method of prolonging the shelf-life of lotus rhizomes (19, 20), application of anti-desiccant materials may benefit long-term storage.

Gum acacia (gum arabic, from *Acacia* species), an edible coating, has been used to limit water loss (14) and to form a protective film in encapsulation (3). Gum arabic has a significant effect on protection of probiotic cultures during drying, storage and gastric transit (4). This biopolymer may also improve water retention when applied to the surface of plant tissue during storage. Hydrogel, a hydrophilic polymer has also been reported to increase water retention when applied to soil or planting media (1).

In this study, Terra-Sorb® fine Hydrogel™, peat moss and gum acacia were investigated to determine the effect on the longevity of lotus propagules under low temperature storage (5C (42F), 95% RH). Changes in moisture retention and sugar and starch content of lotus propagules in cold storage were also examined.

Materials and Methods

Containerized rhizomes of lotus 'Embolene' were harvested at Paterson Greenhouse at Auburn University on 13 Feb. 2005. Healthy propagules (rhizomes with shoots) were cut with two or three internodes remaining and soaked in 1% ZeroTol™ (CropKing, Inc., Serville, OH) for three seconds for surface sterilization and placed into two-gallon (7.6 liter) zip-lock bags (1 treatment/bag) after the following treatments were applied: (a) control stored as is; (b) wrapped with moist sphagnum moss; (c) dipped in tap water saturated Terra-Sorb® fine Hydrogel™ (Plant Health Care, Inc., Pittsburgh, PA) for three seconds; and (d, e, f) dipped in 10%, 20%, and 30% (w/v) gum acacia (Colloides Natural International, Rouen Cedex, France) for three seconds, respectively. Treated materials were placed in a walk-in cooler at 5C (42F) and 95% RH. Ten samples were collected

initially, five of which were weighed and dried in a 70C (158F) oven for 72 h and then weighed for dry mass. After weighing, dried samples were ground in a Thomas® Wiley® mini-mill (Model #: 5KH39QN5525A, Mexico) and stored dry at room temperature in sealed containers for analysis. Rhizome moisture content (%) was determined by 100 * (mass of fresh rhizomes – mass of dry rhizomes)/mass of fresh rhizomes. The remaining five samples were individually planted in 29 liter (#7) plastic containers to evaluate the viability of propagules. Thereafter, ten samples of each treatment were randomly collected every 15 days during 45 days of storage, five for carbohydrate analysis and five for planting. Samples treated with hydrogel and gum acacia were gently surface-wiped with tissue before weighing. A total of 190 propagules were used in this experiment, 95 for planting and 95 for lab analysis.

Total soluble sugar and starch contents were determined by using the Anthrone Reagent and Nelson methods, as described by Li et al. (7) and Owens et al. (12). Sugars were extracted from 20 mg of ground rhizome tissue with 1 mL 80% ethanol in 2 mL micro-centrifuge tubes. Tubes were shaken occasionally for 10 min at 25C (77F) and centrifuged at 14,000 rpm for 5 min at 5C (42F). The supernatant was retained. Ethanol extraction was repeated twice and the combined supernatants diluted to a final volume of 5 mL with 80% ethanol. Sugar concentrations in the ethanol extracts were determined with anthrone (16) and glucose as a standard. Optical density of supernatants was read at 625 nm in a Bio-Tek SynergyHT with software KC4^{TB} V3.1 (Bio-Tek® Instruments, Inc., Winooski, VT). The ethanol extracted residue was placed in a 60C (140F) oven for 12 h to evaporate any residual ethanol before starch hydrolysis. Water (500 µL) was added to

each tube, and the tubes were heated in a boiling water bath for 10 min to gelatinize starch. The pH of the solution was adjusted to 5.1 by adding 400 μL 0.2 M sodium acetate buffer. Starch was digested by adding 0.2 U of amyloglucosidase (Sigma-Aldrich, Inc., St. Louis, MO; product A1602-25MG from *Aspergillus niger)* and 40 U of α-amylase (Sigma-Aldrich, Inc., St. Louis, MO; product A2643*)* in 100 μL of 0.2 M Na acetate buffer (pH 5.1). Tubes were incubated at 55C (133F) for 24 h with occasional shaking. Tubes were centrifuged at 14,000 rpm for 5 min and the supernatant diluted to 1:100 of dilution in 15 mL glass tubes. Tubes were then incubated at 37C (99F) for 30 min in a water bath, afterwards 500 μL of copper reagent (reagent D) was added. The solution was heated for 15 min in an 80C (176F) water bath and then cooled down to room temperature for 15 min. 500 μL of arsonomolybate reagent (reagent C) was added and vortexed until all foaming ceased. Finally, 3.5 mL of water was added and vortexed. The samples along with standards containing 0, 20, 40, 60, 80, and 100 μg mL^{-1} of glucose solution were read at 540 nm in a Bio-Tek SynergyHT. Starch concentration was calculated as 0.9 × glucose concentration. For each treatment, four samples and four replicates were analyzed. For each sample, 20 mg of ground dried lotus rhizome was used for analysis.

Survival rates of propagules in containers were recorded on 1 Apr. 2005, one month after the last sampling and planting day. Means of moisture and carbohydrates were examined by Tukey's Multiple Comparisons Test (HSD, $\alpha = 0.05$). Two-Way ANOVA was used for determination of interaction and the main factor. Models of the relationship between moisture or carbohydrates and storage time were analyzed by regression analysis. All procedures are run by SAS 9.1 (SAS Institute, Cary, NC).

Results and Discussion

During 45 days of storage, all propagules lacked decay or visible diseases regardless of the treatment used. No effect of treatment or time on viability of lotus propagules was detected during storage. Between 80 to 100% of the planted propagules survived in all treatments. Total survival rate was 94%. Therefore, lotus propagules could remain highly viable for at least 45 days when stored in a 5C (42F), 95% RH cooler. Death of a few propagules might be attributed to damage of primary leaf shoots prior to planting or stress from environmental factors (low temperature and possible disease) after planting.

Moisture in rhizome samples ranged between 73.5% and 77.4%, and the difference was not significant among treatments during storage based on Tukey Multiple Comparison procedure (HSD, $\alpha = 0.05$, Table 1). An average water content change of -0.2 to 4.8% was observed in this study, which was similar to water change of -1.7 to 3.65% in edible lotus rhizomes recorded by Zhan and He (22) during 9 days of storage in plastic bags at 5C (42F). Wang and Li (18) reported only 0.59 to 0.98% of water loss in edible lotus after 30 days of storage in vacuum polythene bags at 4 to 10C (39 to 50F), and Wang and Zhang (19) recorded 0.71 to 0.74% of water loss in cooler at 3 to 5C (37 to 45F) (80 to 85% RH) and 2.5 to 4.9% of water loss at room temperature (8 to 20C (46 to 68F), 70 to 80% RH) after 30 days of storage. Xu et al. (20) observed water loss between 5.27% and 10.23% for four cultivars of edible lotus stored for 15 days at 15C (59F). These studies indicated low temperature played a critical role on water maintenance and decay resistance of stored lotus rhizomes (9).

In our study, except for treatments of 30% gum acacia and sphagnum moss, water content of lotus rhizomes generally decreased initially and then increased slightly, but not statistical significantly (Table 1). Increased moisture content in the latter storage period was unclear but possibly explained by: (a) fast degradation of carbohydrates or increased respiration rate but less water expenditure; or (b) less water loss from tissue as moisture presence was adequate in the sealed bag. A similar reversal in water content in lotus rhizomes was reported during 9 days of short-term storage in polythene bags at 5C (42F) (22). In our study, the highest water content and a similar change trend were found in rhizomes receiving either control or Hydrogel^{TM} treatment. A continued decreasing trend was observed in the 30% gum acacia (G3) treatment with a linear relationship observed between moisture (y) in tissue and storage time (x): $y = 76.879 - 1.709 x$ ($P < 0.0001$, $R^2 = 0.6796$). However, no strong linear or quadratic relationships were found between moisture and time for other treatments. Gum acacia had no effect on water retention, and, in fact, higher concentrations of gum acacia had a negative effect on water maintenance of lotus rhizomes compared to the control (Table 1).

For carbohydrate analysis, total sugar among the individual samples of lotus propagules differed widely (2.5 to 36.5 mg g^{-1} on a dry mass basis, detailed data not shown). During 45 days of storage, the total soluble sugar decreased drastically from the first to the 30th day (50 to 83% decreased within the first 15 days) then increased from the 30th to the 45th day (Fig. 1). A significant decrease (128.13 to 7.6 mg g^{-1}) of total sugar was also recorded by Liu et al. (8) after 11 days of storage for edible lotus at 30.6C (87F). In our study, based on Two-Way ANOVA procedure, the interaction ($P = 0.057$) between treatments ($P = 0.263$) and time ($P < 0.0001$) was not significant on total sugar

in lotus rhizomes. The strong quadratic relationships were observed between total sugar (y) and storage time (x) for all treatments through regression analysis (Table 2). Although total sugar tended to decrease from the 10 to 30% gum acacia treatments within the last 15 days (16 Mar. to 31 Mar.), no significant difference was found in total sugar of stored lotus rhizomes between treatments of gum concentrations (Fig. 1).

There was no intersaction (P = 0.7104) between time and treatment on starch content in lotus rhizomes after Two-Way ANOVA analysis. Both treatment (P = 0.8399) and time (P = 0.7977) were not significant. Starch in dry rhizomes was 27.5 to 38.9% (Fig 2), which was lower than 62.3 to 68.7% in enlarged edible lotus rhizomes reported by Li et al. (6). Ornamental lotus usually has smaller swollen parts and low starch in rhizomes; therefore, it is not used for vegetable production. The starch content maintained a relatively stable level of between 275 mg g^{-1} and 389 mg g^{-1} on a dry-weight basis throughout storage, without significant differences (HSD, $\alpha = 0.05$) observed for the same treatment over time as well as for all treatments compared to the control (Fig. 2). Therefore, there was no significant treatment effect on starch concentration in lotus rhizomes in this study. A similar result was reported by Zhan and He (22) for 9 days of storage of edible lotus rhizomes at 5C (42F). The increase of starch during storage compared to the initial day for treatments of sphagnum moss, 10% and 20% gum acacia might be attributed to sampling variation because only four samples per treatment were used for carbohydrate analysis.

This study suggested no additional treatment is needed for a short-term cooler storage of lotus propagules besides a good surface sterilization. The 45 days of storage longevity shown in this paper is sufficient to allow production flexibility and the requirements of

either native or international shipping. In a later study, surface sterilized propagules of lotus 'Embolene' retained 100% viability (data nor shown) in the cooler (5C (42F), 95% RH) for more than six months without additional treatments. However, propagules of control without sterilization were easily attacked by pathogens and lost the viability within 1 to 2 months because of rot. It indicated low temperature and good sanitation are critical to maintain the viability of lotus propagules during storage.

Conclusion

Propagules of lotus 'Embolene' retained a high viability after 45 days of cooler storage at 5C (42F) and 95% RH. It indicated lotus propagules can be stored for more than 45 days in cooler after harvest which was supported by the later investigation. Hydrogel^{TM}, sphagnum moss, and three concentrations of gum acacia had no statistically significant effect on storage of lotus propagules in this study. However, this conclusion may be challenged by longer-term storage. Further study is necessary to evaluate effect of biopolymers on extended storage and viability of lotus rhizomes or propagules and their maximum longevity. An additional treatment using biopolymers is not necessary for practical shipping and production practices. Since lotus rhizomes are easily perishable material, good sanitation and low temperature are critical for both storage and shipping.

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Tables

Table 1. Moisture content percentage in propagules of lotus 'Embolene' during cooler storage at 15-d interval.

	Moisture Content (%)			
	Sampling Time			
Treatment	2/14	3/1	3/16	3/31
C	77.2	76.2	75.9	77.4
M	77.2	75.2	75.7	75.2
H	77.2	76.6	75.5	77.3
G_1	77.2	73.9	75.6	76.0
G_2	77.2	75.2	74.6	75.5
G_3	77.2	74.6	73.6	73.5

Means difference was determined by Tukey's Multiple Comparisons (HSD, $\alpha = 0.05$), No significant difference was found between means of moisture in lotus rhizomes for treatments (C = control, M = sphagnum moss, H = hydrogel, G1 = 10%, G2 = 20%, and G3 = 30% gum acacia).

Table 2. Relationships between total sugar (y, mg/g, dry weight) in rhizomes of lotus 'Embolene' and storage time (x, 1 unit/15 days) during 45 days of cooler storage.

Treatment	Model*	
Control	$y = 29.495 - 29.136\ x + 8.331\ x^2$	$P < 0.0001$, $R^2 = 0.845$
Hydrogel™	$y = 28.793 - 25.982\ x + 6.8\ x^2$	$P < 0.0001$, $R^2 = 0.837$
Sphagnum Moss	$y = 30.438 - 22.873\ x + 5.451\ x^2$	$P < 0.0001$, $R^2 = 0.85$
10% Gum Acacia	$y = 27.733 - 23.652\ x + 6.777\ x^2$	$P = 0.0092$, $R^2 = 0.542$
20% Gum Acacia	$y = 29.053 - 25.39\ x + 6.501\ x^2$	$P < 0.0001$, $R^2 = 0.875$
30% Gum Acacia	$y = 30.108 - 22.658\ x + 5.166\ x^2$	$P < 0.0001$, $R^2 = 0.891$

*Models were determined by regression analysis under SAS 9.1.

Figures

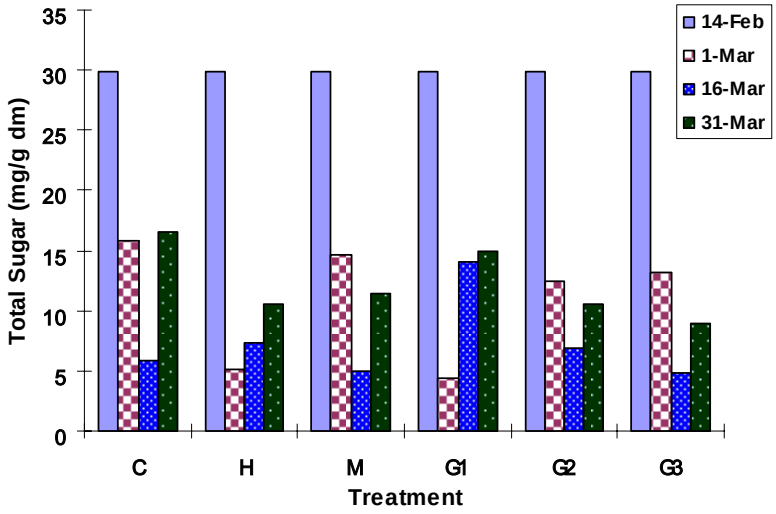

Fig. 1. Changes of total sugar content (on a dry mass basis) in rhizomes of lotus 'Embolene' for six treatments during 45 days of storage. Interaction (P = 0.057) of time and treatment was not significant after analysis of Two-Way ANOVA. Time (P < 0.0001) but treatment (P = 0.263) was the main factor to contribute to change of total sugar in lotus rhizomes during storage (C = control, H = hydrogel, M = sphagnum moss, G1 = 10%, G2 = 20%, G3 = 30% of gum acacia).

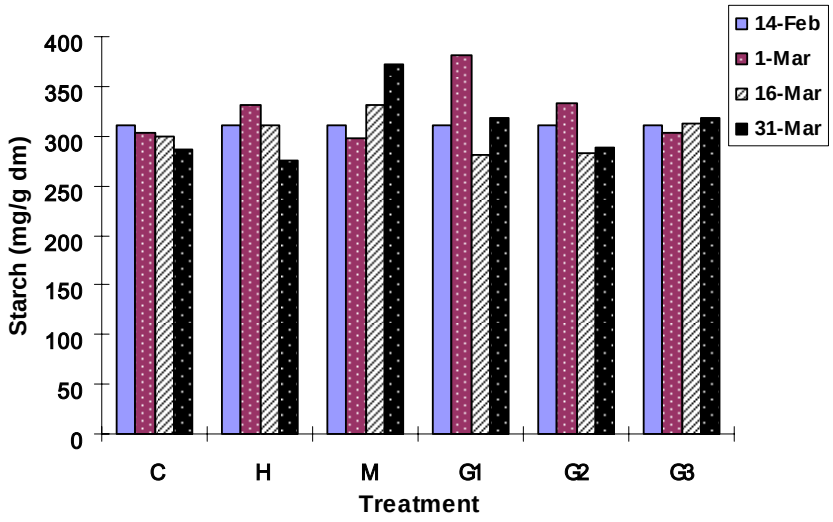

Fig. 2. Changes of starch content (on a dry mass basis) in rhizomes of lotus 'Embolene' for six treatments during 45 days of cooler storage. Interaction (P = 0.7104) of time and treatment was not significant after analysis of Two-Way ANOVA. Both time (P < 0.7977) and treatment (P = 0.8399) were not the main factors to contribute to change of total sugar in lotus rhizomes (C = control, H = hydrogel, M = sphagnum moss, G1 = 10%, G2 = 20%, G3 = 30% of gum acacia).

PART II MICROPROPAGATION OF HERBACEOUS PEONY (*PAEONIA*)

Chapter 1 General Introduction and Literature Review

1 General Introduction

Peonies, of the genus *Paeonia*, are long living perennials belonging to the family Paeoniaceae, and are among the most dramatic and beautiful flowers for gardens. Peonies are also used as pot flowers, cut flowers and dry flowers. *Paeonia* is taxonomically very complex consisting of a relatively small number of species (about 33 species, Rogers, 1995; about 30 species, Hong and Pan et al., 2001; 25 species, Halda and Waddick, 2004). Peonies are divided into two types: herbaceous and tree peony. Herbaceous peony has been cultivated in China for more than 3900 years (Wang and Zhang, 2005; Gao et al., 2001). Tree peony is one of the ten famous traditional flowers in China and has been cultivated for at least 1500 years (Gao et al., 2001). It has been widely cited in the Chinese mythology and honored as 'King of the Flowers'. Peonies have been deeply rooted in the Eastern culture for a long time and spread to other countries. Today, peonies are enjoying a renaissance as popular cut flowers and garden plants and they are back in fashion because of the ease of cultivation in a wide range of climates and the rich diversity of the lovely flowers.

With the complex sequential dormancy of peony seeds, it usually takes two years for germination under natural conditions (Krekler, 1962; Griess and Meyer, 1976). Peony

seedlings must grow for several years before blooming. Peonies can be propagated by division, cutting, grafting, and layering to obtain true-to-type plants. Approaches of propagation of tree peony were well discussed by Zeng et al. (2000) and Zhang et al. (2002). Grafting is more often used in propagation using herbaceous peony or other species as rootstock. Herbaceous peonies are usually propagated by 3–5 eye divisions of underground stock plants. A grower may be able to have stock double every three years by planting in a three-year rotation (Shannon and Kamp, 1959). Nodal stems and underground rhizomes can be used for cuttings (Arino et al., 1981; Antanaitiene and Staniene, 2001; Wang and Zhang, 2005). Layering is seldom used in herbaceous peony but the efficiency has been improved by a new approach of vertical layering with application of rooting chemical like Ukorzeniacz B2 (Czekalski and Jerzy, 2003). There are multiple traditional choices for propagation of peony. However, the limited number produced by these traditional methods can not meet the increasing demands in the market, especially for a quick release of a new cultivar and massive production of a specified variety. The development of micropropagation methods for peonies is necessary to not only overcome this problem but also accelerate peony breeding progress.

The field of plant tissue culture is based on the premise that plants can be separated into their component parts (organs, tissue, or cells), which can be manipulated in vitro and then grown back to complete plants. The first successful plant tissue and cell culture was accomplished by Gottlied Haberlandt near the turn of the 20th century when he reported the culture of leaf mesophyll tissue and hair cells (Steward, 1968; Krikorian and Berquam, 1969). This was a remarkable accomplishment considering that little was known about plant physiology at that time. Unfortunately, the cells that Haberlandt

cultured did not divide and, thus, his ideas of plant development and totipotency did not come to fruition. In the early 1960s, Murashige completed a study while working in Skoog's laboratory leading to a commercial application for tissue culture.

Micropropagation of peony began in the middle 1960s. During the last 40 years, much research on micropropagation of peony has been conducted with plants successfully produced from tissue culture labs. Planteck Biotechnologies Inc., a company based in Quebec, Canada, produced mass numbers of herbaceous Itoh peonies and made the tissue cultured plants available in the market for the first time in 2006 (Whysall, 2006). However, there is still much work to be done in order to make mass production of peony more commercially successful. Optimization of procedure for each stage or step should be studied in depth, including selection of explants, decontamination, screening of medium, application of plant growth regulators (PGRs), induction of callus and shoots, subculture, rooting, and finally transplanting.

2 Literature Review

Tissue culture is one of the most effective approaches for rapid propagation of plants and is also providing a new approach for plant breeding in crops, ornamentals, fruits and vegetables. In peony, callus was induced successfully for the first time by Yamada and Sinotô (1966) from petal culture of *P. japonica*. Large variation in chromosome numbers and characteristics within cells was observed during culture. Demoise (1967) and Demoise and Partanen (1969) induced callus from tree peony and investigated the effects of subculturing and physical conditions of culture on the mitotic cycle kinetics of a population of cells, particularly in relation to the degree of heteroploidy. Since then,

many original studies on in vitro culture of peony have been reported. Several review papers have also been published. The first review on micropropagation of peony was published by Buchheim and Meyer (1992), where research on in vitro culture of peony before 1989 on has been mostly discussed. A summary of in vitro studies conducted on several species of *Paeonia* was included in a table consisting of plant species, inoculum, medium, growth response, and reference. This review is useful for a researcher working on peony tissue culture, although some medium formulations have not been cited correctly. The second review on in vitro culture peony was written by Gabryszewska (2004). The regeneration ability of different organs of both herbaceous and tree peony was reported in this paper. The role of exogenous and endogenous PGRs in differentiation and growth of shoots, roots, and somatic embryos was also discussed. However, the Gabryszewska review is limited because only the work before 1999 was included, and it is published in Dutch and therefore difficult to access for most researchers. In 2007, Beruto and Curir summarized tissue culture of tree peony under a book chapter title, '*In vitro culture of tree peony through axillary budding*', which included most of the results published between 1969 and 2004. The review was well organized by introduction, experimental protocol consisting of stages of tissue culture, and conclusion. During this time, five other review papers on peony tissue culture were published by Chinese researchers (Li and Luo, 2004; Li et al., 2006a; Jia et al., 2006a; Meng et al., 2007; Zhao et al., 2007). However, each of these papers is only 2 to 4 pages long and did not cover as much as information of previous work on micropropagation of peony. With the fast development of internet and database construction in the world and the benefit of language translation software, it is becoming much easier to access and

understand the original research resources published by non-native languages. Through nearly four years of collection, this author has accessed and screened almost all of the original papers on peony in vitro culture and in an effort to provide the most comprehensive, new review possible. In addition to the following individual discussion, 40 years work of peony in vitro culture is summarized in a table (Table 1).

2.1 Explant

Micropropagation is defined as the true-to-type propagation of selected genotypes using in vitro culture techniques. Four basic methods are used to propagate plants in vitro (Trigiano and Gray, 2000). Depending on species and cultural conditions, in vitro propagation can be achieved by: (1) enhanced axillary shoot proliferation (shoot culture); (2) node culture; (3) *de novo* formation of adventitious shoots through shoot organogenesis; and (4) nonzygotic embryogenesis. Currently, the most frequently used micropropagation method for commercial production utilizes enhanced axillary shoot proliferation from cultured meristems.

Many kinds of explants have been used to generate callus, embryos and adventitious shoots for peony in vitro culture. Seeds and immature embryos are good sources as explants because of easy disinfection. Mature embryos were used by Demoise (1967) for the first time to induce callus in *Paeonia suffruticosa*. Thereafter, mature embryos have been widely used in micropropagation of both herbaceous and tree peonies to generate embryos, callus, adventitious shoots, plantlets, and finally, successfully transplanted plants (Demoise and Partanen, 1969; Meyer, 1976a; Lin ,1980; Thomas and Meyer, 1986; Lin et al., 1987; Thomas, 1987; Thomas and Meyer, 1987; Huang, 1987; Brukhin and

Batygina, 1994; Kim and Lee, 1994, 1995; Shin et al., 1996; Zhou and Yao, 2001; Cao, 2003; An, 2005; He, 2006; Jia et al., 2006ab; Yan and Pei, 2006). Immature embryo was also used to induce callus and shoots (Shin et al., 1996; He et al., 2005, 2006; Stanys et al., 2007). Kim et al. (2006) generated plants from desiccated somatic embryos after cryopreservation. Cotyledons have been used for explants to induce callus, embryos and shoots (Kim and Lee, 1996; Shin et al., 1997; Wang and Staden, 2002; An, 2005; Kim et al., 2006; Lang and Luo, 2007). Radicles of both herbaceous and tree peony were investigated by Xie (1987), Wang and Staden (2001), Jin et al. (2005), and Lang and Luo (2007). He et al. (2005) and He (2006) induced shoots from ovules in tree peonies. Ovule wall was used as explant by Orlikowska et al. (1998) in tree peony to induce shoots. The whole seed of herbaceous peony cultivars was used by Jin et al. (2005), from which shoots were multiplied.

Tissue of other reproductive organs is another source of explants for peony tissue culture. Embryos were induced from microspores by Roberts and Sunderland (1977) and Sunderland (1983abc). Microspores also generated callus (Ono and Harashima, 1981). Anthers have been widely investigated as explant in peony tissue culture (Sunderland et al., 1973, 1975; Sunderland, 1974; Sunderland and Dunwell, 1974; Ono and Tsukida, 1978; Lee et al. 1992; Chen, 2006; Kim et al., 2006). Callus, embryoids, embryos, or shoots were successfully generated in some cases. Yamada and Sinotô (1966) induced callus from petal culture of *P. japonica*. Beruto et al. (2004) used both petals and filaments in tissue culture of *Paeonia*, but no adventitious shoots were formed. Explants from flower bases, sepals, petals, and ovary wall, all generated callus successfully by Orlikowska et al. (1998) with a limited number of shoots generated directly from the

bases of both sepals and petals. However, production of callus was very low. Callus and roots have been induced from flower buds by Meyer (1976b). Although portions of flower are more easily sterilized, the efficiency of organogenesis is still low, and also the flower used as an explant has a short period of availability.

Vegetative portions are the major source of explants used in tissue culture of peony (Table 1). It includes the whole dormant buds, sprouting buds, bud tips, shoots, shoot tips, nodal and internodal stems, petioles, leaves, roots, and vitroplantlets. Axillary buds, bud tips and shoot tips, are most frequently used in tissue culture of peony. Higher frequency of shoot induction has been obtained from culture of these explants. Whole dormant buds were used in tissue culture of both herbaceous peonies by Meyer (1976b), Lin (1980), Radtke (1983), Thomas (1987) and Gabryszewska (2006), and tree peonies by Meyer (1976b), Bouza et al. (1993), Kong and Zhang (1998), Chen et al. (2001), and Chen (2005, 2006). Many researchers also used the tips of buds (either dormant or sprouting) and young shoots to induce adventitious shoots in tissue culture of herbaceous peony (Hosoki et al., 1989; Gabryszewska, 1998, 2006; Zhang, 2006; Zhang et al., 2007), and tree peony (Li et al., 1984; Xie, 987; Kong and Zhang, 1998; Zhang et al., 2001; Li et al., 2006bc). Seo et al. (2007) even successfully induced roots from regrowth of desiccated dormant shoots of herbaceous peony after cryopreservation. Segments of leaves and petioles have been investigated but only callus formed in most cases (Li et al., 1984; Orlikowska et al., 1998; Wang and Staden, 2001; Li et al., 2005; Chen, 2005; An, 2005; Zhang, 2006; Lang and Luo, 2007; Zhang et al., 2007). Direct shoot regeneration was observed only on veins of leaf segments within 2 months at rates not exceeding 10% (Orklikowska et al., 1998). Studies on shooting ability have indicated young nodal stems

were more prone to adventitious shoot induction than young internodal stems (Gildow and Mitchell, 1977; Radtke, 1983; Harris and Mantell, 1991; Albers and Kunneman, 1992; Černá et al, 2001; Hu et al., 2003). Root (Meyer, 1976b; Lin, 1980) and root phloem (Lin, 1980) have induced callus but failed to generate shoots. Tissue of vitrioplantlets has been studied by Chu and Li (1993), Chen et al. (2001) and Gabryszewska (2001), and callus and shoots were successfully produced.

In conclusion, embryo tissues are good source to induce embryos and shoots, but the material can be only supplied by the seeded varieties. Callus, embryos can be induced from anthers, but only little quantity is obtained. Flowers used as explants are only available for a short time. Highest frequency of both callus and shoot induction are found in vegetative buds, bud tips and shoot tips. However, underground buds of herbaceous peony are difficult to disinfect and the highest contamination occurs on these explants during tissue culture. Sections of leaves and petioles could also be used in tissue culture of peony, however the amount of callus and adventitious shoots induced are very limited. There is a speculation that nodal stems should be much better than internodal stems to induce shoots because there is an axillary meristem region at the position of bud eye which can often produce multiple shoots directly.

2.2 Disinfection and Contamination

Good sanitation is paramount to successful tissue culture. Just as the media, instruments, and tools must be sterilized, so must the plant tissue be disinfected before it is placed on culture medium. Many different types of materials have been used to surface disinfest explants, but the most commonly used are 1% (v/v) sodium hypochlorite

(commercial bleach contains 5% sodium hypochlorite), 70% alcohol, or 10% hydrogen peroxide. Others include using a 7% saturated solution of calcium hypochlorite, 1% solution of bromine water, 0.2% mercuric chloride solution, and 1% silver nitrate solution (Trigiano and Gray, 2000). The type of disinfectant used, the concentration, and the amount of exposure time (1–30 min) vary depending on the sensitivity of the tissue and how difficult it is to disinfect. In some cases, employing a two or three-step protocol (usually 70% ethanol followed by bleach) or adding a wetting agent such as Tween 20 or detergent helps to increase sanitation effectiveness. In any case, the final step before trimming the explant and placing onto sterile medium is to rinse it several times in sterile, distilled water to eliminate the residue of the disinfesting agent.

Although sterilization of plant tissue is very critical for success of tissue culture, unfortunately less than thirty percent of published papers contain the description of sterilization methods in peony in vitro culture. Data on contamination are limited and only reported by some researchers (Albers and Kunneman, 1992; Kong and Zhang, 1998; Orlikowska et al., 1998; Habib et al., 2000, 2001; Wang and Staden, 2001; Hu et al., 2003; Wu, 2003; Beruto et al., 2004; Chen, 2005, 2006; Jin et al., 2005; Li et al., 2005; Jia et al., 2006ab; Zhang, 2006; Zhang et al., 2006; and Verme P., 2007).

Since there are various types of explants for choice in tissue culture of peony, the disinfection methods of plant tissue are very diverse (Table 2). Ethanol (often 70% or 75%), bleach (10%), and $HgCl_2$ (0.1%) are most commonly used in surface sterilization of peony explants. A preliminary treatment of water wash with addition of detergent is often done before surface sterilization. A one step method was not very effective for decontamination, especially for underground dormant buds of herbaceous peony (Radtke

1983; Hansen et al., 1995). In a two-step method of sterilization, following preliminary water rinse, plant tissue is usually soaked in ethanol for 5–30 sec followed by treatment of other sterilants like bleach (NaOCl) or mercuric chlorite ($HgCl_2$) solution with or without a transition of a water rinse. For the treatments using bleach and $HgCl_2$, Tween 20 as a surface active agent (surfactant) was often added. Besides bleach and $HgCl_2$, 5% potassium iodide (Habib et al., 2000, 2001), 7% calcium hypochlorite (Lee et al., 1988), and 0.5% potassium manganate ($KMnO_4$) (Jin et al., 2005; Yang and Pei, 2006) have also been occasionally used for surface sterilization of peony tissues.

Effect of sterilization is not only dependent on the type of explants and disinfection method, but also dependent on cutting manipulation after sterilization. If the tools (nippers and scalpels) are not frequently sterilized by ethanol dip and flame when moving between individual tissues, inter-contamination may be a problem. For example, contamination was much reduced by removing the seed coat before sterilization of embryos (Wang and Staden, 2001). However, Jin et al. (2005) reported that coat removed embryos had higher contamination (56%) and heavier browning compared with 12% for embryo with seed coat. This result could be possibly explained by unsuitable manipulation or other reasons. Contamination of plant tissue was significantly decreased with the increase of disinfection time but the rates of death and tissue browning also increased (Chen, 2005, 2006; Zhang, 2006; Zhang et al., 2006). Beruto et al. (2004) reported that additional 30 min treatment in 50% plant preservative mixture (PPM) reduced contamination to 30% from 60% for axillary flower buds of tree peony.

Aerial (above soil) explants including flower buds, young shoots, nodes, stems, leaves, petioles and flowers are much easier to disinfect than underground dormant

vegetative buds and roots which have contact with soil. Although given special care and harsh sterilization practices, contamination of underground buds of herbaceous peony is still high; therefore, a novel disinfection method needs to be developed for the future. Treating time of sterilization should be adjusted based on the type, size and age of explants.

2.3 Media and Plant Growth Regulators

The type of tissue culture medium to be used depends on the species to be cultured. Some species are sensitive to high salts or have different requirements for PGRs. The age of the plant also should be considered. Juvenile tissues generally regenerate callus, shoots and roots more readily than adult tissues. Each desired cultural effect has its own unique requirements such as cytokinin (high-cytokinin-low-auxin ratio) for initiation and development of adventitious shoots and auxin for induction of adventitious roots (Trigiano and Gray, 2000).

Murashige and Skoog (1962) medium (MS) is the most suitable and most commonly used basic tissue culture medium for plant regeneration from tissues and callus. It is a "high salt" medium due to its content of K and N salts. LS medium (Linsmaier and Skoog, 1965) is basically MS medium with respect to its inorganic portion. In addition, White's medium (White, 1963), Nitsch's medium (Nitsch and Nitsch, 1969), Gamborg B5 medium (Gamborg et al., 1968), SH medium (Schenk and Hildebrandt, 1972), and later developed woody plant medium (WPM, Lloyd and McCown, 1980) also are commonly used in plant tissue culture.

Media used so far in peony tissue culture have been summarized in Table 1. Half, full strength MS medium and other modified MS media are most commonly used for both herbaceous and tree peony. Additionally, modified LS medium was also often used for herbaceous peony and WPM (half WPM) was used for tree peony. Limited cases reported on application of White medium half SH (SH, 2HS, SH-M) medium, LB medium (Liau and Boll, 1970) (Gildow and Mitchell, 1977), N6 Medium (Chu et al., 1975) (Hu et al., 2003), and Lepoivre medium (LP, Quoirin and Lepoivre, 1977) (Stanys et al., 2007; Albers and Kunneman, 1992). In the very beginning, Steeves medium (Steeves et al., 1955) was used in tree peony culture by Demoise (1967) and Demoise and Partanen (1969). Half strength of N6 Medium was used only once as root induction medium for tree peony by Huang (1987). Meyer (1976b) compared effects of Knop's, Knudson's and LS media and found LS was clearly superior and seemed quite consistent for all the varieties tested in culture of peony embryos. Solid medium was usually used for induction of embryo, callus, adventitious shoots and roots. However, Hosoki et al. (1989) reported higher rooting rate obtained on paper-bridge liquid medium for herbaceous peony.

PGRs exert dramatic effects at low concentration (0.001–10 μM) regulating initiation and development of callus, embryos, shoots and roots on explants and also stimulating cell division and expansion to cause tissue growth. Sometimes, a tissue or an explant is autotrophic and can produce its own supply of PGRs. However, usually PGRs must be supplied in the medium for growth and development of cultured tissue. The most important classes of the PGRs used in tissue culture are auxins and cytokinins. Auxins play a role in many developmental processes, including cell elongation, swelling of tissue,

apical dominance, adventitious shoot and root formation, and somatic embryogenesis. Generally, when the concentration of auxin is low, root initiation is favored; whereas when the concentration is high, callus formation occurs. The most common synthetic auxins used in tissue culture are 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram, PIC). Naturally existing indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are also frequently used.

Cytokinins promote cell division and stimulate initiation and growth of shoots in vitro. Zeatin (ZT), dihydrozeatin (2hZ), kinetin (KT), benzyladenine (BA), thidiazuron (TDZ), and 2-isopentenyl adenine (2-iP) are the most commonly used cytokinins. Gibberellins are less commonly used in plant tissue culture. Of the giberrellins so far described in the literature, gibberellic acid (GA_3) is the most often used, but it is very heat sensitive (90% of the biological activity is lost after autoclaving). Therefore, GA_3 is typically filter sterilized and added to autoclaved medium after it has cooled down. Gibberellins help to stimulate elongation of internodes and have been proven to be necessary for meristem growth for some species.

Most commonly used PGRs in tissue culture of peony are BA, NAA, IAA, 2-iP, 2,4-D, KT, TDZ, GA_3, and IBA (Table 1). Whereas ZT was only investigated by Orlikowska et al. (1998) in herbaceous peony, PC was only used by Radtke (1983) in herbaceous peony and by Beruto et al. (2004) in tree peony, and 2-naphthoxyacetic acid (NOA) was used only once in tree peony (Beruto et al., 2004). BA is the most commonly used PGR and it is usually added to the medium at a concentration of 0.1–2(3) mg l^{-1}. BA could be used alone or together with one or two of other PGRs. The reported concentrations of

PGRs usually are 0.1–3(10) mg l^{-1} for NAA, 0.1–2 mg l^{-1} for IAA, 0.1–6 mg l^{-1} for 2iP, 0.2–2 (5) mg l^{-1} for 2,4-D, 0.1–4 mg l^{-1} for KT, (0.002) 0.01–1 (2.2) mg l^{-1} for TDZ, 0.1–1 mg l^{-1} for GA_3, and 0.1–1 (2) mg l^{-1} for IBA depending on cultivars, explant types and the purpose of culture. TDZ was first reported by Thomas (1987) in tissue culture of herbaceous peony. Thereafter more reports on TDZ have been published (Lee et al., 1992; Orlikowska et al., 1998; Gabryszewska, 1998; An, 2005; Chen, 2006; Shi and Zhang, 2005). GA_3 was mostly used in peony culture to break dormancy when using seeds (embryo) and dormant buds as explants. It was also used in medium for other explants to promote shoot initiation and elongation of the generated shoots. IBA was added to the root induction medium (RIM) to promote root initiation.

2.4 Embryo Culture and Somatic Embryogenesis

Embryo culture, the cultivation of zygotic embryos excised from ovules and seeds under aseptic conditions in a medium of known chemical composition, represents a milestone in efforts to identify the requirements essential for continued growth, differentiation, and morphogenesis of embryos. There is a profound difference in the historical development of plant tissue culture and that of embryo culture. Embryo culture has been known for nearly a century preceding plant tissue culture by many years. On the other hand, embryo culture has progressed slowly with time, while plant tissue culture, after a long period of limited progress, rapidly developed after 1937 (Monnier, 1995).

The establishment of embryo culture as an experimental tool can be dated back to Hannig's work in 1904. Using aseptic techniques, this investigator cultured relatively mature embryos of *Raphanus* and *Cochlearia* in a mineral hormone-free salt medium

supplemented with sugar, and obtained transplantable seedlings. The main application of embryo culture is certainly the rescue of hybrid embryos which abort on the mother plants after particular crosses. The method introduced by Laibach in 1925 has remained the most powerful process to obtain new hybrid plants (LaRue, 1936). Since this pioneering work, embryo culture methods have been widely used to obtain transplantable seedlings from seeds of interspecific and intergeneric crosses previously considered incapable of germination (Raghavan, 2003). Embryo culture also has several other applications such as: the production of haploids (Kasha and Kao, 1970), a good source of somatic embryos (Tétu et al., 1990), a tool to overcome dormancy of recalcitrant seeds (Randoph, 1945), and a refined approach to characterizing embryogenesis and related problems in plants.

Somatic embryogenesis is a promising method because potentially high numbers of regenerates can be obtained, each theoretically originating from a single or few cells. An important contribution was made to the field of experimental morphogenesis when Steward et al. (1958) and Reinert (1959) observed the differentiation of embryo somatic cell cultures of carrot. The development and appearance of these somatic embryos were comparable to carrot's zygotic embryos. The capacity of somatic plant cells in culture to form embryos by a process resembling zygotic embryogenesis is one of the most remarkable features of plants (Thorpe, 1995). The story of embryogenesis in vitro begins with observations by early workers; and thereafter plantlets were often regenerated in carrot callus cultures. However, there are still several questions to be answered after a half century of development: (1) Are there special chemical factors which promote

embryogenesis? (2) Is cell isolation required to induce embryogenesis? (3) An unsolved theory of single vs. multiple cell origin of embryos (Halperin, 1995).

Embryo culture in peony was first reported by Demoise (1967) in his dissertation where callus was successfully induced from embryos of *Paeonia suffruticosa* in modified Steeves medium (Demoise and Partanen, 1969). Thereafter, more reports on peony embryo culture can be found (Table 1). Meyer (1976a) obtained the seedling from embryo culture by overcoming the epicotyl dormancy using 30–40 d of treatment at 2ºC after the cotyledons and roots had been developed. Seeds are the most common used source for isolating embryos. Immature seeds and ovules as explants have been investigated by several researchers. Besides whole embryos, cotyledons, hypocotyls and ovule wall also have been used for callus induction and regeneration of shoots. After transferring geminated embryos to fresh MS medium with BA and NAA, adventitious shoots or shoot clusters have been generated (Huang, 1987). Besides callus and shoots, sometimes somatic embryos were also induced directly from cultured embryos or indirectly from induced callus (Lin et al, 1987; Brukhin and Batygina, 1994; Kim and Lee 1995; He, 2006).

The germination rate of peony embryos is largely dependent on the age of seed. For example, Shin et al. (1996) reported that embryos within 60 days after pollination showed the highest shooting rate in hormone-free medium. When 0.3 mg l^{-1} GA_3 was added to the medium, a high germination could be obtained from embryos 50–100 d after pollination. However epicotyls did not elongate from immature embryos after 50–60 d of pollination. GA_3 was usually used to break the epicotyl dormancy of seeds during embryo culture (Shin et al., 1996). Stratification treatment has a similar effect as GA_3 on

overcoming dormancy of embryo. An (2005) reported the highest shoot induction was found in 30–40 d (40–43.3%) of treatment at 5ºC (40ºF) compared with control (3.3%) and 10 d (10%) and 20 d (23.3%) of treatment. WPM and MS media were better than B5 medium for both induction and growth of shoots. Since an increased number of shoots and leaves formed when embryos were treated with PGRs like GA_3 and BA, growth during the first year could actually be greater than that from seeds germinated in nature (Buchheim et al., 1994).

The embryogenesis of peony can be dated back to the work of Sunderland et al. (1973) who induced embryoids from cultured anthers of a *Paeonia* hybrid. It was tolerably certain that the embryoids were of pollen origin. Androgenetic embryoids were also induced from tree peony two years later (Zenkteler et al., 1975). Rooted plantlets were obtained by Roberts and Sunderland (1977) from high yielding pollen-originated embryos in several peony cultivars. Thereafter, embryos have been not only induced from anther culture (Sunderland et al., 1973, 1975; Sunderland, 1974; Sunderland and Dunwell, 1974; Zenkteler et al., 1975; Ono and Tsukida, 1978; Lee et al., 1992; Kim et al., 2006, Chen, 2006) and microspore culture (Roberts and Sunderland, 1977; Ono and Harashima, 1981; Sunderland, 1983abc), but also often from embryo or cotyledon culture (Lin et al, 1987; Brukhin and Batygina, 1994; Kim and Lee, 1995, 1996; Shin et al., 1997; An, 2005; He, 2006; Kim et al., 2006). Embryogenesis is providing a new approach to seedling production of peony by using anthers and embryos in vitro culture. For both embryo in vitro culture and somatic embryogenesis of distant cross-breeding tree peonies, He (2006) reported more details in her PhD dissertation. There are no reports on embryogenesis on other tissues. Much study is necessary in this field in the future.

2.5 Induction and Multiplication of Callus and Shoots

2.5.1 Callus induction and multiplication

The purposes of callus production in peonies are: (1) use as material for physiological and genetic research (Ono and Harashima, 1983; Sunderland et al., 1984; Ono and Takahara, 1985); (2) medicinal chemical production such as paeoniflorin (Yamamoto et al., 1982abc) and paeonol (Shi and Zhang, 2005); and (3) source for shoot induction in tissue culture. Callus has been widely reported to be induced from all types of explants including embryos, buds, leaves, petioles, stems, flower parts, rhizomes and roots (Table 1). However, callus induction from tissue of mature plants usually fails. Callus generation from embryos has been already discussed in the section addressing embryo culture. The focus of this section is callus induction from other tissues and callus multiplication.

The earliest successful callus induction of peony in vitro was possibly done by Yamada and Sinotô in 1966. Callus was induced from the base of petals from *P. japonica* and huge variation in the chromosome numbers of cells was observed. There is a difference in the induction ability of callus between types of explants. Meyer (1976b) reported that callus generated from root culture of herbaceous peony grew slow. Underground bud eyes produced callus slowly but far more rapidly than root slices. Tissue pieces excised from spring flowers were easiest to result in callus formation. Callus was successfully initiated using sections of etiolated stems as the initial explant. However, attempts to induce callus from explants (stems, leaves, roots, and floral parts) from green plants were completely unsuccessful and were characterized by the production of intense brown colorations in both explants and the medium (Gildow and

Mitchell, 1977). Ono and Tsukida (1978) obtained haploid callus within the anthers of *P. lactiflora* in MS medium containing 1–2 mg l^{-1} NAA + 0.1 mg l^{-1} KT, with or without 1 mg l^{-1} 2,4-D. Orlikowska et al. (1998) compared effects of explant type on callus induction in *P. mlokosewitschii* and *P. tenuifolia* cultured in ½ MS + 1 mg l^{-1} BAP. The most effective callus formation was observed at the bases of vegetative buds and less intensive induction was seen on sepals, petals, ovary walls, stem tops and flower bases. In *P. rockii*, underground dormant buds and terminal buds of vitroplants were the best explants which had a 100% callus induction rate compared with 60% on petioles, and 31% on blades. However, tissues including pistils and stamens from productive organs were not successful for callus induction in MS + 0.5–3 mg l^{-1} BA + 0.1–3 mg l^{-1} NAA (Chen et al., 2001). Chen (2005, 2006) reported that in tree peony culture petioles were better than leaves for callus induction in WPM medium. Also, petioles near the axil were easier to generate callus than the middle petioles and petioles near leaf blades. Zhang (2006) investigated effects of three types of explants from growing herbaceous peony and found young stems had the highest callus induction rate (92%) compared with leaves (84%) and petioles (12.5%). In addition, patterns of placement on medium also influenced induction rate and growth of callus: the face-up placed leaf sections with 87% induction rate generated much more callus with better quality than the face-down leaf sections with 73% induction rate. When the petioles were placed on medium vertically no callus occurred. There is also significant difference in callus induction between vegetative tissues and embryos. Lang and Luo (2007) reported callus was much easier to be induced from cotyledons and radicles which had 96% and 90% of induction rate respectively while only 36% of petioles and 43% of leaf sections generated callus. On the

other hand, callus formed within 14–20 d from embryo culture but 30–45 d from petiole and leaf culture.

Callus has been induced when explants inoculated on a wide variety of basal media such as B5 (An, 2005), LB (Gildow and Mitchell, 1977), MS (Radtke, 1983; Xie, 1987; Chen et al., 2001; Zhang et al., 2001, 2007; Hu et al., 2003; An, 2005; Shi and Zhang, 2005; Zhang, 2006; Lang and Luo, 2007), ½MS (Li et al., 1984; Orlikowska et al., 1998; Chen, 2005; Li et al., 2005; Lang and Luo, 2007), Steeves medium (Demoise, 1967; Demoise and Partanen, 1969), SH (Gildow and Mitchell, 1977), SH-M (Gildow and Mitchell, 1977), modified SL (Lin et al, 1987), WPM (An, 2005), and ½WPM (Lang and Luo, 2007). The effect of basal media on callus induction is little studied in peony culture. An (2005) compared effects of four types of basal media containing 0.2 mg l^{-1} NAA + 0.5–6 mg l^{-1} BA on callus induction of tree peony. A consistent result showed that the highest callus induction rate occurred in WMP followed by MS and then by B5 but no callus formed on N_6 medium for all three types of explants: petiole, leaf and embryo. Lang and Luo (2007) found ½WPM was the best basal medium for inducing callus from both cotyledons and radicles of tree peony when cultured in basal media of WPM, ½WPM, MS or ½MS, all of which contained 1 mg l^{-1} 2,4-D + 1 mg l^{-1} BA + 500 LH. Among ½SH, SH, 2SH and SH-M media, the highest growth of callus was found in ½SH medium with an approximate 11-fold increase in fresh weight compared with 10-fold increases in SH and SH-M and only a 4-fold increase in 2SH after 30 d culture (Gildow and Mitchell, 1977).

PGRs play a more critical role on callus induction of peony compared with basal medium type. Callus could not be generated when peony is cultured in PGR-free medium

(Ono and Tsukida, 1978; Zhang, 2006). NAA and 2,4-D were more frequently used to induce callus in peony culture when applied alone or in a combination with other PGRs. NAA (0.1–2.5 mg l^{-1}) was widely used in both herbaceous peony (Meyer, 1976b; Ono and Tsukida, 1978; Radtke, 1983; Lin, 1980; Lin et al., 1987; Zhang, 2006; Zhang et al., 2007) and tree peony (Gildow and Mitchell, 1977; Li et al., 1984; Xie, 1987; Chen et al., 2001; Chen, 2005; An, 2005; Li et al., 2005; Shi and Zhang, 2005). Until the beginning of the 21st century, 2,4-D was not commonly used in callus induction medium for peony (Chen et al., 2001; Hu et al., 2003; Beruto et al., 2004; Chen, 2005, An, 2005; Li et al., 2005; Shi and Zhang, 2005; Zhang, 2006; Zhang et al., 2007; Lang and Luo, 2007). Callus also has been induced using other PGRs like TDZ, BA, PC, 2-iP and IAA usually when applied in a combination. Several cases reported that callus was induced in medium with additional BA or TDZ alone (Orlikowska et al., 1998; Hu et al., 2003). Application of a single PGR such as IAA, KT, NOA or TDZ did not lead to callus induction (Ono and Tsukida, 1978; Beruto et al., 2004). However results might be dose- or genotype-dependent. For example, one study reported 80–90% of explants formed callus in MS medium with 2,4-D or BA alone (Hu et al., 2003).

There are large differences in both induction and multiplication of callus between types, concentrations, and combinations of PGRs used in the medium. Callus was more easily induced in medium with 2,4-D than BA (Hu et al., 2003) and 2,4-D was considered the main factor on induction of callus from young leaf tissues (Li et al., 2005). Radtke (1983) found, in MS medium, application of 1 mg l^{-1} PC + 5 mg l^{-1} produced callus more readily than that of 2.5 mg l^{-1} NAA + 5 mg l^{-1} 2-iP for herbaceous peony. More callus was observed on medium with 2-iP + PC and TDZ + 2,4-D although it was also induced

in a TDZ-alone PGR medium (Beruto et al., 2004). Li et al. (1984) reported the best combination for callus induction from embryos was modified LS + 2.5 mg l^{-1} NAA + 0.4 mg l^{-1} 2-iP, but a higher concentration of NAA and 2-iP prevented callus formation and even killed the explants.

Sugar in medium is necessary and there have been no reports of callus formation when explants are cultured in sugar-free medium. The highest callus induction rate (100%) has been obtained at 30 g l^{-1} for both sucrose and glucose at the range of 0–70 g l^{-1}. Sucrose has been found to be better than glucose in both quality and growth of callus (An, 2005). Agar content in the medium also affects callus induction and growth. Agar at 6–7 g l^{-1}contributed the highest rate of callus induction (95%) compared with 72% at 8 g l^{-1} and 8 % at 9 g l^{-1} (An, 2005).

The type and concentration (mg l^{-1}) of PRGs in subculture medium of callus may be different from those in callus induction medium. Lin et al. (1987) reported the best combination for callus induction was modified LS + 2.5 NAA + 0.4 2-iP but callus grew most vigorously on a modified SL medium with 0.5 NAA + 5 2iP during subculture. Xie (1987) found MS + 2 BA + 0.2 NAA + 500 LH optimal for callus induction and MS + 2 BA + 0.5 IAA the best for subculture in tree peony. Chen (2005) obtained the highest rate of callus induction in ½MS + 0.5 2,4-D + 1 NAA + 0.2 IAA and the highest rate of callus proliferation in ½MS+ 0.3 NAA + 2 BA + 0.5 IAA. An (2005) used tree peony leaf to induce callus reporting WPM + 2 BA + 0.2 NAA to be the best medium for callus induction and WPM + 0.5–1 2,4-D + 0.5–1 TDZ better for callus multiplication. In herbaceous peony, both induction and multiplication of callus performed well in ½MS + 0.5/2 NAA + 0.5–1 2,4-D+ 0.2 BA (Zhang, 2006)

Low temperature may improve initiation of callus formation. A comparison of callus initiation from etiolated stems at 15ºC and 25ºC showed a much higher induction rate (40%) from tissue at the low temperature compared with 15% at higher temperatures (Gildow and Mitchell, 1977). However, it was reported that highest callus multiplication occurred at higher temperature (29ºC) among the range of 20–32ºC. The average callus weight increased in the dark but light increased the average weight of paeonol (Shi and Zhang, 2005).

Browning is a major problem, not only for initial explants (see 2.8 Browning) but also for callus culture. In herbaceous peony culture, generated callus eventually turned brown and died even after several transfers to fresh medium (Radtke, 1983; Zhang, 2006). Browning problem is also serious in callus culture of tree peony, especially under exposure of light. Culture in the darkness and application of the absorbent or antioxidant in the medium may alleviate this problem (Zhang and Luo, 2006). A 15-d interval of subculture was optimal to decrease callus browning (Lang and Luo, 2007)

2.5.2 Indirect shoot induction via callus

Induction of adventitious shoots from callus in peony is usually difficult. Meyer (1976b) attempted to produce shoots from callus culture; however, generated callus grew slow and no bud activity was found even after two years of subculture with frequent transfers. An attempt at shoot induction on callus also failed in *P. rockii* (Chen et al., 2001). Zhang (2006) induced callus from young stems, leaves and petioles of herbaceous peony, but no adventitious shoots were obtained from subcultured callus. Radtke (1983) reported excessive browning was the major problem to cause the death of both explants

and induced callus, and to prevent formation of shoots from callus on all parts of the plant. Only one bud from the cross of 'Westerner' × 'Good Cheer' formed an adventitious shoot on callus after three months culture on shoot induction medium (SIM): MS + 0.3 IAA + 15 2-iP. Callus was easily induced from excised embryos of herbaceous peony on modified LS medium. Although roots were observed and somatic embryos were generated during subculture of callus, no adventitious shoots were initiated on callus (Lin et al., 1987). Beruto et al. (2004) induced callus from axillary buds, filaments and petals of *P. suffruticosa*. Adventitious shoots only generated from callus came from filaments and petals after 8 weeks cultured on medium with TDZ. Yang and Pei (2006) obtained very limited number of shoots from subcultured callus of embryos in MS + 1 BA + 0.1 NAA.

On the other hand, the success of adventitious shoots originating from callus has been reported in several cases. Li et al. (1987) obtained shoots on callus from bud tips of tree peony after 4-5 wk cultured in ½MS + 0.2–1 KT + 0.5–1 BA + 0.1–0.5 GA_3. Shoots also have been induced from callus in MS + 0.2–0.5 IAA + 2 BA, which originated from young leaves and petioles. Petiole-originated callus resulted in a higher shooting rate (60–70%) compared with leaf-originated callus (20–30%), and each section of callus generated about 10 shoots. Xie (1987) induced shoots from callus generated from axillary bud tips and embryos in tree peony culture in MS + 2BA + 0.5 IAA. After one and half years of culture, adventitious shoots regenerated from callus which originated from sepals, petals, ovary walls, stem tips and flower bases (Orlikowska et al., 1998).

Shooting ability of callus is medium and PGR dependent. For example, in herbaceous peony, Hu et al. (2003) reported that MS + 3.5 BA was the best for shoot

formation of callus (0.9 shoots/callus section) compared with MS + 5 2,4-D (0.07 callus section) and N6 + 3.5 2,4-D (0.12 shoots/callus section). An (2005) compared the effect of PGRs on shooting from callus of tree peony in WPM medium containing 0–1 TDZ + 0–1 2,4-D and found shooting only occurred in WPM + 0.5 TDZ (45% shooting rate) and WPM + 0.5 TDZ + 0.25 2,4-D (10% shooting rate) after 8 wk of culture. Also, rooting ability was callus source dependent. Adventitious shoots could be induced on callus originated from embryo but not from young leaves and petioles.

2.5.3 Direct shoot induction

Indirect shoot induction through a callus phase is often difficult and takes a long time. Direct shoot induction is more prevalent for peony in vitro culture. Early efforts on tissue culture of peony resulted in success of callus formation but no shoots have ever been obtained routinely (Zenkteler et al., 1975; Meyer 1976ab; Radtke, 1983; Thomas and Meyer, 1986; Lin et al., 1987).

High rates of shoot induction were reported for the first time by Li et al. (1984) in tree peony culture with up to 26 shoots per explant (axillary bud tip) obtained after 4–5 wk cultured in ½MS + 0.2–1 KT + 0.5–1 BA medium. In herbaceous peony, excised shoot tips elongated and formed 3–5 axillary shoots at most nodal positions cultured in ½MS + 0.5 BA +1 GA_3 and more shoots were obtained after subculture in the same medium (Hosoki et al., 1989). Thereafter, more and more successful cases were often seen in both herbaceous peony (Albers and Kunneman, 1992; Hansen and Zhang, 1995; Gabryszewska, 1998, 2001, 2006; Orlikowska et al., 1998; Habib et al., 2001; Jin et al., 2005; Zhang, 2006; Zhang et al., 2007) and tree peony (Harris and Mantell, 1991; Albers

and Kunneman, 1992; Bouza et al., 1993, 1994b; Kong and Zhang, 1998; Černá et al., 2001; Zhang et al., 2001; Chen, 2005, 2006; Li et al., 2006bc).

2.5.4 Mechanism of shoot induction

Information on mechanism of shoot induction is extremely limited in peony in vitro culture. Bouza et al. (1993) reported that the different effect of BA and 2-iP on in vitro propagation of *P. suffruticosa* was correlated with different hormone contents. During the first five days of culture, the activity of BA-treated explants was higher than that of 2-iP-treated explants. High BA levels were detected in BA-treated explants, which were correlated with the absence of or low IAA content. Low 2-iP levels measured in 2-iP-treated explants were correlated with high endogenous IAA content. BA enhanced phylogenesis and axillary bud development during in vitro culture allowing good propagation while 2-iP did not.

2.5.5 Factors affecting shoot induction

Explant type

Terminal or axillary buds, tips of buds/shoots and nodal stems have been widely reported to be most effective (up to 100% induction rate) to directly induce adventitious shoots in peony culture possibly because of the apical/axillary meristematic region (Table 1), whereas other types of explants such as roots, stems, petioles, leaves, and parts of flower buds have been difficult to regenerate shoots directly. The shooting ability of explants is significantly influenced by type and concentration of PGRs in the medium. Orlikowska et al. (1998) investigated a variety of explants from field-growing shoots of *P.*

mlokosewitschii and *P. tenuifolia*, which included vegetative buds from the bases of shoots, bases of petioles, internodes, fragments of leaves with main veins, the highest part of shoots being in contact with the flower bud, the bases of flowers, the bases of sepals and petals, ovary wall, pistils and anthers. Direct shoot regeneration was only observed at a low ratio (≤ 10%) from tissues on the bases of petioles and petals, and leaf veins which were already cultured two months in ½MS + 1 BAP.

Basal medium

MS, ½MS, and WPM basal media have been mostly used successfully for direct shoot induction of peonies. WPM medium was only reported suitable in tree peony culture. In addition, shoots were obtained in Lepoivre medium containing 1 BAP + 0.1 GA_3 (Albers and Kunneman, 1992).

PGR

BA (BAP) is the most often used cytokinin to induce shoots from the initial explants. It was usually added to medium in a combination with GA_3 (Li et al., 1984; Hosoki et al., 1989; Albers and Kunneman, 1992; Bouza et al., 1994b; Hansen and Zhang, 1995; Kong and Zhang, 1998; Orlikowska et al., 1998; Habib et al., 2001; Jin et al., 2005; Zhang, 2006; Zhang et al., 2007). Kong and Zhang (1998) reported BA was significantly better than KT for multiplication of shoots. However, high concentration of BA (≥ 2 mg/L) often caused a higher rate of vitrification (Černá et al., 2001; Zhang et al., 2001). Optimal concentration of BA was 0.5–1 mg l^{-1} for shoot induction of peony. TDZ has shown a much stronger ability on shoot induction (Trigiano and Gray, 2000). However, TDZ has

been only recently used for this purpose in peony culture (Gabryszewska, 1998, 2001, 2006; Orlikowska et al., 1998; Beruto et al., 2004). Axillary shoot formation was significantly enhanced by the addition of TDZ at a very low concentration to medium containing a mixture of BAP + 2-iP + KT. The combination of TDZ with other cytokinins in medium can be more effective as compared to TDZ used alone for multiplication (Gabryszewska, 1998). Other PGRs such as KT, NAA, IAA, 2-iP, ZT, and NOA are usually used in combination with BA or TDZ.

Single type of cytokinins can induce adventitious shoots, but the efficiency will be improved with a combination of two or more types. Gabryszewska (1998) reported that all tested cytokinins (BAP, 2-iP, KT, and TDZ), when used separately, could stimulate axillary bud development; however, the multiplication rate was very low. Bouza et al. (1994b) compared the effect of five various hormones on in vitro multiplication and elongation of tree peony. MS medium with ZT, 2-iP, isopentenylam-inopurine (iPA) and KT at 4, 8, 16, 20 μM did not induce organogenesis, whereas 4 μM BAP was effective. GA_3 alone in the culture medium allowed neither shoot elongation nor leaf and axillary bud formation or development. The combination of BAP with GA_3 increased the multiplication rate as compared with BAP alone and allowed shoot elongation.

GA_3

GA_3 plays a very critical role and is often used in peony tissue culture. It not only elongates shoot stems but also increases the induction rate of shoots. Li et al. (1984) induced shoots from bud explants in GA_3-free medium and found leaves of the shoots grew fast but stems elongated very slow. These shoots might not be good for root

induction. When shoots were transferred to a medium containing 0.2–0.5 mg l^{-1} GA_3 stems of shoots elongated. Later, Hosoki et al. (1989) confirmed GA_3's effect on elongation of shoots. Beruto et al. (2004) reported explants of tree peony developed clusters of axillary shoots but no stem-elongated shoots were observed in medium WPM + 4.44 μM BA. It indirectly indicated shoot stems could not be elongated in medium without GA_3. Zhang et al. (2007) compared the effect of GA_3, BA and NAA on shoot induction of dormant buds in herbaceous peony. The result indicated shoot induction rate significantly increased with GA_3.

Other factors

There are other environmental factors such as temperature and light quality to influence induction and multiplication of shoots. Albers and Kunneman (1992) investigated effects of the factors, including temperature, KT, BAP, 2-iP, GA_3, activated charcoal (AC), liquid medium, day length, sugar, cold treatment, addition of low concentrations of auxins, and concentrations of macroelements, on improving multiplication. The only significant effect on the length of shoots was a cold treatment at the end of the subculture cycle. In liquid media, explants elongated but also became vitrified. Since the information of both treatments and data was unavailable, the conclusion may be doubtful. Gabryszewska (2001) found application of ABA or fluridone to cytokinin-free medium had no effect on the number of induced axillary shoots but influenced leaf formation on non-dormant and dormant shoots grown under all light qualities (white, red, blue). However, addition of ABA with cytokinins inhibited the leaf production in non-dormant as well as dormant shoots. Fluridone, an inhibitor of

ABA-synthesis, applied during culture, promoted leaf formation and axillary shoot production. The highest number of leaves and shoots were found on the explants grown under red light for both types of shoots. Development of dormancy in tissue-cultured peony shoots can be caused by ABA. Dormancy was also induced when cultured at high temperatures at 20ºC and 25ºC but not at 15ºC (Gabryszewska, 2006). Rates of growth and multiplication were reduced and senescence and death of leaves took place. Cooling treatment reactivated further growth and development of peony shoots at all temperatures. Cooled explants grown at 15ºC produced the highest numbers of shoots and leaves during the first subculture after cooling. Shoot formation and emergence of leaves decreased with increased temperature for both cooled and non-cooled explants. Zhang et al. (2001) compared the effect of both light intensity (1000 - 3000 lx) and temperature (20–30ºC) on performance of tree peony during 4 wk culture. The best culture condition was 2000 lx, 10 h/d at 25 ± 1ºC.

2.5.6 Subculture

Adventitious shoots could be proliferated by subculture in the same medium used for initial shoot induction or in shoot multiplication medium (SMM) with different compositions of PGRs. Li et al. (1987) obtained 4–5 folds of shoots every two months when generated shoots were subcultured in the same shooting medium every 1–2 months for tree peony. Hosoki (1989) developed a longitudinal shoot-split method for subculture purpose of tree peony in the same medium used for induction of shoots. However it was reported that the medium with alternative PGRs could improve shoot multiplication. Habib et al. (2001) found that successive subculture in low-BAP (0.1 mg l^{-1})-high-GA_3 (1

mg l^{-1}) containing medium caused progressive decline in the proliferation rate, over the course of several months. When subcultures were alternated each month between medium containing low-BAP (0.1 mg l^{-1})-high-GA_3 (1 mg l^{-1}) and high-BAP (1 mg l^{-1})-low GA_3 (0.1 mg l^{-1}), shoot proliferation was improved with less elongation. Li et al. (2006) obtained 100% shoot induction rate in MS + 2 BA + 0.2 NAA + 200 LH (lactalbumin hydrolysate), and found subculture had the highest multiplication rate (11.8) in MS+1 BA + 0.5 KT + 0.1 NAA + 200 LH and the lowest in MS+2 BA + 2 KT + 0.2 NAA + 200 LH. Multiplication of shoots is also sugar source and dose dependent. In medium ½MS + 1 BA + 0.5 KT, glucose was the best followed by fructose, sucrose and maltose (Zhang, 2006). For sucrose, 35 g l^{-1} of concentration was suitable for shoot multiplication.

The time span of subculture intervals or cycles affects shoot multiplication. In fern-leaf peony, induced shoots were routinely divided every 15–20 d (Hansen and Zhang, 1995) and 3.39-fold proliferation rate was obtained in *P. lactiflora* at a 60-d cycle (Zhang et al., 2007). A three-week subculture may be optimal. For example, Harris and Mantell (1991) found a three-week subculture interval gave shorter doubling times (21.7 d) than either 4- or 5-wk subculture intervals (24.8 and 27.0 d, respectively). Performance of shoots usually decreases with increased subculture cycle. Gabryszewska (2006) reported multiplication rate of both cooled and non-cooled explants decreased in the third subculture independently of temperature. Furthermore, length of the final subculture interval used immediately prior to the rooting treatment was found to be critical for determining the subsequent rooting responses of microshoots during subculture. Shoots selected from a five-week shoot multiplication subculture regime gave the best rooting

performance, while those from a four-week regime gave the lowest rooting response (Harris and Mantell, 1991).

2.6 Rooting

2.6.1 Introduction

Considerable progress has been recently made in understanding adventitious root formation using physiological studies. It is recognized that rooting is a process consisting of distinct phases. Rooting formation possibly includes three phases: dedifferentiation, induction, outgrowth of roots (De Klerk et al., 1999), or induction, initiation, and expression (Moncousin and Gaspar, 1983; McDonald and Wynne, 2003) based on the microscopical observations or biochemical characteristics, most notably peroxidase activity and auxin content.

Rooting is a critical step to success of tissue culture. The cost for rooting stage usually ranged from 35% to 75% of the total production costs (Trigiano and Gray, 2000). Root formation seems relatively easy for peony in tissue culture. Up to 90–100% rooting rate was obtained by Hosoki et al. (1989), Zhou and Yao (2001), and Li et al. (2006bc). The earliest information on rooting of in vitro peony was reported by Meyer (1976a) who obtained rooted plantlets from embryo culture. Roberts and Sunderland (1977) established rooted plantlets from embryos generated from anther culture.

Roots can be induced from callus (Meyer, 1976b), embryos (Roberts and Sunderland, 1977), and vitroshoots by most researchers. Before rooting formation, callus formed within a few days and root primordia usually generated after 10–20 d of inoculation (Li et al, 1984; Zhou and Yao, 2001; Yang and Pei, 2006). Roots occurred in liquid culture

within 2 wk (Gildow and Mitchell, 1977) while it took 4–7 wk in agar medium (Gildow and Mitchell, 1977; Xie, 1987; Zhou and Yao, 2001; An, 2005; Chen, 2006; Yang and Pei, 2006) depending on genotype, type of basal medium, PGRs, and culture condition.

2.6.2 Mechanism of rooting

The physiology of adventitious rooting in tissue culture of other species has been well discussed before (De Klerk et al., 1999; De Klerk, 2002; McDonald and Wynne, 2003). However, the mechanism of rooting has been not much investigated in peony tissue culture. Bouza et al. (1992) reported that shoots used for rooting had a moderate mitotic activity which was accompanied by low ABA production. When transferred to root induction medium (RIM), disappearance of mitotic activity and production of high levels of ABA suggested that exogenous auxin caused a shock to vitroplants and consequently, an inhibition of shoot development. During root induction, exogenous IBA was quickly absorbed by the explants and was transformed into IAA and IAA conjugates. In parallel, an important increase of abscisic acid (ABA) production by explants was observed. Rooting capacity of explants was favored by a preliminary accumulation of endogenous IAA only when levels of BA absorbed from multiplication medium had decreased (Bouza et al., 1994c). Main shoots coming from a 5-weeks subculture fulfilled these hormonal conditions and were the best microcuttings for rooting (87% rooting rate). Main shoots coming from medium contained high BA levels had a low rooting capacity (25–55% rooting rate). Root induction was associated with an early peak of IBA followed by a 10-fold lower peak of endogenous ribofuranosyl-isopentenyladenine. Root development was efficient, especially in a medium containing activated charcoal, which led to an almost 3-

fold decrease of IAA contents in roots. The omission of exogenous BAP in RIM and the addition of IBA in high concentrations resulted in inactivity of terminal buds of shoots. Although mitotic index (MI) in terminal buds rose soon after transfer to the root development medium (RDM), shoot development was inhibited and ex vitro establishment was disturbed.

2.6.3 Factors affecting rooting

Basal medium

Adventitious roots have been produced in most of the rooting media reported in peony tissue culture (Table 1). MS basal medium including MS medium (Li et al., 1984; Xie, 1987, Harris and Mantell, 1991, Kim and Lee, 1995; Cao, 2003; Chen, 2005; Yang and Pei, 2006), modified MS medium (Bouza et al., 1994ac), half strength MS medium (Hosoki et al., 1989; Hansen and Zhang, 1995; Shin et al., 1996; Kong and Zhang, 1998; Zhang et al., 2001; Habib et al., 2001; Zhou and Yao, 2001; Chen, 2005; He, 2006, Li et al., 2006bc; Zhang et al., 2006), and ¼MS medium (Chen, 2005) were the most commonly used media for root induction of peony in vitro. Gildow and Mitchell (1977) induced roots in many kinds of modified SH media (½SH, SH, 2SH, SH-M) without PGRs. Modified SL medium was also used by Buchheim et al. (1994) to successfully induce roots from embryos of herbaceous peony. The WPM basal medium including modified WPM (Beruto et al., 2004; Chen, 2005) and ½WPM medium (An, 2005) were recently used for rooting only in tree peony. Lepoivre medium was only used by Albers and Kunneman (1992) for both herbaceous and tree peony. Stanys et al. (2007) obtained roots in the White medium from cultured embryos of herbaceous peony. Most researchers

used solid medium for rooting culture, but roots actually developed faster in liquid medium (Gildow and Mitchell, 1977). High frequency of rooting (57–100%) was obtained by Hosoki et al. (1989) on paper-bridge liquid medium supplemented with 1 mg l^{-1} IBA in herbaceous peony.

Although roots have been induced from the basal media discussed above, rooting of peony responds to media significantly different when other factors, such as agar, sugar, PGRs, and cultural conditions are fixed. Chen (2005) compared effects of ½MS, MS, ¼MS and WPM on rooting performance of tree peony. Results indicated that the best medium was ½MS (49% rooting rate) followed by MS for root induction when supplanted with 1 IBA + 1 IAA and 20 g l^{-1} sugar, while ¼MS medium was the worst for rooting. WPM medium with 1 IBA + 1 IAA + 40 g l^{-1} sugar was better for root growth. Stanys et al. (2007) found embryos formed roots twice more often in MS medium than in White medium. Stratification of seeds significantly stimulated rooting and root growth in White medium. However, it had no influence on rooting in MS medium.

Sugar

Sugar content in medium also affects rooting of peony in vitro. Albers and Kunneman (1992) found the highest rooting percentage produced in Lepoivre medium with 30 g l^{-1} of saccharose for both herbaceous and tree peony. The highest rooting rate was obtained at 20 g l^{-1} of sugar and the lowest rooting rate was found at 10 g l^{-1} of sugar. Rooting decreased as sugar concentration increased from 20–40 g l^{-1} in the medium.

PGR

Like many kinds of other plants in tissue culture, in vitro rooting of peony is heavily dependent on PGRs used in medium, although in a few cases, roots have successfully formed in PGR-free medium (Beruto et al. 2004). Among auxins, IBA is the most commonly used in rooting medium followed by NAA and IAA (Table 1). All three of these PGRs have been used separately or in combination. The optimal concentration for IBA and IAA is 0.1–1(2) mg l^{-1} when added in medium. However, much higher concentrations of IBA solutions are usually used in a short-time treatment like a quick dip. There are three major regimes to induce roots: (1) shoots or germinated embryos are directly inoculated in the RIM with low concentrations of auxins and there is no transfer to PGR-free medium (Albers and Kunneman, 1992; Kong and Zhang 1998; Zhou and Yao, 2001; Chen, 2005; Li et al, 2006bc; Yang and Pei, 2006; Zhang et al., 2006); (2) shoots or germinated embryos are treated in RIM with high level of auxins for a period of time then transferred to PGR-free medium (Xie, 1987; Bouza et al., 1994a); (3) after a quick dip in high concentration (0.1–1 g l^{-1}) of auxin, shoots and geminated embryos are immediately inoculated in PGR-free medium (Li et al., 1984; Bouza et al. 1994a; Hansen and Zhang 1995; Habib et al., 2001).

The relationship between rooting capacity of microcuttings and concentration of auxins in medium was shown to be a linear correlation ($P < 0.01$) in tree peony culture (Beruto et al., 2004). Rooting also took place without IBA (±10%), but increased to 50% with IBA at 49.2 μM. Rooting percentage increased somewhat when shoots were transferred onto hormone-free medium for two additional months, this being particularly significant when lower IBA concentrations (less than 49.2 μM) were used during auxin

treatment. In herbaceous peony, Zhang et al. (2006) obtained 50%, 60% of rooting rates in ½MS +1 mg l^{-1} IBA, 2 mg l^{-1} IBA respectively, but found only 25% of shoots rooted in medium with 5 mg l^{-1} IBA. The number and length of roots also significantly decreased with increased concentration of IBA. Similar results were obtained by An (2005) in medium containing IBA or NAA with 0, 0.5, 1, 2, and 4 mg l^{-1}. No roots formed in PGR free medium for tree peony culture, rooting rate increased with concentration of IBA and NAA firstly and reached a peak (60%) at 2 mg l^{-1} IBA and (45%) at 1 mg l^{-1} NAA then steeply decreased at 4 mg/L level of both auxins.

Reasons for large differences in rooting rate are very complicated. It is dependent on genotype, quality of material, composition of medium, type and concentration of PGRs, treatment method, and condition of pretreatment and culture. Li et al. (1984) reported roots were only induced from the shoots of tree peony when treated with a dip in IBA or directly cultured in the medium containing IBA, but not from similar treatments of IAA or NAA. Rooting rate was above 90% when shoots were treated for 30 min of soaking in 50–100 ppm of IBA, but only up to 66% of rooting rate from ½MS medium with 0.1–2 mg l^{-1} IBA. In addition, much callus was produced when IBA concentration was higher. Lin et al (1987) successfully induced roots from callus in modified LS medium with NAA and 2-iP in embryo culture. Hosoki et al. (1989) reported roots were induced from medium with either NAA or IBA in herbaceous peony. Xie (1987) reported roots were formed in PGR-free MS medium after 30 d of treatment in ½MS with 1 IAA +1 IBA.

Comparative effect of IBA and NAA on rooting capacity has been investigated by An (2005) in tree peony. It was shown that IBA gave higher rooting rate than NAA for all treatments with a range of concentration of 0, 0.5, 1, 2, and 4 mg l^{-1}. The highest rooting

rate occurred at 1 mg l^{-1} NAA and 2 mg l^{-1} IBA, respectively, then root rate steeply dropped to 10–15% at the level of 4 mg/L. Comparison of IBA and IAA on rooting has been studied as well. Kong and Zhang (1998) concluded that IBA seemed a little better than IAA based on higher rooting rates (65–79%) found in IBA than in IAA (62–69.4% rooting rate) at a range of 0.5, 1, and 2 mg l^{-1}.

The difference in rooting rate has not only been seen when a single auxin is used but also when a combination of auxins is applied in medium. For example, Li et al. (2006ac) obtained 100% rooting rate in ½MS + 0.1 NAA. Other higher rooting rates (82.4, 83.2%, 84.8%) were observed in treatments of 0.5 NAA, 0.1 NAA + 0.5 IBA, and 0.5 mg/L IBA respectively. When total concentration ≥ 1 mg l^{-1} (by directly adding up both NAA and IBA concentrations), rooting rate dropped to 31–74%. It indicated the concentration of NAA and IBA should be no more than 1 mg/L to obtain a better result in rooting.

Usually auxins promote root induction and cytokinins inhibit rooting while favoring induction of shoots. The antagonistic relationship of auxins and cytokinins has also been seen in peony tissue culture. In embryo culture of herbaceous peony, Buchheim et al. (1994) reported that root growth was reduced as concentration of BA in medium increased. Roots formed on embryos cultured on 10 μM BA were shorter (3–15 mm), stubby, and did not form lateral roots; whereas roots formed on embryos grown on cytokinin-free medium were longer (about 50 mm), thin, and generated lateral roots. Stanys et al. (2007) found that additional BAP (0.25, 0.5 mg l^{-1}) significantly decreased rooting rate and length of root in White medium for both stratified and unstratified embryos and decreased rooting in MS medium for unstratified embryos only. In embryo

culture of tree peony, increase of the ratio of IAA-BAP improved rooting and the reverse ratio benefited shoots induction (He et al. 2005).

Rooting capacity of shoots also is affected by other PGRs. In embryo culture of herbaceous peony, cotyledon and root growth appeared to be inhibited by high concentration of ABA in modified SL medium (Buchheim et al., 1994). GA_3 has a positive effect on rooting: 86% roots formed on somatic embryos in 0.3 mg l^{-1} GA_3 treatment compared with 61.5% in GA_3-free MS medium after more than three weeks culture at 4ºC (Shin et al., 1997).

Genotype

The genotypic difference in peony in vitro rooting has been mentioned by several researchers. Habib et al. (2001) reported that the cultivars 'Paula Fay' and 'Cytherea', but not 'Prairie Moon', rooted quite well in medium containing 2.5 mg l^{-1} IBA. In embryo culture, 63.6% and 72.6% of rooted plantlets were obtained from *P. otssi* and *P. rockii*, respectively (He, 2006).

Pretreatment

Chilling pretreatment and culture in the dark are beneficial for rooting. For example, Beruto et al. (2004) reported chilling microcuttings before transfer to root induction medium, had a beneficial effect on rooting percentage when no hormones were supplied in tree peony culture. Stratification had an effect on rooting but it interacted with other factors. In herbaceous peony embryo culture, stratification significantly stimulated

rooting and root growth in White medium, however, it had no influence on rooting in MS medium (Stanys et al., 2007).

Root initiation in 'Paula Fay' was favored in darkness compared with light (Habib et al., 2001). These results are consistent with other reports of improved rooting in the dark (Kunneman and Albers, 1989). However, it was also reported that rooting was inhibited by 1–4 d of short darkness treatment and then increased when the treatment time was elongated (Chen, 2005).

Other pretreatments may also benefit rooting in peony tissue culture. Bouza et al. (1994a) found that rooting percentage and mean number of roots on elongated microcuttings increased if axillary buds were removed from their bases before transfer to RIM. Overall, non-elongated shoots gave better rooting response than elongated shoots.

Subculture

Subculture has an important effect on rooting stage. The length of the final subculture interval used immediately prior to rooting treatment was found to be critical for determining the subsequent rooting responses of microshoots (Harris and Mantell, 1991). Shoots selected from a five-week shoot multiplication subculture regime gave the best rooting performance, while those from a four-week regime gave the lowest rooting response. The percentage survival of rooted microshoots of *P. suffruticosa* has been low. After four months, the percentage survival of rooted shoots from *P. suffruticosa* was 0%, 0% and 27% (taken from 3, 4, 5-wk subculture duration, respectively). The large number of roots per rooted shoot may partly account for the higher survival rate of the weaned plantlets from the five-week subculture treatment. However, Chen (2006.) found the

highest rooting rate occurred in shoots with less than 5 subcultures after 45 d of culture for tree peony.

Light quality

Besides a positive role of darkness treatment on rooting, effect of light quality on rooting of in vitro cultured plants has been widely studied. Red light showed both positive (Bertazza et al, 1995; Powyszyńska and Gabryszewska, 2003) and negative (Kvaalen and Appelgren, 1999; Niemi et al., 2005) contributions to rooting. In peony tissue culture, a beneficial effect of darkness treatment for a short period on rooting is reported by several researchers (Bouza et al., 1994a; Habib et al., 2001; An, 2005; Zhang et al., 2006bc), but information on comparative effect of light quality on rooting is very limited. Only Albers and Kunneman (1992) reported rooting of shoots with dark treatment was better than with white and red light treatments.

Temperature

Low temperature treatment was favorable for rooting of peony (Albers and Kunneman, 1992; An, 2005). Beruto et al. (2004) even reported IBA improved rooting but was not really necessary provided shoots were pre-treated at 2ºC for 7 d. Kim and Lee (1995) found roots were well formed from germinated embryos of *P. albiflora* in medium containing glutamine at both high (25 ± 2ºC) and low (15 ± 2ºC) temperatures, but high temperature treatment caused more albino plants.

CO_2

There is less information about effect of carbon dioxide (CO_2) on rooting of peony. Chen (2006) treated shoots with application of CO_2 (at 450 and 3000 ppm level) and found no effect on root number. However the treatment with higher levels of CO_2 significantly increased root length. It is consistent with other reports that high levels of CO_2 stimulated root growth in other plants (DiIorio et al., 1992) but inconsistent with the increased root number at high levels of CO_2 (Fsichella and Morini, 2003).

Others

Addition of activated charcoal (AC) is very effective for rooting improvement of in vitro cultured peony. Bouza et al (1994ac) found root development was efficient, especially in a medium containing AC. Positive effect of AC on rooting is supported in other plants (Van Staden, 2002; Gübbük and Pekmezci, 2004). Browning often happens during root induction. Besides AC, the use of absorbent polyvinylpyrrolidone (PVP) in medium can alleviate this problem during root induction (Zhang and Luo, 2006).

2.6.4 Summary

Rooting is a critical step for obtaining regenerated plants. The fact that the effects of auxins on root primordium elongation are different from those on initiation of root initials is widely accepted (Soh et al., 1999). The process of adventitious root formation can be divided into several stages. However, there is a lack of agreement on the number and nature of stages as well as the terminology used. It seems certain that there are at least two stages for rooting in peony culture: root induction and root growth. The factors

which affect rooting are very diverse and complicated. The genotype, type of basal medium, type and concentration of agar, sugar and PGRs, AC, temperature, light, subculture (age) and pretreatment like chilling, all interact with rooting of microshoots. The inherent PGR level of plant tissue may be the decisive factor in rooting. Incongruous conclusions are often seen in previous studies, many of which might be inconclusive. The reason for this phenomenon is possibly explained by genotype difference, absence of repetitive experiments, misused statistical analysis especially when data is based on a very small sample size or when the outliers have not been treated reasonably. On the other hand, the environmental heterogeneity, such as size of culture vessel and place on the culture shelf, is also often omitted by many researchers. Rooting of peony in vitro has been already proven successful but the procedures need to be optimized for reproducibility.

2.7 Transplanting

Transplanting is the final step of plant tissue culture. High survival rate of transplanted vitroplants is a basic requirement of successful commercial production through tissue culture. In peony tissue culture, success of transplanting has been reported by some researchers. However, after transferring to soil or other media, the young plants often grow slowly because of bud dormancy and survive at a low percentage because of easy infection from pathogens. Earlier efforts of transplanting generated no more than 50% survival rates (Meyer, 1976b; Li et al., 1984; Hosoki et al., 1989; Harris and Mantell, 1991; Albers and Kunneman, 1992; Kong and Zhang 1998). Thereafter, higher survival

rates (70–90%) have been obtained by several researchers (Habib et al., 2001; Zhou and Yao, 2001; Beruto et al., 2004; An, 2005; He, 2006; Kim et al., 2006)

Medium type

The following types of media have been successfully used in transplanting of in vitro cultured peonies: a mixture of 3:1:1 coarse sand-loam soil-peat moss (Meyer, 1976b), soil (Li et al., 1984; Kim, 2006), porous soil (Hosoki et al., 1989), peat-based potting mixture (Habib et al., 2001), 1:1 peat-perlite (Albers and Kunneman, 1992; Beruto et al., 2004; Li et al., 2006), 1:2 and 1:3 peat-perlite (He, 2006), 1:1 peat-sand (Albers and Kunneman, 1992), 1:1 peat-vermiculite (Li et al., 2006), 1:2 perlite-vermiculite (He, 2006), 1:1:1, 1:1:2 and 2:1:1 of perlite-vermiculite-peat (He, 2006), vermiculite (Kong and Zhang, 1998), sand bed (Zhou and Yao, 2001), 2:2:3:2:1 of garden soil, perlite, wood sawdust, chicken litter and horse manure (An, 2005). The type of medium has an effect on performance of transplanted plants. For example, growth in fertilized peat-perlite (1:1) was better than in fertilized peat-sand (1:1) during the first 3–4 wk (Albers and Kunneman, 1992). Sterilized muck soil (48% survival rate) was better than vermiculite (15% survival rate) in tree peony at 15–20ºC (Kong and Zhang, 1998). He (2006) compared a series of medium for transplanting and the highest survival rates were found in 2:1:1 of perlite-vermiculite-peat (80%) and 2:1 of perlite-vermiculite (81%) and the lowest survival rate (33.3%) in 1:1 mixed soil-sand for *P. rockii*. Li et al. (2006) reported that there was no difference in survival rate between 1:1 peat-vermiculite and 1:1 peat-perlite for tree peony but 1:1 peat-vermiculite significantly increased plant height after 30 d of culture.

Sterilization

Infection from pathogens is the major problem of death of plants during weaning. About 50% of plantlets planted in soil were lost due to infection during weaning (Albers and Kunneman, 1992). Therefore, any of medium used for transplanting should be sterile or sterilized to obtain high survival rates.

Cold treatment

Plantlets transferred directly to greenhouse had 100% mortality, primarily due to poor growth performance (Habib et al., 2001). A chilling treatment was often used in peony tissue culture to break dormancy during transplanting and thereafter accelerate plant growth. When plantlets were given a cold treatment for 4 months in cooler (4 ºC) before being transferred to peat-based potting mixture and covered with a transparent plastic dome, up to 80% of these plants were well established after 8 wk culture in growth room under the conditions of 16 h light with 65 mmol m^{-2} s^{-1} and 21ºC (Habib et al., 2001). He (2006) also mentioned that it was necessary to treat plantlets 2–3 months at 2–4ºC before transplanting to improve survival rate. After breaking dormancy at 4ºC for 6 wk, most of seedlings from somatic embryos developed into healthy plants in growth chamber (Kim et al., 2006).

Acclimation

The performance of transplanted plants largely depends on acclimatization procedures involving adaptation of plantlets to ex vitro conditions of significantly lower relative humidity and higher light intensity. Therefore, a period of gradual acclimation

must be taken during transplanting. For example, Li et al. (1984) exposed plantlets to 3–4 d acclimation under plenty light before being transplanted to neutral soil. Hosoki et al. (1989) placed transplanted plantlets under a plastic cover at 18–20ºC and 16 h illumination of 52 $\mu EM^{-2} S^{-1}$ cool white fluorescent lamps. Beruto et al. (2004) reported that a gradual acclimatization of plantlets in closed containers placed in culture room resulted in good quality plantlets and 80 ± 10% of transplanted plants survived. An (2005) increased light exposure of plantlets in the open tubes for 3–4 d before transplanting and used a plastic cover for two weeks to maintain high relative humility after transplanting. By this acclimation procedure, 80% survival rate has been obtained for *P. rockii*.

Light

The information about effect of light on performance of transplanted peony in vitro plants is extremely limited. Albers and Kunneman (1992) reported that increased day length (8, 12, 16 h) at a light intensity 45 mmol s^{-1} m^{-2} during the first 3 wk resulted in increase of shoot length and leaf size, but the difference in shoot length disappeared with extended weaning.

Temperature

Temperature shows significant impact on transplanted plants especially on survival rate. Low temperature is beneficial for the survival of rooted plantlets after transplanting. Kong and Zhang (1998) obtained 36% survival rate of plants at 15–20ºC but only 8 % at 25–30ºC in vermiculite medium. However, Albers and Kunneman (1992) found no difference in temperature treatments (5, 10, 15ºC) for growth of transplanted plants. It

was mentioned that an environment with changeable temperatures benefited growth of transplanted plants (He, 2006).

Summary

Medium used for transplanting purpose must be sterile. A chilling treatment before transplanting may improve survival rate and growth of plants. A gradual acclimatization is a critical step of transplanting and it significantly affects performance of transplanted plants. After being transplanted, in vitroplants of *Paeonia* are very sensitive to fungi and bacteria, which spread quickly among the plantlets if a nonsterile environment exists. Therefore, individual acclimatization in separate pots or multi-plots was suggested to reduce disease development (Albers and Kunneman, 1992). Low temperature, high humility and long day length also may benefit transplanting.

2.8 Browning

2.8.1 Introduction

Browning is one of most commonly seen phenomena and one of the most serious alternation processes in the food industry (Arias et al., 2007). It is estimated that over 50% of fruit and vegetable crops are lost due to postharvest deteriorative reactions resulting mostly from browning (Martinez and Whitaker, 1995). Browning has been broadly studied especially in postharvest physiology of fruits and vegetables (Kader et al., 1997). Browning results from both enzymatic and non-enzymatic oxidation of phenolic compounds. It usually impairs the sensory properties of products because of the associated changes in color, flavor, and softening (Martinez and Whitaker, 1995).

Browning in tissue culture was a major obstacle in establishment of explants, which subsequently made in vitro techniques more complicated (Murkute Mayakumari, 2003). Browning is one of major problems in peony in vitro culture and it has occurred in peony tissue culture with all types of explants. Roberts and Sunderland (1977) induced embryos from peony pollen in the dark but illuminated culture was hampered by brown pigment formation. Gildow and Mitchell (1977) found that any small callus formation was invariably stopped by production of an intense dark brown coloration which permeated plant tissue and diffused into culture medium. Radtke (1983) investigated tissue culture of herbaceous peony using several types of explants and found the main obstacle to success was the phenolic compounds which caused medium to brown and inhibited tissue growth. Hansen and Zhang (1995) also mentioned browning of culture medium became a severe problem and plants had to be transferred almost on a daily basis over a few weeks whenever the stem of a shoot was cut during subculture of fern leaf peony. Shi and Zhang (2005) reported browning occurred after 20 d of root culture of tree peony. Much more information was provided by Wu (2003) in her master thesis '*Research on browning mechanism and factors influencing explant browning in preliminary culture of peony in vitro*'. Since browning is a major problem and extremely important in peony tissue culture, it will be discussed in more detail in the following paragraphs.

2.8.2 Mechanism of browning

Browning in peony in vitro culture is a serious problem, researchers have tried to decode its secret. Radtke (1983) proposed that the phenolic compounds may be produced in response to the internal bacteria, or they may be a wounding response of peony tissue.

In most situations, researchers just discussed the possible relationships between browning and other factors. The secret of browning in peony tissue culture still remains. The mechanism of browning in peony tissue culture was recently studied in more detail by Wu (2003) for tree peonies. A positive relationship was revealed between the content of total phenol and browning index. Total phenol content in plants was the major factor to contribute to browning. Browning happened more easily in cultivars with higher levels of total phenol. Browning was caused by oxidization of the total phenol by catalysis of polyphenol oxidase (PPO) while oxygen existed. The total phenolic content decreased and PPO was active during browning. Both PPO activity and total phenol content positively related with browning index in the early stage while negatively related in the late stage, which brought up a two-phase theory on explant browning: browning initiation and browning development.

A positive correlation was found between membrane permeability, malodialdehyde (MDA) content, and browning index. But, the correlation between superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), which prevented oxidative damages, and membrane permeability was negatively related. It suggested that an increase of membrane permeability and destruction of protective systems were the major factors to accelerate browning. The higher browning rate shown under strong light culture was possibly because of activity increase of PPO.

In summary, the mechanism of browning and resistance has been widely studied in postharvest fruits, vegetables and other foods. Browning of foods results from both enzymatic and non-enzymatic oxidation of phenolic compounds as well as from Maillard reaction that occurs when mixtures of amino acids and reducing sugars were heated

(Mcevily and Iyenger. 1992). Because of its involvement in adverse browning of plant products, PPO had received much attention from researchers in the fields of plant physiology and food science (Yoruk and Marshall, 2003). PPO activity plays a dominant role in enzymatic browning (Kader et al., 1997). In peony tissue culture, PPO was involved in enzymatic browning, and a nonenzymatic browning may be also responsible for deterioration of explants.

Browning is affected by a series of factors, which include type and age of explants, genotype, sampling time, pretreatment, method and time of sterilization, medium, PGR, light, temperature, and transfer frequency and generation of subculture. Although Albers and Kunneman (1992) doubted the statement of Radtke (1983) who believed that exuded phenolic compounds inhibited growth of explants. In fact, based on the results from the majority of researchers, serious browning often inhibited tissue growth and multiplication and thereafter easily caused explants to die if they were not transferred to the fresh medium frequently.

2.8.3 Factors affecting browning

Explant type and age

The browning in peony tissue culture is related to the type of explants and sampling time. Kong and Zhang (1998) found different browning rates among explant types (dormant bud and sprouting shoots) of tree peony as well as sampling times. Dormant buds between August and November had a better response or lower browning than sprouting buds in March. Terminal buds showed less browning than axillary buds (Wu, 2003). Lang and Luo (2007) reported low browning rates in embryo (0–16%) and radicle

culture (23–43%), and high browning rates in leaf (43%) and petiole culture (50%). Old plant tissue may produce higher browning, for example, 44–100% browning rate occurred in mature leaves and 27.5–90% for young leaves of tree peony (An, 2005). In preliminary embryo culture, the browning rate was much higher in immature embryos (1.5–67%) than in mature embryos (0–22%) (He, 2006).

Explant age also affects browning of explants. Wu (2003) found the lowest browning rate (25–45%) in explants when taken during the middle of April and May, and the highest browning rate (70–100%) in explants when sampled during March, June to October. The effect of sampling time has also been mentioned by Chen (2005) and He et al. (2005).

Genotype

Browning is genotype dependent, for example, Wu (2003) reported the bud explant of 'Feng Dang' contributed to the highest browning rate compared with that of three other cultivars in tree peony culture.

Pretreatment

Given different pretreatments either naturally or artificially, browning of explants could be affected. Wu (2003) compared bud explants from plants which were under forcing growth in greenhouse, potted plants at room temperature, and plants in field, separately. The result indicated buds from field had the lowest browning rate (50%) while explants from greenhouse caused highest browning (90%). Browning of explants is also related to environmental differences of mother plants. For instance, petiole explants from

tree peony plants exposed to high light intensity showed worse browning than those from plants under shady cover (Chen, 2005; He et al., 2005). A pretreatment of water rinse before sterilization is often used to remove dust and some pathogens. This procedure may also affect browning of explants. Wu (2003) reported explants treated with icy water - icy water or icy water - PVP before sterilization had the lowest browning (60%) whereas a treatment of icy water with powdery detergent caused all explants browning. Seed coat removed embryos were more prone to browning (Jin et al., 2005).

Method and time of sterilization

Peony browning in vitro is affected by sterilization methods and treatment time. Jia et al. (2006) reported different browning rates between sterilization methods in embryo culture of tree peony. Similar results were also found in leaf culture (An, 2005). Wu (2003) investigated effects of sterilization methods on browning with three sterilants ($HgCl_2$, NaOCl, HQS) under the same time of 8 min. The highest browning rate (100%) occurred in the treatment of high concentration of NaOCl. Browning usually increased at prolonged sterilization time. In dormant bud culture of herbaceous peony, 27%, 38%, 44%, and 50% of browning rates were recorded when treated explants in 0.1% $HgCl_2$ for 8, 10, 12, and 14 min, respectively (Zhang et al., 2006).

Medium

Effect of medium on browning is complicated since when PGRs are usually added. However, when the same type and concentration of PGR(s) were added, the results are still comparable. Several papers reported that browning was affected by medium type. An

(2005) observed 24% browning rate in ½MS, 32% in WPM, and 48% in MS. Lang and Luo (2007) found the lowest browning rate in modified half strength WPM medium followed by ½MS, while MS medium contributed to the highest browning of cultured tree peony. However, Chen (2005) and He et al. (2005) reported there was no difference in browning rates between the media of WPM, ½MS, and MS, which all caused 100% browning in callus culture, but the browning degree was different with WPM < ½MS < MS. It was confirmed that high macro-element medium and high level of PGRs could increase the degree of browning (Wu, 2003). Browning was also affected by agar content. Browning increased as agar decreased in medium (Wu, 2003).

PGR

Besides basal medium and agar, browning is related to type and concentration of PGRs. Li et al. (2005) reported large differences (0–100%) of browning in MS medium with various combinations of PGRs (BA, 2,4-D, KT, NAA) in leaf culture of tree peony. Browning increased as concentration of cytokinins (BA, 2-iP, and GA_3) in medium increased (Wu, 2003). He et al. (2005) found 100% browning rate in MS medium with 0.5 - 2 BA + 0.5 - 2 NAA in petiole culture of tree peony. The reason of browning caused by cytokinins possibly was that cytokinins accelerated synthesis of phenolic compounds and improved activity of PPO (Wu, 2003).

Light

The relationship between light and browning has been much discussed in tissue culture of other species. Habib et al. (2001) reported shoot tip explants of herbaceous

peony mostly always exhibited medium browning within 2–3 h when incubated under light. However, less than 15% of explants placed in the dark for 48 h developed browning. Light was implicated in increased browning and probably resulted in increased phenol production. However, Lang and Luo (2007) found no large difference in browning between light (54%) and darkness (53%). Chen (2005) and He et al. (2005) also mentioned that there was no difference in browning rate between treatments of darkness and light but browning degree appeared worse under light. It implicated light intensity did not switch on ignition of browning but accelerated browning process. Therefore, weak light can alleviate browning especially when cultured at low temperature (An, 2005).

Temperature

Low temperature is beneficial for alleviation of browning while high temperature accelerates browning in peony tissue culture. The etiolated explants cultured at 15ºC showed no dark coloration compared with a severe browning problem occurred at 25ºC (Gildow and Mitchell, 1977). Lang and Luo (2007) also reported browning increased with temperature, and 36%, 49%, and 53% of browning rates were recorded at 4, 14, and 24ºC, respectively. Weak light and low temperature (18–20ºC) could reduce the browning problem (An, 2005). However, a short period of low temperature treatment in the dark only temporarily inhibited browning of explants and browning became worse later as the explants were exposed to the normal culture conditions (Wu, 2003).

Other Factors

Besides the above mentioned factors, other factors may contribute to browning. For instance, Zhang et al. (2001) found higher browning in the explants when they were inserted too deep in medium. Browning increased with subculture generations or cycles (Lang and Luo, 2007). Browning rate was recorded with 40%, 53% and 62% for the 1st, 2nd, and 3rd culture generation, respectively; therefore, browning increased with subculture generations. Increase of transfer frequency not only reduced browning but also improved the survival and growth of explants in peony tissue culture (An, 2005; Wu, 2003). On the other hand, He (2006) reported low browning rate (0–22%) in preliminary culture of mature embryos and higher browning rate (4.2–30%) in subculture after embryos germinated.

2.8.4 Strategy to prevent or alleviate browning

So far, it seems impossible to prevent tissue browning completely in peony in vitro culture. However, approaches to reducing browning have been investigated. The addition of 0.3% AC in medium was found effective in preventing tissue and medium browning in tree peony (Zhang et al., 2001). He et al. (2005) found 0.1 g l^{-1} vitamin C (VC) and 3 g l^{-1} AC added in medium could prevent browning effectively. More recently, a series of antioxidants or absorbents including VC and AC, others like polyvinylpyrrolidone (PVP), phytagel, sodium hyposulfite ($Na_2S_2O_3$), and diethyldithiocarbamate (DDTC), have been evaluated in their effects on reducing browning in peony culture (An, 2005; He et al., 2005; Chen, 2005; Zhang and Luo, 2006; Lang and Luo, 2007). Among which, PVP and phytagel followed by VC showed the ability to reduce browning, while $Na_2S_2O_3$, and

DDTC were ineffective to diminish browning and even made it worse. Considering side effects of high concentrations on shoot multiplication, 3–5 g l^{-1} is optimal for PVP. The positive role of PVP in browning reduction was also supported by Yang and Pei (2006) who reported 0.05% of PVP prevented browning effectively and improved growth of microplantlets. VC was effective for browning control, but it also significantly inhibited growth and multiplication of shoots and caused yellowing of tree peony during culture. Explants died when 0.9 g l^{-1} of VC was used in the medium (Zhang and Luo, 2006). Besides a positive effect on browning reduction for phytagel, the highest shoot multiplication also occurred in the phytagel added medium (Zhang and Luo, 2006).

2.9 Vitrification

2.9.1 Introduction

The term “vitrification” as applied to plant tissue culture was first used by Debergh et al. in 1981. In the literature, vitrification also is termed as: hyperhydration or hyperhydricity, translucency, hyperhydric transformation, glauciness, waterlogging, and glassiness refer to a physiological and morphological disorder in tissue culture grown plants (Ziv, 1991; Taji et al., 1996). Although Debergh et al. (1992) recommended that “vitrification” should no longer be used to indicate plant material with an abnormal morphological appearance and physiological function, and it should be substituted by the term ‘hyperhydricity’, this term is still widely cited in current journal publications. Vitrification is especially common if the plants have too much water available, this being usually in liquid medium, or if solid medium has low agar concentration. The vitrified plants appear glassy, have reduced or retarded growth, a bushy habit and thickened and

malformed stems and leaves with hypertrophy of cortical and pith parenchyma cells (Taji et al., 1996). Vitrification of plants is a serious problem since it can affect shoot multiplication and culture vigor and can impede the successful transfer of micropropagated plants to in vivo conditions thereby limiting the application of the in vitro techniques as a means for mass propagation.

Vitrification has been widely reported in plant tissue culture. High humidity in the culture vessels and excess of cytokinins in the medium were considered to be the major factors resulting in vitrification (Kataeva et al., 1991). Other factors such as sucrose concentration and culture generation also affected vitrification (Zhu et al., 2006). The interaction between gel concentrations, cytokinine levels and temperatures in vitrification of *Olearia microdisca* in vitro has been investigated by Williams and Taji (1991).

Vitrification increased with increasing cytokinin concentrations and temperatures, and decreased with gel concentrations. Because of too much water the explants became easily vitrified in liquid medium during subculture (Albers and Kunneman, 1992). The high concentration of BA in medium often resulted in vitrification. For example, Zhang et al. (2001) and He et al. (2005) found vitrification in medium with 2 mg l^{-1} BA. Cérna et al. (2001) observed the highest number of vitrified shoots (62%) in medium with the addition of 2 mg l^{-1} BA in nodal stem culture of tree peony. A similar result was seen in other species. When 3 mg l^{-1} of BA was used, as high as 95% vitrification rate occurred in the seedlings of *Prunus cerasus* (Gao et al., 2006).

2.9.2 Mechanism of vitrification

Recently, the morphological, physiological and biochemical mechanism of vitrification has been investigated by Xie et al. (2004) in Chinese onion and Park et al. (2004) in potato shoot in vitro. Ambient conditions possibly affected gene expression which led to a series of changes in vitro shoots (Yang et al., 2005). Vitrification is a complex phenomenon which depends on several factors (physical, chemical, and biochemical) acting together. If the influence of cytokinins, ammonium ions and gelling agent are well recognized to promote vitrification, attention should be paid to other factors such as relative humidity, temperature, light intensity and quality. All those parameters have an indirect effect on the physiochemical conditions of the culture environment and therefore on the nutrient transfer from culture medium into plant which affects plant growth and vitrification (Pâques, 1991).

Once the cultured peony plantlets have been vitrified, it was usually hard to recover. The highest recovery of vitrification was only obtained in the medium without PGRs or with PGRs at low concentrations (An, 2005). However, it was possible to avoid vitrification or to reverse it if the phenomenon was not too advanced. Unfortunately the majority of the proportions to obtain this result also decreased the proliferation rate (Debergh et al., 1981).

2.9.3 Factors affecting vitrification

Medium

An (2005) reported 16% of vitrified shoots when cultured in WPM with PGRs compared with 30% in MS with PGRs and 80% in WPM without PGRs. It implicated

that vitrification was related to types of basal media and addition of PGRs at low concentrations was beneficial for reducing vitrification.

Explant age

Bouza et al. (1994) demonstrated that both multiplication rate (MR) and vitrification were affected by explant age, and the youngest explants gave a low MR and were only sometimes vitreous during subculture. Shoots being used for two or three passages were the most productive and did not show vitrification, whereas the older shoots (4–5 generations) became less productive and were more frequently vitreous. The relationship between vitrification and subculture age was also mentioned by Zhu et al. (2006).

Temperature

Vitrification is related to temperature and high temperature usually causes severe vitrification. In tissue culture of peony, nearly no vitrified microplantlets were observed at 10–26ºC, but vitrification increased from 65% to 85% as temperature went up from 26–30ºC to above 30ºC (Chu and Li, 1993)

Light

There is less information about effect of light on vitrification of peony in vitro. It was only reported that vitrification occurred when cultured explants were exposed to 12 h per day of light but no vitrification was found at 8 h per day (Zhang et al., 2001).

Genotype

Vitrification is species and genotype dependent. It was observed that the percentage of hyperhydric shoots was significantly different among cultivars of peony (Beruto et al., 2004). The large differences (0–30%) in vitrification between cultivars of tree peony were reported by He (2006).

2.10 Necrosis and Disorder

Necrosis is a serious problem and has been often found in plant tissue culture (Karhu, 1997; Xing et al., 1997; Podwyszyńska and Goszyńska 1998; Thomas, 2001; Liu et al., 2005). Necrosis usually begins at the tips of leaves and shoots then spreads to other regions even causes the ultimate death of shoots. In peony tissue culture, necrosis of shoots has been mentioned by several researchers. Bouza et al. (1994b) reported the frequency of apical and leaf necroses in BAP-GA_3 containing MS medium increased dramatically (from 28% to 91% of shoots) if a low concentration Ca^{2+} (3 mM) was used. Apical necroses were considerably reduced if the culture medium contained 6 mM of Ca^{2+} instead of 3 mM. Orlikowska (1998) reported $AgNO_3$ was also helpful in recovery of explants from necrosis. Necrosis is associated with type and concentration of PGRs, for example, the lowest number of leaf necroses was found on the media containing TDZ in high concentrations or BAP in a very low concentration in culture of herbaceous peony (Gabryszewska, 1998). Genotypes showed significant difference in necrosis, in tree peony in vitro culture. The percentage of necrotic explants ranged from 0% for ‘Old Pink’, ‘Huang Yu’, and ‘Shi Yuang Bai’ to 35% for ‘Japanese Red’ (Beruto et al., 2004). Necrotic rate also showed differences between media with combination of PGRs. For

example, no necrotic explants were found in 0.5 TDZ + 2 2,4-D medium but with 27–93% rate in other combinations of medium for both filaments and petal culture of tree peony (Beruto et al., 2004).

The reason for apical necrosis remains unclear. However, several explanations have been given by previous researchers for the occurrence of shoot tip necrosis (Kulkarni and D'Souza, 2000). Exudation of phenolics has been reported to cause necrosis of shoot tips in in vitro culture of many tree species (Bellarosa, 1988; Standardi and Romani, 1990). However this hypothesis was not supported by the finding in *Butea* tissue culture (Kulkarni and D'Souza, 2000). In some cases shoot tip necrosis was considered to be a physiological disorder associated with rooting. Necrosis was to a large extent avoided but the rooting rates fell considerably when a low concentration of BA was added to the rooting medium (Vieitez et al., 1989; Kataeva et al., 1991). It indicated that the apical necrosis of plantlets was the result of a deficiency of cytokinins from the rooting medium linked with the root-inducing auxin treatment. The tissue necrosis was more often reported to be considered a Ca-related physiological disorder. Supplementation of calcium in the medium significantly reduced apical necrosis (Vieitez et al., 1989; Abousalim and Mantell, 1994; Bouza et al., 1994b; Podwyszyńska and Goszyńska, 1998; Matin et al., 2007). Shingha et al. (1990) have reported that an increase in calcium concentration in the culture medium of *Cydonia oblonga* prevented apical necrosis. It was also reported the apical necrosis was associated with endophytic bacteria. Endophytic bacteria were detected in plantlets with a leaf-tip necrosis problem in statice culture, but the plantlets freed from endophytic bacteria by subculture on antibiotic-amended medium did not develop leaf-tip necrosis (Liu et al., 2005).

Other disorders of tissue development were seldom mentioned in peony tissue culture. Gabryszewska (1998) reported the high concentrations of BAP and TDZ induced leaf deformation but kinetin and 2-iP allowed the development of normal leaves. GA_3 often causes over elongation of shoots and produces line leaves. Buchheim et al. (1994) reported that application of GA_3 resulted in a significant increase in epicotyl growth of cultured embryos. However, spindly petioles, deformed leaves, and curled elongated cotyledons also have been developed after GA_3 application, especially at 2.25 mM and 3 mM.

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Table 1. Summary on in vitro culture of peony (1967- 2007)

Explant	Species	Medium (mg l^{-1} for PGRs)	Result	Reference
Ovule wall	*P. mlokosewitschii*	IM: ½ MS + 1BA	Callus, roots, shoots	Orlikowska et al. 1998
	P. tenuifolia	MM: MS + combinations of BA , KT, ZT, TDZ/ 2iP, IAA / NAA , GA_3		
Ovule	*P. rockii, P. otsii*	½ MS (MS) + 0-2 BA + 0-1 IAA + 0-1 GA_3	Shoots, roots, plantlets	He et al. 2005
	P. rockii, P. delavayi × *suffruticosa*	½ MS + 0-2 BA + 0-1 IAA + 0-1 GA_3 (+ 100 Vc)	Shoots, roots, plantlets	He 2006; He et al. 2006
Immature embryo	*P. lactiflora*	MS + 0.3 GA_3	Shooting, roots, plants	Shin et al. 1996
	P. lactiflora	MS+ 0.25-0.5 BAP + 0-1.4 IAA or White + 0.25-0.5 BAP	Callus, roots, microplants	Stanys et al. 2007
	P. rockii, P. otsii	½ MS (MS) + 0-2 BA + 0-1 IAA + 0-1 GA_3	Shoots, roots, plantlets	He et al. 2005
Mature embryo (from seed)	*P. albiflora*	MS + 0.5 2,4-D (+ BA or +BA + NAA)	Embryos, callus, roots, plantlets	Kim & Lee 1994, 1995
		RM: MS + 0.5 BA + 0.3-1 GA_3, MS + 500-100 Glu.		
	P. anomala	IM: ½ MS + 1 NAA + 0.5 or 1 BA	Embryogenesis, callus, shoots, roots	Btukhin & Batygina 1994
	P. lactiflora	Mod. LS	Embryo growth, leaves, roots, plantlets	Meyer 1976a
		Mod. LS + 0.5 NAA	Embryos	Lin 1980
		Mod. LS	Embryos	Lin 1980, Lin et al. 1987, Thomas 1987
		Mod. LS + 0.5 NAA + 5.0 2-iP	Embriods	Thomas & Meyer 1986
		Mod. LS + 2.2 TDZ	Callus, shoot meristems	Thomas 1987
		IM: Mod. LS + 2.5 NAA + 0.4 2-iP	Callus, roots	Lin et al. 1987
		MM: Mod. LS + 0.5 NAA + 5 2-iP		
	P. lactiflora	Mod. LS + 0-2.5 BA+ 500 Casein + 0-0.7 GA_3	Roots, shoots, plantlets	Buchheim et al. 1994
	P. lactiflora	MS + 0.3 GA_3	Shooting, roots, plants	Shin et al. 1996
	P. lactiflora cvs.	½ MS + 0.5-1 BA (+ 0.2 NAA)	Radicel elongation, cotyledon opening	Jin et al. 2005
	P. rockii	IM: ½ MS + 1 BA + 1 IAA	Callus, shoots, roots, plants	Zhou & Yao 2001
		MM: ½ MS + 1 BA + 0.2 IAA		
		RM: ½ MS + 0.2 IAA		
	P. rockii	MS+ 0.1-1 BA (+ 0.1-1 NAA)	Embryo germination, roots, plantlets	Cao 2003
	P. suffruticosa	Steeves (1955) + 2,4-D + CH	Callus	Demoise 1967, Demoise & Partanen 1969
	P. suffruticosa	Mod. LS	Radicle expansion	Meyer 1976a
	P. suffruticosa	IM: MS + 0.5 BA + 1 IAA, MS + 1BA + 0.01 NAA	Embryo growth, shoots multiplication, no roots.	Huang 1987
		MM: MS + 1BA		
		RM: MS (1/2 N_6) + 1-2 IAA/ IBA		

	P. suffruticosa cvs.	IM: WPM + 2.5 BA + 0.1-0.2 2,4-D RM: ½ WPM + 2 IBA	Callus, shoots, roots, plants	An 2005
	P. suffruticosa	½ MS + 1 IAA + 1 GA_3 + 100 Vc, ½ MS + 1 IAA + 0.1 BAP + 0.5 GA_3	Embryo, germination, roots, plants	He 2006; He et al. 2006
	P. suffruticosa	IM: MS + 2.5 BA + 1 NAA MM: MS + 2BA + 0.5 NAA	Embryo growth, callus, shoot clusters	Jia et al. 2006
	P. suffruticosa	IM: MS + 0.5 BA + 2 2,4-D MM: MS + 1 BA + 0.1 NAA RM: MS + 0.5 IAA	Callus, shoots, roots, plantlets	Yang & Pei 2006
Seed	*P. lactiflora cvs.*	½ MS + 0.5 BA + 1 GA_3	Germination, shoot multiplication	Jin et al. 2005
Cotyledon	*P. albiflora*	MS + 0.2 BA + 0.2 2,4-D MS + 0.5 BA + 1.0 GA_3	Embryos, roots, plantlets	Kim & Lee 1996
	P. lactiflora	MS MS + 0.5-5 2,4-D	Embryos, roots Callus	Shin et al. 1997
	P. lactiflora	MS + 0-2 ABA, MS + 0.3 GA_3	Embryos, roots, plants	Kim et al. 2006
	P. suffruticosa cvs.	IM: WPM + 0.5 TDZ (+ 0.25 2,4-D) RM: ½ WPM + 2 IBA	Callus, shoots, roots, plants	An 2005
Somatic embryo (desiccated)	*P. lactiflora*	MS + 0.3 GA_3	Plant regeneration	Kim et al. 2006
Cotyledon + embryonic axis	*P. suffruticosa cvs.*	IM: WPM + 1 2,4-D or 1 BA or 1 GA_3 MM: WPM + 0.5-1 2,4-D + 0.5-1 BA	Callus, callus multiplication	Wang & Staden 2001
	P. suffruticosa	Mod. ½ WPM + 1 2,4-D + 6 BA	Callus	Lang & Luo 2007
Hypocotyl	*P. lactiflora cvs.*	½ MS + 0.5-1 BA (+ 0.2 NAA)	Petiole elongation, leaf opening	Jin et al. 2005
	P. suffruticosa	IM: MS + 2 BA + 0.2 NAA + 500 LH MM: MS + 2 BA + 0.5 IAA RM: ½ MS + 1 IAA + 1 IBA	Callus, shoots, roots, plantlets	Xie 1987
Microspore	*P. hybrids*	MS	Embryos, roots, plantlets	Roberts & Sunderland 1977
	P. delavayi, P. hybrids	MS	Embryos	Sunderland 1983a, b, c
	P. lactiflora	MS + 2 NAA + 10% CM	Callus	Ono & Harashima 1981
	P. lactiflora	MS	Callus, haploid suspension cell line	Ono & Takahara 1985
Anther	*P. albiflora*	IM: MS + 0.2 NAA + 0.5 BA + 0.4 TDZ (callus) IM: MS + 0.2 NAA + 0.0002 TDZ (embryo) IM: MS + 0.5 BA (0.002 TDZ) (shoot+root)	Callus, Embryo, shoot, root	Lee et al. 1992
	P. cv.	MS + 1-2 NAA + 0.1 KT + (1 2,4-D), or coconut milk	Callus	Ono & Tsukida 1978
	P. hybrids, P. decora,	MS + low auxin, kinetin + high auxin	Callus, embryos	Sunderland et al. 1973

	P. triernata			
	P. hybrida	MS + 0.1 NOA	Embryos, roots, plantlets	Sunderland 1974, Sunderland & Dunwell 1974
	P. hybrids, P. decora, P. triernata	MS	Callus, embryos, roots, plantlets	Sunderland et al. 1975
	P. lutea, P. suffruticosa	MS (LS) + IAA + 2,4-D + BAP + CH	Embryoids	Zenkteler et al., 1975
	P. lactiflora	MS + 2 PAA, MS + 0.3 GA_3	Embryos, roots, plants	Kim et al. 2006
	P. suffruticosa cvs.	MS + 0.5 BA + 3 NAA + 1 2,4-D + 0.05 TDZ	Callus	Chen 2006
Petal (from young bud)	*P. japonica*	-	Callus	Yamada & Sinotô 1966
Petal and Filament	*P. suffruticosa cvs.*	IM: MS + 6 2iP + 1 PIC; 0.5 TDZ + 2 2,4-D; 2.27 TDZ; 0.49 NOA	Callus, shoot regeneration	Beruto et al. 2004
Flower (petal, sepal, pistil, and anthers)	*P. mlokosewitschii* *P. tenuifolia*	IM: ½ MS + 1BA MM: MS + combinations of BA , KT, ZT, TDZ/ 2iP, IAA / NAA , GA_3	Callus, shoots	Orlikowska et al. 1998
Flower bud	*P. lactiflora, P. suffruticosa*	Mod. MS + 2.5 KT + 2.5/10 NAA	Callus, roots	Meyer 1976b
Dormant bud (or bud tip)	*P. anomala*	MS + 0.5/1 BA + 0.5/1 IAA + 1 GA_3	Shoot growth	Zaripova & Baiburina 2005
	P. lactiflora, P. suffruticosa	Mod. MS + 2.5 KT + 2.5/10 NAA	Callus, roots	Meyer 1976b
	P. lactiflora	Mod. MS + 0.2 2-iP + 1 NAA	Callus	Lin 1980
	P. lactiflora	MS + 2.5 NAA + 4 2-iP, or MS + 0.3 IAA+ 15-30 2-iP	Callus, shoots	Radtke 1983
	P. lactiflora	Mod. LS + 1.1 TDZ	Shoot meristems	Thomas 1987
	P. lactiflora	IM: ½ MS + 0.5 BAP + 1 GA_3 RM: ½ MS + 1 IBA (liquid)	Multiplication and expansion of axil, buds, roots, plants	Hosoki et al. 1989
	P. lactiflora	IM: ½ MS + 0.5 BAP + 1 GA RM: ½ MS + 1 IBA (liquid)	Shoots, roots, plantlets	Hansen & Zhang 1995
	P. lactiflora cvs.	IM: ½ MS + 1.0/0.1 BA + 0.1/1.0 GA_3 RM: ½ MS + 2.5 IBA	Callus, shoots, roots, plants	Habib & Donnelly 2001
	P. lactiflora cvs.	½ MS + 0.5 BA + 1 GA_3	Shoots multiplication	Jin et al, 2005
	P. lactiflora	IM: ½ MS + 0-1 BA + 0-2NAA + 0-1 MM: GA_3, ½MS + 0.5-2 BA + 0-1 NAA+ 0-1 KT RM: ½ MS + 1-5 IBA (1IBA + 0-2 IAA) + 0.3% AC	Callus, shoot, roots, plantlets	Zhang 2006
	P. lactiflora	MS + 1 BAP + 1 2iP + 1 KT + 0.01 TDZ	Shoot multiplication	Gabryszewska 2006

	P. lactiflora	IM: ½ MS + 1 BA + 1 GA_3 MM: ½ MS + 1 BA + 1 KT RM: ½ MS + 1 IBA	Callus, shoots, roots, plantlets	Zhang et al. 2007
	P. mlokosewitschii	IM: ½ MS + 1BA	Callus, shoots	Orlikowska et al. 1998
	P. tenuifolia	MM: MS + combinations of BA , KT, ZT, TDZ/ 2iP, IAA / NAA , GA_3		
	P. rockii	MS + 1.5 BA + 2 NAA	Callus	Chen et al. 2001
	P. suffruticosa	IM: ½ MS + 0.2-1 KT + 0.5-1 BAP + 0.1-0.5 GA_3 MM: Mod. MS + 0.2-0.5 IAA + 2.0 BA RM: ½ MS + 0.1-2.0 IBA	Callus, shoot meristems, roots, plants	Li et al. 1984
	P. suffruticosa	IM: MS + 2 BA + 0.2 NAA + 500 LH MM: MS + 2 BA + 0.5 IAA RM: ½ MS + 1 IAA + 1 IBA	Callus, shoots, roots, plantlets	Xie 1987
	P. suffruticosa	MS + 0.9 BA	Shoots	Bouza et al. 1993
	P. suffruticosa	IM: Mod. MS + 1 BA + (0.5 GA_3) MM: Mod. MS + 1 BA + 0.5 GA_3 RM: ½ MS + 1 IBA	Shoots, shoots, roots, plants	Kong & Zhang 1998
	P. suffruticosa	IM: MS + 0.5-1 BA + 0.1-0.2 NAA RM: MS + 0.1-0.5 NAA or 0.2-0.5 IBA + 0.2-0.5 IAA	Callus, shoots, no roots	Zhang et al. 2001
	P. suffruticosa cvs.	RM: ½ MS + 1-1.5 BA + 0.5-1 NAA MM: MS + Ca^{2+}(WPM) +2 BA + 0.3 IAA, MS + NAA + Ca^{2+} + 1 BA + 0.1 IAA RM: ½MS + Ca^{2} + 1 IBA + 1 IAA	Callus, shoots, roots, plantlets	Chen 2005
	P. suffruticosa	IM: MS + 0.5/1 BA RM: MS + 2.0 IBA	Callus, roots, plantlets	Zhang & Luo 2006
	P. suffruticosa cvs.	MS + 3 BA + 0.05 NAA + 5 2,4-D + 0.05 TDZ	Callus	Chen 2006
Sprouting bud or shoot (tip)	*P. lactiflora*	MS + BAP + 2iP + KT + TDZ	Shoots	Gabryszewska 1998
	P. suffruticosa	IM: Mod. MS + 0.9 BA MM: Mod. MS + 0.9 BA + 0.7 GA_3 RM: Mod. MS + 5-15 IBA (before autoclaving)	Shoots, roots, plantlets	Bouza et al. 1994a
	P. suffruticosa	IM: Mod. MS + 0.9 BA (+ 0.5-1 GA_3) + 6 mM Ca^{2+}	Shoots	Bouza et al. 1994b
	P. suffruticosa	IM: MS + 0.5-1 BA + 0.1-0.2 NAA RM: MS + 0.1-0.5 NAA or 0.2-0.5 IBA + 0.2-0.5 IAA	Callus, shoots, no roots	Zhang et al. 2001
	P. suffruticosa cvs.	IM: WPM + 1 BA	Shoot clusters, shoots, roots, plants	Beruto et al. 2004

		RM: WPM + 0-10 IBA		
	P. suffruticosa	IM: MS+ 2 BA + 0.2 NAA + 200 LH MM: MS + 1 BA + 0.5 KT + 0.1 NAA + 200 LH RM: ½ MS + 0.1 NAA+ 200 LH	Shoots, roots, plants	Li et al. 2006
Desiccated dormant shoot tips	*P. lactiflora*	MS + 0.5 BA + 1 GA_3, ¼MS +0.1 NAA	Re-growth, roots, plantlets	Seo et al. 2007
Leaf	*P. lactiflora*	MS + 0.5 2,4-D + 0.5 BA	Callus	Zhang et al. 2007
	P. suffruticosa cvs.	MS + 4 2,4-D + 0.2 KT + 0.4 NAA + 200 Casein	Callus	Li et al. 2005
Leaf and petiole	*P. lactiflora*	IM: Mod. MS + 0.5 BA + 0.5 NAA	Callus	Zhang 2006
	P. mlokosewitschii	IM: ½ MS + 1BA	Callus, shoots	Orlikowska et al. 1998
	P. tenuifolia	MM: MS + combinations of BA , KT, ZT, TDZ/ 2iP, IAA / NAA , GA_3		
	P. suffruticosa	IM: Mod. MS + 2 BA + 0.1-0..5 NAA+ 500 LH MM: Mod. MS + 0.2-0.5 IAA + 2.0 BA RM: ½ MS + 0.1-2.0 IBA	Callus, shoot meristems, roots, plants	Li et al. 1984
	P. suffruticosa	WPM + 1 2,4-D + 1 BA + 1 GA_3	Callus	Wang & Staden 2001
	P. suffruticosa cvs.	RM: ½ MS + 0.5 2,4-D + 1 NAA + 0.2 IAA MM: ½ MS + 2 BA + 0.3 NAA + 0.5 IAA	Callus	Chen 2005
	P. suffruticosa cvs.	IM: WPM + 0.5-1 TDZ + 0.5-1 2,4-D MM: WPM + 1 TDZ + 0.5 2,4-D	Callus	An 2005
	P. suffruticosa	Mod. ½ WPM + 1 2,4-D + 6 BA	Callus	Lang & Luo 2007
Petiole	*P. lactiflora*	Mod. MS + 5 2-iP + 2.5 NAA	Callus	Radtke 1983
Young internodal stem	*P. lactiflora*	Mod. MS + 0.5 BA + 0.5 NAA	Callus	Zhang 2006
	P. lactiflora	Mod. MS + 5 2-iP + 2.5 NAA	Callus	Radtke 1983
	P. mlokosewitschii	IM: ½ MS + 1BA	Callus, shoots	Orlikowska et al. 1998
	P. tenuifolia	MM: MS + combinations of BA , KT, ZT, TDZ/ 2iP, IAA / NAA , GA_3		
	P. suffruticosa	IM: SH-M (LB) + 0.2/2 2,4-D or 10 NAA MM: ½ SH (SH, 2SH, SH-M) + 0.2 2,4-D+ 0.1 KT RM: Mod. ½ SH (SH, 2SH, SH-M)	Callus, roots	Gildow & Mitchell 1977
	P. saffruticosa	Mod. MS + 1 BAP or 0.2 NAA	Shoots	Černá et al 2001
Young nodal stem	*P. lactiflora*	IM/MM: LP + 1 BAP + 0.1 GA_3 RM: LP + 0.1-2 IAA (IBA, NAA)	Shoots, roots, plants	Albers & Kunneman 1992
	P. lactiflora	IM: MS + 3.5 BA or 5 2,4-D; N6 + 3.5 2,4-D	Callus, shoots	Hu et al. 2003
	P. suffruticosa	IM: MS + 1 BAP + 1 2iP	Shoots, roots, plants	Harris & Mantell 1991

		RM: MS + 1 IBA		
Root	*P. lactiflora,*	Mod. MS + 2.5 KT + 2.5/10 NAA	Callus	Meyer 1976b
	P. suffruticosa			
	P. lactiflora	Mod. LS + 0.5-2 2,4-D	Callus	Lin 1980
Root phloem	*P. ostii*	IM: MS + 1 2,4-D or 1.5 NAA	Callus	Shi & Zhang 2005
		MM: MS + 1 2,4-D + 5×10^{-6} TDZ or 1.5 NAA + 0.005 TDZ		
Vitro-shoot	*P. hybrida (herbaceous)*	MS + 1 BAP + 1 KT + 0.01 TDZ	Shoot growth and multiplication	Gabryszewska 1998
		MS + 2 BAP + 2 KT + 0.02 TDZ		
		MS + 4 BAP + 4 KT + 0.04 TDZ		
	P. lactiflora	MS + 1 BAP + 1 2iP + 1 KT + 0.01 TDZ	Shoot growth and multiplication	Gabryszewska 2001
	P. rockii	MS + 1.5 BA + 2 NAA	Callus	Chen et al. 2001
	P. suffruticosa	MS + 0-1 BAP + 0/1 NAA	Callus, shoots, vitrification	Chu & Li 1993

Media: B5 - Gamborg B5 (Gamborg et al., 1968); IM –shoot/callus initiation medium; LP – Lepoivre medium (Quoirin and Lepoivre, 1977); LS – Linsmaier and Skoog medium (Linsmaier and Skoog, 1965); MM – shoot/callus multiplication medium; Mod – modified medium; MS – Murashige and Skoog medium (1962); NT – Nitsch medium (Nitsch and Nitsch, 1969); N6 – N6 medium (Chu et al., 1975); RM – root induction medium; SH – Schenk and Hildebrandt medium (1972); Steeves – Steeves medium (Steeves et al., 1955); White – White's medium (1963); WPM – Woody plant medium Lloyd and McCown, 1980); ½ MS, ½ WPM – half strength MS and WPM. PGRs and other chemicals: AC – activated charcoal; BA – benzyladenine; CH – casein hydrolysate; CM – coconut milk; GA_3 – gibberellic acid; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; KT – kinetin; LH – Lactalbumin Hydrolysate hydrolysate; NAA – 1-naphthaleneacetic acid; NOA – 2-naphthoxyacetic acid; PIC – picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid); TDZ – thidiazuron; Vc – vitamin C; ZT – Zeatin; 2hZ – dihydrozeatin; 2-iP – 2-isopentenyl adenine; 2,4-D – 2,4-dichlorophenoxyacetic acid.

Table 2. Summary on sterilization methods of peony in vitro culture

Reference	Plant Tissue	Method	Contamination	Death rate
Zenkteler et al. 1975	H: flower bud	30 sec 70% ethanol then 6-12 min chlorine water	-	-
Meyer 1976a	H: seed (embryo)	Water rinse and 15-20 min 10% Chlorox, remove seed coat, soak embryo 20 min in 10% Chlorox	-	-
Meyer 1976b	H: roots, underground buds, flower	20 min 10% Chlorox	High for bud and roots	-
Gildow & Mitchell 1977	H: etiolated shoots	10 min 1% aqueous sodium hypochlorite, three times water rinse	-	-
Radtke 1983	H: dormant bud	20 min 0.5% sodium hypochlorite with a few drops of surfactant, 3 times rinse in sterile water	Very high	
	flower, stem		-	-
	axillary buds from stem		-	-
Li et al. 1984	T: bud shoot tip, leaf, petiole	75% Ethanol 5-10 sec, 5% Antiformin (NaOCl) 7-10 min, 3-4 time water	-	-
Huang 1987	T: seed	Saturated bleach solution 10 min, 0.1% $HgCl_2$ 5 min, water rinse	-	-
Lin et al. 1987	H: seed	0.5% NaOCl (10% Clorox) and 0.1% Tween 20 for 20 min, air-dried 16C (80F) 24 hr; then Half embryo-0.5% NaOCl (10% Clorox) and 0.1% Tween 20 for 15 min water rinse	-	-
Lee et al. 1988	H: flower bud (anther)	95% alcohol 4-5 sec; 7% calcium hypochlorite 15 min, water 3-4 times	-	-
Hosoki et al. 1989	H: axillary bud	Diluted sodium hypochlorite (active chlorine 0.7%), 2 times water rinse	-	-
Harris & Mantell 1991	T: nodal stems	30 sec in 80% ethanol → tap water rinse 30 sec → 10% Jeyes 'Brobat' bleach (equal to 0.45% w/v sodium hypochlorite) with 0.3 ml Tween 20 – 10% bleach → 4 rinses in water	-	-
Albers & Kunneman 1992	H: plant and axillary bud	Rhizomes immerged in fungicide (2-5 g/l benomyl, 30 min) – 20C in dark- twice a week of spray with fungicide (50 mg/l rifampicine, trimethoprim and chlorotetracycline) – nodal section: 5-10 min tap water rinse, soap solution (20-40 ml/l Burtan) 1 min, 96% ethanol 1 min, tap water 1 min, 5% NaOCl with a few drops of Tween 20 for 10 min, 3 times water rinse. rifampicine 20 mg/l and 20 mg/l trimetroprim added in medium	Negligible to 22%	-

Bouza et al. 1994	T: axillary bud	6% Mercryl laurylé (Menarini-Rungis, France) 10 min-NaOCl solution 20min	-	-
Brukhin & Batugina 1994	H: fruits (immature seeds)	Dipping in alcohol + light fire	-	-
Buchheim et al. 1994	H: seeds	20min in 0.53% NaOCl + 0.1% Tween 20, 3 times water rinse	-	
Hansen and Zhang 1995	H: axillary bud	10% commercial bleach (5% NaOCl) 15 min, 4 times water rinse	33.3%	-
Kim & Lee 1995	H: seeds (embryo)	95% ethanol seconds, 7% calcium hypochlorite (w/v) 10 min, 3-4 times water rinse	-	-
Kim & Lee 1996	H: seeds (embryo	95% ethanol 4-5 sec, 7% calcium hypochlorite (w/v) 10 min, 5 times water rinse	-	-
Shin et al. 1996	H: seeds (embryo)	70% ethanol 30 sec, 1% NaOCl 10 min, 4-5 times water rinse	-	-
Gabryszewska 1998	H: vegetative buds	3% Cloramin T 20 min, 3 times sterile water rinse.	-	-
Kong & Zhang 1998	T: dormant bud	Tap water rinse, 70% ethanol 20 sec, 5% Antiformin (NaOCl) 20 min, 0.1% $HgCl_2$	12-17%	20-36%
	T: young stem	(1% Tween 20) 10 min, 6 times sterile water rinse.	28.3%	82%
Orlikowska et al. 1998	H: vegetative and flower buds, internodes, leaves	2 h tap water rinse, shaken 30 min kitchen detergent, water rinse, 10 min 0.1% $HgCl_2$, 3 times sterile water rinse	10% (1st year) High (2nd year)	-
Habib et al. 2000	H: dormant bud	20 min 5% potassium iodide, 10 min 10% bleach, water rinse.	low	-
Cerna et al. 2001	T: internodal stem	30 min autoclaving (PS 20-A) 120C	-	-
Habib & Donnelly 2001	H: dormant buds	5 min running tap water, 15 or 20min 10% commercial bleach (6% NaOCl), sterile water rinse.	> 40%	-
		10 or 20 min KI (5% active I), 10min 10% bleach (6% NaOCl), sterile water rinse.	10-15%	-
Wang & Staden 2001	T: buds, leaves, roots	(1) 20min running tap water, 7-10 sec 70% ethanol, 15, 20 and 25 min in 0.5, 1.0, 3.5% active NaOCl commercial bleach, 3 times sterile water rinse.	Bud: 86% Leaf: 76-96%	-
		(2) 20 min running tap water, 7-10 sec 70% ethanol, then 3, 5, 7 min 0.1% (w/v) $HgCl_2$, 3 times sterile water rinse.	Bud: 62% Leaf: 72-94%	
		(3) 5, 7, 9 min mixture of 20% ethanol and 0.075% $HgCl_2$, 3 times sterile water rinse. Tween 20 (1 drop/50ml) added for each strilant.	Bud: 40% Leaf: 52-96% Roots: ≥67%	
	Seeds (embryos)	(1) 2 min 70% ethanol then 30 min 3.5% NaOCl (active) or 10 min 0.1% (w/v) $HgCl_2$, (2) seed coat removed, 7 min mixture of 20% ethanol and 0.075% $HgCl_2$. (3) combination of (1) and (3)	100% (1) 14% (2) 14% (3)	-

Zhang et al. 2001	T: axillary and maid bud	Tap water rinse, 20 min detergent, water rinse 10 min, 75% ethanol 30 s, 0.1% $HgCl_2$ 8-10 min, 3-4 times water rinse.	-	-
Zhou & Yao 2001	T: seeds (embryo)	70% ethanol 1min, 2 times sterile water rinse, 0.1% $HgCl_2$ 8 min, 5 times sterile water rinse.	-	-
Wang & Staden 2002	T: seed	7 min mixture of 20% ethanol and 0.075% $HgCl_2$ (1 drop/50 ml Tween 20), sterile water rinse	-	-
Chen et al. 2003	T: dormant bud	Different concentration of $HgCl_2$	-	-
Cao 2003	T: embryo	75% ethanol then 0.1% $HgCl_2$, 4 times sterile water rinse	-	-
Hu et al. 2003	H: young stem	30 sec 70% ethanol then 8-12 min 0.1% $HgCl_2$, 4-5 times sterile water rinse	2-8%	-
Wu 2003	T: main bud, nodal stem	2 h running water rinse, 20 sec 75% ethanol then 8 min 0.1% $HgCl_2$, 4 times sterile water rinse	10% (bud) 15-20% (stem)	-
Beruto et al. 2004	T: axillary and flower buds	3 min 0.5% (w/v) $HgCl_2$, 15 min NaOCl (1% Cl), sterile water rinse (or additional 30 min 50% (v/v) dip in PPM	60% (-PPM) 30% (+PPM)	-
Chen 2005, Chen 2006	T: dormant buds	Running water rinse, 20 min water with detergent and 84 sterilant, 10 min running water rinse, 30 sec 75% ethanol, 5, 7, 9 min 0.1% $HgCl_2$, sterile water rinse	20% (5 min) 10% (7 min) 10% (9 min)	0% 10% 30%
	T: young petiole	Running water rinse, 20 min water with detergent and 84 sterilant, 10 min running water rinse, 3, 5, 7, 10 min in 0.1% $HgCl_2$, sterile water rinse	50% (3 min) 41.6% (5 min) 33.3% (7 min) 33.3% (10 min)	Normal growth Normal growth End browning End browning
He et al. 2005	T: young petioles	20 min water soaking, 1 h running water rinse, 30 sec 75% ethanol, 5 min 0.1% $HgCl_2$, 4-5 times sterile water rinse	-	-
Kim et al. 2004	H: seed (embryo)	1 min70% ethanol, 15 min 1% sodium hypochlorite, 3 times sterile water rinse	-	-
An 2005	T: young leaf, stem, petiole, cotyledon	5 sec 75% ethanol, 10 min of 0.1-0.2 % $HgCl_2$ or 10-20% NaOCl, 5 times sterile water rinse	-	-
Jin et al. 2005	H: seeds	Running water, 10 min 0.5% $KMnO_4$, 30 sec 70% ethanol, 10 min 0.1% HgCl, 4-5 times sterile water rinse	16-52% 95% (+coat), 20% (-coat)	-
	H: dormant bud	30 min running water, 15 sec 70% ethanol, 15 min 0.1% NaOCl, 3-5 times sterile water rinse	30-57% (field) 27-100% (4C)	-

Li et al. 2005	T: young leaf	6-7 h running water, 4 min 0.1% $HgCl_2$, 5-6 times sterile water rinse	0-33.3%	-
Shi & Zhang 2005	T: roots	Running water rinse →30 sec 75% ethanol,→12 min 0.1% $HgCl_2$ →3-4 times sterile water rinse	-	-
He 2006	T: ovule	24 h running water rinse, 30-60 sec 70% (v/v) ethanol, 6 min 0.1% $HgCl_2$, 3 times of 3 min sterile water rinse	0	-
		24 h running water rinse, 30-60 sec 70% (v/v) ethanol, 3 min 2% NaOCl, 3 times of 3 min sterile water rinse	Low	-
	T: pistil	24 h running water rinse, 30-60 sec 70% (v/v) ethanol, 12 min 2% NaOCl, 3 times of 3 min sterile water rinse	High	-
		24 h running water rinse, 30-60 sec 70% (v/v) ethanol, 4,10 min 0.1% $HgCl_2$, 3 times of 3 min sterile water rinse	High	-
Jia et al. 2006	T: seed (embryo)	Tap water rinse, 30 sec 75% ethanol, 8-10 min 0.1% $HgCl_2$, 3-4 times sterile water rinse	9-28%	-
Kim et al. 2006	H: flower bud, seed (embryo)	1 min 70% ethanol, 15 min 1% (v/v) sodium hypochlorite, 4 times sterile water rinse	-	-
Li et al. 2006	T: shoot tip	10 min detergent water, running water rinse, removing scale, 30 sec 70% ethanol, 1 min sterile water rinse, 15 min NaOCl (50 g/L), 3 times sterile water rinse	-	-
Yang & Pei 2006	T: seed (embryo)	30 min 0.5% $KMnO_4$, sterile water rinse, remove seed coat, 75% ethanol, 0.1% $HgCl_2$, 4 rinses in sterile water	-	-
Zhang 2006	T: axillary but tip, petiole	20-30 min running water rinse, 20 min water with detergent, 30 min running water rinse, 30 sec 75% ethanol, 8, 10, 12, 14 min 0.1% $HgCl_2$, 6-7 times sterile water rinse	55.56% (8 min) 39.28% (10 min) 34.48% (12 min) 28% (14 min)	27.27% (8 min) 37.50% (10 min) 43.75% (12 min) 50% (14 min)
	T: petiole	20-30 min running water rinse, 20 min water with detergent, 30 min running water rinse, 30 sec 75% ethanol, 8, 10, 12, 14 min 0.1% $HgCl_2$, 6-7 times sterile water rinse	46.34% (8 min) 35.9% (10 min) 25.64% (12 min) 27.27% (14 min)	31.71% (8 min) 51.28% (10 min) 56.41% (12 min) 65.91% (14 min)
	T: leaf	20-30 min running water rinse, 20 min water with detergent, 30 min running water rinse, 30 sec 75% ethanol, 2, 4, 6 min 0.1% $HgCl_2$, 6-7 times sterile water rinse	39.47% (2 min) 27.78% (4 min) 34.15% (6 min)	26.32% (2 min) 27.78% (4 min) 36.59% (6 min)

Zhang et al. 2006	H: dormant bud	20 min water with detergent, 30 min running water, 30 sec 75% ethanol, 8,10, 12, 14 min 0.1% $HgCl_2$, 6-7 times rinse in sterile water	55.56% (8 min) 39.28% (10 min) 34.48% (12 min) 28% (14 min)	3.7% (8 min) 3.6% (10 min) 10.3% (12 min) 16% (14 min)
Zhang & Luo 2006	T: axillary buds	1 h tap water rinse, 30 sec 70% ethanol, 14 min 5% NaOCl, 4 times sterile water rinse	-	-
Lang & Luo 2007	T: seed (embryo)	30 sec 70% ethanol, 10 min 1% NaOCl	-	-
Stanys et al. 2007	H: seed (embryo)	2 min 96% ethanol, 10 min 0.1% calomel, 2 times sterile water rinse	-	-
Pizarro-Verme 2007	H: dormant bud	10 or 15 min 3% (5%) NaOCl with Tween 20, water rinses	53-62%	-
Zhang et al. 2007	H: dormant bud (tip), leaf	20 min water with detergent, 30 min running water, 30 sec 75% ethanol, 4 min (for leaf) 10 min (for bud) 0.1% $HgCl_2$, 6-7 times rinse in sterile water	-	-

Note: H – herbaceous peony; T – tree peony; '-' – data are unavailable.

Chapter 2 Comparison of Direct Shoot Induction Ability of Different Explants in Herbaceous Peony (*Paeonia lactiflora* Pallas.)

Abstract

Shoot induction ability of explants of herbaceous peony was investigated in solid MS medium with combinations of BA, TDZ and GA_3. Callus was readily induced from stem and petiole explants within two days of culture but failed to generate shoots. Adventitious shoots were successfully produced from meristematic regions only: bud eyes on nodal stem sections, and junctions of petioles and petiolules. No shoots were induced from internode sections, petiole without junctions, or leaf sections. Nodal sections were the most efficient explants. There were up to 20 shoots in one explant generated within 20 days of culture. TDZ was more effective than BA to induce shoots. The 100% shoot induction rate was obtained in medium with 0.1–3 mg l^{-1} of TDZ. However, higher concentrations of TDZ inhibited shoot stem elongation and only large leaf clusters were produced. BA-containing medium supplemented with TDZ did not increase shoot induction rates but made shoots shorter and stronger. The very short pretreatment of explants with 20 mg l^{-1} TDZ solution was very effective to induce adventitious shoots directly, but both shoot number and shoot length decreased as treatment time increased. GA_3 was beneficial for shoot and stem elongation.

Key words Nodal stem · Meristematic region · Shoot elongation · Benzylaminopurine · Gibberellic acid · Thidiazuron

Introduction

Peonies, of the genus *Paeonia*, are long living perennials belonging to the family Paeoniaceae, and are among the most dramatic and beautiful flowers for gardens. Peonies are also used as pot flowers, cut flowers and dry flowers. Herbaceous peony (*Paeonia lactiflora* Pallas.) has been cultivated in China for more than 3900 years (Wang and Zhang, 2005). Today, peonies are enjoying a renaissance as popular cut flowers and garden plants and they are back in fashion because of the ease of cultivation in a wide range of climates and the rich diversity of flowers. With the complex sequential dormancy of peony seeds, it usually takes two years for germination under natural conditions (Krekler, 1962; Griess and Meyer, 1976). Peony seedlings must grow for several years before blooming. Peonies can be propagated by division, cutting, grafting, and layering to obtain true-to-type plants. Herbaceous peonies are usually propagated by 3-5 eye divisions of underground stock plants. A grower may be able to double their stock plants every three years by planting in a three-year rotation (Shannon and Kamp, 1959). Nodal stems and underground rhizomes can be used for cuttings (Arino et al., 1981; Antanaitiene and Staniene, 2001; Wang and Zhang, 2005). Layering is seldom used in herbaceous peony but the efficiency has been improved by a new approach of vertical layering with application of a rooting chemical like Ukorzeniacz B2, a mixture of PGRs (Czekalski and Jerzy, 2003). There are multiple traditional choices for propagation of peony. However, the limited number of plants produced by these traditional methods

can not meet the increasing demands in the market, especially for a quick release of a new cultivar and massive production of a favorite variety. The development of micropropagation methods for peonies is necessary to not only overcome this problem but also accelerate peony breeding progress.

Microprogation of peony began in the middle 1960s. Callus was induced successfully for the first time by Yamada and Sinotô (1966) from petal culture of *P. japonica*. Demoise (1967) and Demoise and Partanen (1969) induced callus from tree peony. During the last 40 years, much research on micropropagation of peony has been conducted with plants successfully produced from tissue culture labs. Many advances have been made and several paper reviews on tissue culture of peony have been published (Buchheim and Meyer, 1992; Gabryszewska, 2004; Li and Luo, 2004; Li et al., 2006; Jia et al., 2006; Meng et al., 2007; Beruto and Curir, 2007; Zhao et al., 2007). Only since 2006 has one company, Planteck Biotechnologies Inc. (based in Quebec, Canada) mass-produced herbaceous peonies and made tissue cultured plants available to the market (Whysall, 2006). More research is needed to make mass production of peony more commercially successful. Optimization of procedures are necessary, from selection of explants, decontamination, screening of medium, application of plant growth regulators (PGRs), induction of callus and shoots, subculture, rooting, and to final transplanting. The major goal of this study is to evaluate shoot induction ability of peony explants and PGRs.

Materials and Methods

Materials: Three cultivars of herbaceous peony were used in this study: 'Xi Shi Fen' ('XSF'), 'Yang Fei Chu Yu' ('YFCY') and 'Fen Ling Hong Zhu' ('FLHZ'). Containerized plants of 'XSF' and 'YFCY' were moved from outside to a 4ºC cooler in Nov., 2005. One week before experiment, 'XSF' was moved from the cooler to the greenhouse for several days of forcing growth and then moved back to the same cooler to avoid over growth when stems elongated but young leaves were not open. 'YFCY' remained in the cooler and had elongated shoots when explants were taken. Some plants in this cultivar were also treated in the greenhouse for forcing growth. 'FLHZ' was introduced from China and potted in Nov. 2005. This cultivar were placed outside until initial shoots emerged. Five types of explants were evaluated including stem sections (5–8 mm long) with or without nodes, petioles (5 mm long) without junction, fork petioles (5–8 mm long) consisting of top main-petiole section and basal sections of three petiolules (Fig. 2), and sections (16–25 mm^2) of young leaves (unexpanded or just expanded).

To the basal medium, half or full strength MS medium (Murashige and Skoog, 1962) with vitamins; 30 g l^{-1} of sugar (D-sucrose, ultra pure, S829), 12 g l^{-1} of agar (A111), 3 mM of calcium chloride dihydrate ($CaCl_2$) and different combinations of PGRs including benzylaminopurine (BA), gibberellic acid (GA_3), and thidiazuron (TDZ) (PhytoTechnology Laboratories, Inc., Shawnee Mission, KS, USA. www.phytotechlab.com), were added. GA_3 was dissolved in 70% ethanol and added to medium after autoclaving.

Treatments: Three experiments were conducted simultaneously beginning in January, 2006. In experiment I, the response of 5 explant types of 'XSF' (Table 1) to different PGRs were evaluated: medium (1) ½ MS + 1 mg l^{-1} BA + 0.1 mg l^{-1} GA_3; (2) ½ MS + 0.1 mg l^{-1} BA + 1 mg l^{-1} GA_3; (3) ½ MS + 1 mg l^{-1} BA + 3 mg l^{-1} TDZ; and (4) ½ MS + 1 mg l^{-1} BA + 3 mg l^{-1} TDZ + 1 mg l^{-1} GA_3. In the second experiment, four types of explants from 'YFCY' and 'FLHZ' (Table 2) were used to evaluate their shoot induction ability in ½ MS medium with the following concentrations of TDZ: (A) control; (B) 0.1 mg l^{-1}; (C) 0.5 mg l^{-1}; (D) 1 mg l^{-1}; and (E) 3 mg l^{-1}. After 12 d of culture, explants were transferred to fresh medium with ½ MS + 1 mg l^{-1} GA_3. In the third experiment, 'XSF' explants was pretreated with high concentration TDZ at 20 mg l^{-1} (90 μM) for 2 min (I), 15 min (II), or 60 min (III) at 25ºC. Explants were then inoculated onto full strength PGR-free MS medium. After 15 d of culture, explants were transferred to the fresh medium with ½ MS + 0.1 mg l^{-1} BA + 1 mg l^{-1} GA_3 for shoot elongation or medium with ½ MS + 1 mg l^{-1} GA_3.

Surface sterilization, inoculation and culture: Young elongated shoots were washed in tap water three times and cut into 2–4 cm long sections. Sections were soaked in 70% ethanol for 8–10 sec followed by 15 min of sterilization with 10% commercial bleach, to which Tween 20 (1 drop per 100 ml) was added. Plant material was rinsed three times for 5 min in autoclaved distilled water. After sterilization, explants were cut into shorter sections (3-8 mm); inoculated on Petri dishes, and moved to 25 × 95 mm tubes or small plastic culture jars after initial culture. Culture vessels were maintained under cool

fluorescence light, 60 lux, with a 16 h light cycle at 25 ± 1ºC.Data were collected after 3, 7, 15, and 30 d of culture, respectively.

Results

Contamination of explants was problematic only on YFCY' stem sections originating form the cooler, not from forcing-growth treated plants. Less than 10% of explants were contaminated on 'XSF' and 'FLHZ'. With a treatment of 8–10 sec in 70% ethanol before sterilization with 10% bleach, the contamination of explants was largely decreased compared to the one-step sterilization method (using 10% bleach only) in previous experiments (data not shown).

Stems and some petioles generated callus within one or two days of culture but little callus was produced on leaf blades even following two to three months of culture (Table 1, 2, 3). Explants inoculated on medium with PGRs produced more callus than those on medium without PGRs. Also, callus was pale white and wet in medium with BA as only PGR, it grew fast in the beginning but stopped growth and turned brown after 15 days of culture even when transferred to fresh medium. Attempts to induce adventitious shoots from this type of callus were unsuccessful because of browning problems. On media with TDZ, callus was green and rigid. It grew slow and had less browning problems. Whether transferred to fresh medium or not, this type of callus continued to grow. However, callus failed to differentiate into adventitious shoots.

Shoot primordia usually developed on nodal stems within three days of culture and were followed by shoot generation in all PGR containing media. Shoots elongated to 2 cm length within two weeks of culture. New shoots or shoot clusters were only induced

from meristematic regions: eye of lateral buds (Fig. 1), junction of rachis and petioles (Fig. 2), and junction of petiolule and leaflets (Fig. 3). Adventitious shoots were not induced on young stems without nodes, petioles without junctions, and sections of leaf blades. Young nodal stems were the most efficient explants for shoot induction with nearly 100% shoot induction rate obtained (Table 1, 2, 3). Each node can produce several to over 20 shoots in 20 d. However, most shoot primordia on fork petioles did not develop into normal shoots, and at most only 3 elongated shoots on one explant formed following 30 d of culture. Callus from explants failed to form shoots.

Addition of BA to medium resulted in shoot induction of peony. A higher concentration (1 mg l^{-1}) of BA was more effective than a lower concentration (0.1 mg l^{-1}) (Table 1). Application of TDZ to BA-containing medium did not significantly increase shooting rate but made shoots visually shorter and stronger. GA_3 had a significant effect on shoot elongation, but shoots induced on medium with high GA_3 concentrations were thin and too weak for root induction. TDZ showed very strong shoot induction ability and 100% shooting rate was obtained on the nodal stems of 'YFCY' with treatments of TDZ ranging from 0.1 to 3 mg l^{-1} (Table 2). Shooting rates of 'FLHZ' were low. It was not clear if this large difference was caused by genotype or by pretreatment of stock plants. Shooting rate of 'FLHZ' also decreased at TDZ concentrations larger than 0.5 mg l^{-1}. Pretreatment with high concentrations of TDZ inhibited stem elongation of induced shoots significantly and resulted in larger leaf clusters (Fig. 4) after transfer to shoot elongation medium (½ MS + 0.1 mg l^{-1} BA + 1 mg l^{-1} GA_3).

TDZ shock treatments of 'XSF' resulted in similar shoot induction rates between the time lengths of treatments (Table 3). Treatments with 2, 15, and 60 min of TDZ soak

resulted in 100% shooting rates for explants of both nodal stems and fork petioles. However, no shoot initiation was observed on petioles without junctions and on stem sections without nodes. Although fork petioles also showed higher shoot-induction ability in shock treatment with high concentrations of TDZ, the induced shoot primordia were difficult to form normal shoots, and only three shoots at most could be produced on a segment. No difference in shoot number and shoot length was observed between 2 and 15 min of shock treatments. Longer shock treatment (60 min) of 20 mg l^{-1} TDZ did inhibit elongation of adventitious shoots (Table 3). However, other experiments showed that normal elongated shoots could be obtained from nodal stem sections of 'XSF' even if explants were treated with 20 mg l^{-1} TDZ at 8ºC for 10 h (Fig 5). This difference was possibly caused by either the difference in treatment temperature or the different response of meristematic tissue to TDZ in different growing seasons.

Discussion

Many kinds of explants from herbaceous peony are known to generate callus, embryos and adventitious shoots in vitro. Mature and immature embryo tissues are good sources to induce embryos and shoots (Meyer, 1976a; Buchheim et al., 1994; Kim and Lee, 1994, 1995; Shin et al., 1996; Kim et al., 2006; Stanys et al., 2007) but can only be supplied by seeded varieties. The portions of flowers are more easily sterilized. However, the efficiency of organogenesis is very low, and also the flower has a short period of availability (Meyer, 1976b). Vegetative portions are the major source of explants used in tissue culture of peony and high frequency of shoot induction has been obtained from these explants. The highest frequency of both callus and shoot induction are found in

vegetative buds and bud or shoot tips (Radtke, 1983; Hosoki et al., 1989; Hansen and Zhang, 1995; Gabryzewska, 1998, 2001; Habib and Donnelly, 2001; Jin et al., 2005; Zhang et al., 2007), and nodal stem sections (Albers and Kunneman, 1992; Hu et al., 2003). Segments of leaves and petioles have been investigated but only callus was induced in most cases (Radtke, 1983; Li et al., 1984; Orlikowska et al., 1998; Li et al., 2005; Chen, 2005; An, 2005; Zhang, 2006; Lang and Luo, 2007; Zhang et al., 2007). Direct shoot regeneration was only observed on veins of leaf segments within 2 months at rates not exceeding 10% (Orklikowska et al., 1998).

Previous studies (data not shown) as well as present study indicated that adventitious shoots were not induced directly from non-nodal stem sections, petiole without section, leaf sections without main vein. Whereas up to 100% shoot induction rate was obtained from bud eyes of nodal stem sections and from the junctions of fork petioles, but two of more than 300 leaf sections generated shoots. Therefore, meristematic regions play a critical role in direct shoot induction of herbaceous peony. Although high frequency of shoot initiation was also induced on fork petioles by the addition of TDZ in medium, a very limited number of normal shoots formed. Thus, axillary buds and bud or shoot tips, and stem nodes are among the best vegetative explants to directly induce shoots in tissue culture of herbaceous peony. TDZ has shown a much stronger ability on shoot induction in other plants (Trigiano and Gray, 2000). But, it has only been reported in a few cases of peony tissue culture (Gabryszewska, 1998, 2001, 2006; Orlikowska et al., 1998; Beruto et al., 2004). Axillary shoot formation was significantly enhanced by the addition of TDZ at a very low concentration to medium containing a mixture of other PGRs. TDZ influenced the existing meristems specifically and subsequently *de novo* organogenesis

was triggered in the neighboring cells (Metha et al., 2005). The combination of TDZ with other cytokinins in medium can be more effective as compared to TDZ used alone for multiplication (Gabryszewska, 1998). The high concentrations of TDZ led to inhibition of stem elongation. The inhibitive effect of TDZ on shoot or stem elongation has also been reported in other tissue cultured plants (Murthy et al., 1998; Ledbetter and Preece, 2004; Lee, 2005). TDZ shock method was very effective in direct induction of adventitious shoots in tissue culture of herbaceous peony. However, the optimal concentration of TDZ and treatment time need further study. GA_3 was necessary for elongation of shoot stems (Figueiredo et al., 2001) and has been often used in peony tissue culture. The present study also showed that GA_3 was beneficial for shoot elongation.

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Tables

Table 1. Callus and shoot induction of 'Xi Shi Fen' on MS medium containing BA, TDZ and GA_3.

Explant type and number/treatment		Treatment	Calli induced 3 d	Number of explants induced shoots 7 d	15 d	30 d	Shoot induction rate (%) 30 d
Leaf	27	(1) to (4)	-	0	0	0	0
Petiole	15	(1) to (4)	-	0	0	0	0
Fork petiole	10	(1)	+	0	0	5	50
	10	(2)	+	0	0	6	60
	10	(3)	+	0	0	5	50
	8	(4)	+	0	0	NA	NA
Stem (- node)	10	(1) to (4)	+	0	0	0	0
Stem (+ node)	10	(1)	+	3	7	10	100
	10	(2)	+	5	8	8	80
	10	(3)	+	6	10	10	100
	15	(4)	+	8	8	14	93

Note: '- node' – without node; '+ node' – with node; 'NA' – The data are unavailable for evaluation because of heavy contamination of explants. Treatments: (1) ½ MS + 1 mg l^{-1} BA + 0.1 mg l^{-1} GA_3; (2) ½ MS + 0.1 mg l^{-1} BA + 1 mg l^{-1} GA_3; (3) ½ MS + 1 BA + 3 mg l^{-1} TDZ; and (4) ½ MS + 1 mg l^{-1} BA + 3 mg l^{-1} TDZ + 1 mg l^{-1} GA_3.

Table 2. Callus and shoot induction of 'Yang Fei Chu Yu' (YFCY) and 'Fen Ling Hong Zhu' (FLHZ).

Cv.	Explant type and number per treatment		Treatment	Calli induced (3 d)	Number of explants induced shoots			Shoot induction rate (%)
					7 d	15 d	30 d	
YFCY	Stem (- node)	8	A to E	+	0	0	0	0
	Stem (+ node)	8	A	+	0	0	0	0
		8	B	+	4	8	8	100
		8	C	+	6	8	8	100
		8	D	+	7	8	8	100
		8	E	+	6	8	8	100
	Petiole (- junct)	20	A to E	+	0	0	0	0
	Leaf	50	A to E	-	0	0	0	0
FLHZ	Stem (- node)	8	A	+	0	0	0	0
	Stem (+ node)	8	B	+	4	4	5	≥ 60
		8	C	+	0	6	6	≥ 70
		11	D	+	2	4	4	≥ 40
		13	E	+	2	3	5	≥ 38
	Petiole(- junct)	8	A to E	+	0	0	0	0
	Leaf	20	A to E	-	0	0	0	0

Note: 'junc' – junction, 'cont' – contamination, '-node' – without node, '+node' – with node, 'cont'- contamination, '≥' – shooting rates should be higher than the actual observed values because shoot induction ability of the explants was possibly inhibited by contamination.
Treatments: A: ½ MS; B: ½ MS + 0.1 mg l^{-1} TDZ; C: ½ MS + 0.5 mg l^{-1} TDZ; D: ½ MS + 1 mg l^{-1} TDZ; and E: ½ MS + 3 mg l^{-1} TDZ .

Table 3. Callus and shoot induction of 'Xi Shi Fen' by soaking shock treatment with TDZ**.**

Explant type and number per treatment		Treatment	Callus induction (3 d)	Number of explants induced shoots			Shoot induction rate (%, 30d)	Mean shoots /explant	Mean LS length (mm)
				7 d	15 d	30 d			
Petiole	10	I to III	+	0	0	0	0	0	0
Fork petiole	6	I	+	0	0	6	100	-	-
	6	II	+	0	0	6	100	-	-
	10	III	+	0	0	10	100	-	-
Stem (- node)	10	I to III	+	0	0	0	0	0	0
Stem (+ node)	10	I	+	0	8	10	100	3	20.4
	10	II	+	0	7	10	100	2.8	19.1
	10	III	+	2	9	10	100	2	15.8

Treatments: Explants were soaked in 20 mg l^{-1} TDZ for 2 min (I), 15 min (II), and 60 min (III), respectively, then inoculated on PGR-free MS medium. After 15 d of culture, explants were transferred to ½ MS + 0.1 mg l^{-1} BA + 1 mg l^{-1} GA_3 for shoot elongation and multiplication.
Note: '-node' – without node, '+node' – with node, 'prim' – primordium, '-' – data unavailable.
Mean shoot number and the average length of longest shoots (LS) were calculated after 30 d of culture.

Figures

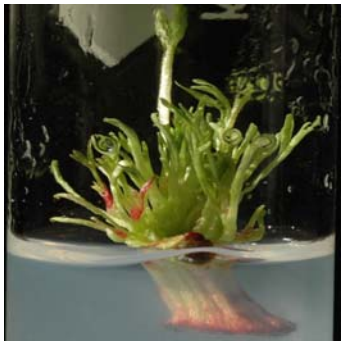

Fig. 1. Up to 20 tiny shoots induced from bud eye after 20 d treatment with 0.1–1 mg l^{-1} TDZ.

Fig. 2. Shoots generated on junction of petiole and petiolules.

Fig. 3. Shoots generated on junction of petiolule and leaflets.

Fig. 4. Leaf cluster formed on single stem node treated 14 d on medium containing 3 mg l^{-1} TDZ.

Fig. 5. Shoots obtained from stem nodes treated 10 h with TDZ (20 mg l^{-1}) before being inoculated in medium.

Chapter 3 Optimal Concentrations of Benzylaminopurine, Thidiazuron and Gibberellic Acid for Shoot Induction of Herbaceous Peony (*Paeonia*)

Abstract

Two varieties of herbaceous peony were used to evaluate the effects of BA, TDZ and GA on shoot induction of nodal stem explants in solid MS medium. Callus was readily induced from both sides of nodal stem segments within 2 days especially in BA treatments. However, shoots failed to generate through callus. Shoots were directly induced from the axillary bud eye region in the medium with BA or TDZ alone or in combination with GA_3. Higher concentrations of BA often caused vitrification and deformation of shoots or leaves. TDZ had a much stronger effect on shoot induction than BA. Up to 100% of explants induced shoots in TDZ treatments. However, a 12-day pretreatment in the medium containing TDZ (0.1–5 mg l^{-1}) was too long and compact leaf clusters developed. GA_3 had a positive effect in shoot induction and it also promoted elongation of both explants and induced shoots.

Key words: *Paeonia lactiflora* · Nodal stem · Direct shoot induction · Leaf cluster · Vitrification

Introduction

Peony (*Paeonia*) is one of the most valuable ornamental plants. Because of a complex sequential dormancy in peony seeds, it usually takes two years for germination under natural conditions (Krekler 1962; Griess and Meyer 1976). Peony seedlings must grow for several years before blooming. Peonies can be propagated vegetatively by division, cutting, grafting, and layering to obtain true-to-type plants. However, traditional propagation methods can not produce a large number of plants in a short time period to meet the increasing market demands, especially for newly developed cultivars. Development of micropropagation methods for peonies is necessary not only for mass production but also for acceleration of breeding programs. In vitro culture of peony has been studied for more than 40 years and many advances have been made (Buchheim and Meyer 1992; Gabryszewska 2004; Li and Luo 2004; Li et al. 2006; Jia et al. 2006; Meng et al. 2007; Beruto and Curir 2007; Zhao et al. 2007). Plantlets have been successfully produced from various types of explants, and established in-vitro plants are reported in many cases (Li et al. 1984; Hosoki et al. 1989; Harris and Mantell 1991; Shin et al. 1996; Kong and Zhang 1998; Habib and Donnelly 2001; Zhou and Yao 2001; Beruto et al. 2004; An 2005; He 2006; Kim et al. 2006; Li et al. 2006). However, only one company, Planteck Biotechnolgoies Inc., based in Quebec, Canada, makes tissue cultured peonies commercially available (Whysall 2006). Much research is necessary to make commercial tissue culture of peony more successful. In this study, nodal stem explants of herbaceous peony were used to evaluate the effects of 6-benzyl aminopurine (BA), thidiazuron (TDZ) and gibberellic acid (GA_3) on shoot induction in half strength Murashige and Skoog (MS) medium (1962).

Materials and Methods

Containerized plants of 'Xi Shi Fen' ('XSF') and 'Lian Tai' ('LT') were moved to the cooler (4ºC; 95% RH) on Oct. 2006 for 6 months of storage. Plants had elongated stems with tight young leaves when the experiments began. Single-node stem segments were used as explants. The first experiment started on March 10, 2007. 'XSF' was used to investigate optimal concentrations of BA on shoot induction. Shoot induction medium (SIM) was ½ MS + BA (0.1, 0.2, 0.5, 1, 2, and 5 mg l^{-1}) + GA_3 (0.1 mg l^{-1}) and the shoot elongation medium (SEM) was ½ MS + 0.1 mg l^{-1} BA + 0.1 mg l^{-1} GA_3. The second experiment began on March 26. 'XSF' and 'LT' were used to investigate optimal concentrations of TDZ on shoot induction. SIM was ½ MS + TDZ (0.1-5 mg l^{-1}), and SEM or shoot multiplication (SMM) was ½ MS + 0.1 mg l^{-1} BA + 1 mg l^{-1} GA_3. The third experiment started on April 17, 2008. 'XSF' and 'LT' were used to investigate effects of GA_3 (0–2 mg l^{-1}) in combination with 1 mg l^{-1} BA or TDZ on shoot induction.

Young elongated shoots were cut from stock plants and washed with tap water three times. After removing top 2-3 nodes (usually with tiny flower buds), shoots were then cut into 2-4 cm long sections. Shoot sections were soaked in 70% ethanol for 10 sec followed by sterilization with 10% commercial bleach with 1 drop per 100 ml of Tween 20 for 20 min. Plant material was rinsed three times for 5 min in sterile water. After sterilization, explants were cut into 3-8 mm sections and placed on 100 × 20 Petri dishes or in 25 × 95 mm tubes. Considering position effect of stem, each treatment had the same number of explants in the same stem positions: basal-position node stem segments with bract (BN), middle-position node segments (MN) and upper-position node stem segments (UN). Culture vessels were placed on a culture shelf with cool fluorescence light (60 lux) with a

16-h light cycle at 25 ± 1ºC. After 14 d of culture, explants were transferred to SMM or SIM medium.

Results and Discussion

Effect of BA on shoot induction

Callus quickly generated at two sides of stem segments within 2 days of culture. Callus was usually pale white or slightly green. More calli were produced in the treatments of medium with BA at 0.2–2 mg l^{-1} (Table 1, Fig. 1). The length of the explant-stem was influenced by BA concentration. Explants on medium with 2 mg l^{-1} BA produced the greatest length (1.93 cm) while high concentration of BA (5 mg l^{-1}) inhibited explant growth. Also, higher concentrations of BA (2–5 mg l^{-1}) caused a gradual loss of green color of explants, which turned light pink-red, whereas explants with other treatments grew normally and maintained a healthy appearance. The initiation media with 0.2–2 mg l^{-1} BA were more favorable for induction of callus and adventitious shoots (Table 1). Highest induction rate of shoots or shoot primordia was found in the treatment of ½ MS + 2 mg l^{-1} BA + 0.1 mg l^{-1} GA_3 following 25 d of culture. The shooting rate decreased in the treatment of medium with 5 mg l^{-1} of BA. Only a single shoot was induced on lateral bud eyes of explants on media with the lowest (0.1 mg l^{-1}) and highest (5 mg l^{-1}) concentrations of BA, while multiple shoots or shoot clusters were often induced in other treatments. Higher dose of BA usually caused morphological disorder of the tissue. Higher rates of vitrification and shoot deformation were observed in treatments of 2–5 mg l^{-1} of BA. When comparing induction ability of callus and shoot,

growth of explants and shoots, and color of explants, 1 mg l^{-1} of BA in ½ MS + 0.1 mg l^{-1} GA_3 medium was the best choice for initial shoot induction of 'XSF'.

For elongation and proliferation of shoots, when shoot-induced explants were transferred to fresh medium ½ MS + 0.1 mg l^{-1} BA + 1 mg l^{-1} GA_3, phenolics quickly exuded into the medium whether or not callus was removed from explants. If callus was not removed, browning became much more severe. Browning was mainly related to callus and wounding of explants. When callus and most of the explant stem were removed and only a small part was left attached to the base of induced shoots or shoot clusters, browning decreased. Thus, browning might be a consequence of interaction of senescent callus and wounding area of explants with the medium. The degree of browning was dependent on size of callus and wounding region.

Effect of TDZ on shoot induction

Different concentrations of TDZ did not affect stem length of explants from 'XSF' and 'LT' (Table 2). However stem thickness of shoots induced from BN explants increased as the concentration of TDZ in medium increased. Explants cultured on medium with 0.1, 0.5, and 2 mg l^{-1} TDZ produced 2.6, 3.23, and 3.63 mm of stem diameter, respectively (LSD, $\alpha = 0.05$, $P = 0.02$). Callus formation was genotype and explant size dependent. More callus formed in cultivar 'XSF' (larger explants) than 'LT' (smaller explants). Callus on medium with TDZ as the only PGR was green and more rigid. MN explants generated more callus than BN explants (Fig. 1). After transfer to fresh medium, callus continued to grow and no obvious browning occurred. However, callus did not develop adventitious shoots even after more than two months of culture.

Shoot initiation rates during the first 14 d of culture were generally higher on medium with 0.1–1 mg l^{-1} TDZ and was lower in treatments with higher concentrations (2–5 mg l^{-1}) (Table 2). 1 mg l^{-1} of TDZ was found to be optimal for shoot induction. 'XSF' was more favorable for shoot induction than 'LT'. More shoots or leaf clusters were induced from lateral bud eyes of 'XSF', whereas only single to several shoots were induced from 'LT'. Although nearly 100% of explants finally produced shoots or leaf clusters in all treatments, the treatment with the highest concentration of TDZ (5 mg l^{-1}) visually inhibited later elongation of shoots and leaves. Shooting response to medium containing TDZ was different among types of explants. For BN, a primary single shoot grew out and elongated first (Fig. 1), then shoot primordium was induced from the bud-eye region. Some of these elongated shoots developed into terminal flower buds. A similar case occurred on some UN explants but initiation of shoot primordium at the bases was not delayed much when the first major single shoot developed and elongated. In both cultivars, a limited number of shoots with elongated stems were produced after transfer to medium with ½ MS + 0.1 mg l^{-1} BA + 1 mg l^{-1} GA_3. Only leaf-clusters developed especially from cultivar 'XSF' (Fig. 2). This suggested that 14 d of initial culture on TDZ containing medium might be too long or explants should be treated in the medium with a combination of TDZ and GA_3 at the shoot initiation stage because of a shoot elongation role of GA_3.

Effect of GA_3 on shoot inductions

Callus generation on two sides of nodal stem segments of 'XSF' in media with BA (0-2 mg l^{-1}) was high but no visual difference in quality and amount of callus occurred

among treatments. GA_3 had a significant effect on elongation of explant stems. Stem length increased with increased GA_3 concentrations in the medium with 1 mg l^{-1} BA (Table 3, Fig. 3). GA_3 also promoted shoot initiation and elongation. Shoot induction ability was increased by GA_3 applied at 0.1–1 mg l^{-1} in the medium with 1 mg l^{-1} BA. However, a higher concentration of GA_3 at 2 mg l^{-1} inhibited shoot induction. A similar result also was found in the TDZ-media. Elongation of explants in both 'XSF' and 'LT' increased in the medium with GA_3 compared to control. However, shoot elongation of 'XSF' and 'LT' did not consistently increase upon increasing concentrations of GA_3 (Table 4, Fig. 3). The highest increase of mean explant length was found on the medium with 2 mg l^{-1} GA_3 for 'XSF' but with 0.1 mg l^{-1} GA_3 for 'LT'. This could be due to genotype effect or different interactions between GA_3 and BA or GA_3 and TDZ. Highest shooting initiation rates were found at 0.2–0.5 mg l^{-1} GA_3 for both cultivars. However, effect of GA_3 on shooting rate was less in TDZ-medium than in BA-medium. Explants on TDZ-medium with 0.1 mg l^{-1} GA_3 even produced a lower rate of shoot induction than those in non-GA_3 control. This indicates that TDZ plays a major role during shoot induction. On TDZ-containing medium, the bud eye region of explants quickly expanded. The angle between the stem section and basal petiole continued to enlarge over time and became more than 90^o even 180^o following one month of culture (Fig 4). Shoot induction and shoot formation were genotype dependent, 'XSF' showed higher rates than 'LT'. Large leaf or shoot clusters formed on 'XSF' explants while only a single shoot or very little shoot-clusters generated from 'LT'. Unfortunately, very few normal shoots with elongated stems were obtained from both cultivars.

General discussion

Many types of explants, including immature embryos, seeds, dormant buds, young shoots, bud or shoot tips, stems, leaves, portions of flower buds or flowers, have been used for in vitro culture of peony (Tian 2008). One type of cytokinin can induce adventitious shoots, but the efficiency of shoot induction was improved using a combination of two or more types of PGRs. Gabryszewska (1998) reported that BAP, 2-iP, KT, and TDZ, used separately, could stimulate axillary bud development. However, the multiplication rate was very low. BA is the most often used cytokinin for shoot induction of peony. Optimal concentrations of BA were 0.5–1 mg l^{-1} and high concentrations of BA (≥ 2 mg l^{-1}) often caused a higher rate of vitrification (Černá et al. 2001; Zhang et al. 2001). Similar results were obtained in our study that 0.2–2 mg l^{-1} BA was optimal for direct shoot induction of nodal stem segments and higher concentrations of BA often caused vitrification and shoot or leaf deformation. TDZ treatment results in higher shoot induction than BA treatment (Mondal et al. 1998; Trigiano and Gray 2000; Lee 2005). Pretreatment of explants with TDZ was reported to be more effective to improve shoot induction of rose (Singh and Syamal 2001), *Curcuma longa* (Prathanturarug et al. 2003, 2005), and *Curculigo orchioides* (Thomas 2007). Optimal concentrations of TDZ and pretreatment time were dependent on genotype and type of explants. TDZ has also been used in peony in vitro culture (Gabryszewska 1998, 2001, 2006; Orlikowska et al. 1998; Beruto et al. 2004) but all explants were treated continuously in the medium containing low concentrations (0.01–0.8 mg l^{-1}) of TDZ or in a combination with other PGRs. Axillary shoot formation was enhanced by addition of TDZ at very low concentrations (0.01–0.04 mg l^{-1}) to medium containing a mixture of

BA + 2iP + Kinetin (Gabryszewska 1998). In the present study, TDZ showed higher ability of shoot induction in both cultivars when explants received either a 14-d pretreatment in medium with TDZ alone or a continuous treatment in medium with TDZ and GA_3 (Table 2, 4). However, only large leaf clusters developed instead of normal shoots with elongated stems. Explants treated by high concentrations of TDZ often produced deformed leaves. Inhibitory effect of TDZ on shoot or stem elongation has been reported in other plant species (Murthy et al. 1998; Ledbetter and Preece 2004; Lee 2005; Ramanayake et al. 2006). Shoot length of bamboo was significantly reduced in the presence of TDZ as compared to BA, and repeated subculture in the medium with TDZ caused compact shoot clusters that could not be separated during subculturing (Ramanayake et al. 2006). Since normal shoots were obtained by shock treatments of TDZ with high concentration (20 mg l^{-1}) in other studies (data not shown), the time of TDZ pretreatment in the present study was too long. Further study is necessary to determine the optimal pretreatment time and concentrations of TDZ for shoot induction of peony.

GA_3 not only plays an important role in dormancy release but also is necessary for elongation of shoot stems in tissue culture of some species (Figueiredo et al. 2001). GA_3 has been often used in peony tissue culture combined with other PGRs to promote shoot induction or elongation. The combination of BA with GA_3 in the medium increased the multiplication rate of peony compared with BA alone and allowed shoot elongation which was also supported by the present study. However, GA_3 alone in the culture medium allows neither shoot elongation nor leaf and axillary bud formation or development (Bouza et al., 1994). The present study indicated that development of shoot

stem could be completely inhibited by TDZ. Under this situation, the role of GA_3 can not be expressed on stem elongation of induced shoots.

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Tables

Table 1. Effect of BA on callus formation and shoot regeneration of 'Xi Shi Fen' following 25 d of culture on solid MS media with BA and GA_3.

Treatment	BA (mg l^{-1})	GA_3 (mg l^{-1})	Explant No.	Callus induction	Shooting (%)	Explant length (cm)	Explant color
A	0.1	0.1	24	+++	41.1	1.67 bc	Green
B	0.2	0.1	24	++++	46.7	1.62 bc	Green
C	0.5	0.1	23	++++/+++	58.8	1.84 abc	Green
D	1	0.1	24	++++/+++	62.1	1.87 abc	Green
E	2	0.1	24	++++/+++	77.8	1.93 a	Pink-green
F	5	0.1	24	+++/++	20	1.59 c	Red-green

Mean comparisons were determined by LSD ($\alpha = 0.05$). '+' – represents the amount of callus induced.

Table 2. Effect of TDZ on callus and shoot induction of 'Xi Shi Fen' (XSF) and 'Lian Tai' (LT).

Cv	TDZ (mg l^{-1})	Explant No.	Explant length (mm, 14 d)	Callus induction	Shoot induction rate (%)		Shoots per explant (20 d)
					14 d	20 d	
'XSF'	0.1	12	9.2 a	+++	89	100	Leaf/shoot cluster
	0.2	12	8.8 a	+++	55	91	Leaf/shoot cluster
	0.5	12	9.1 a	+++	75	91	Leaf/shoot cluster
	1	12	9.6 a	+++	89	100	Leaf/shoot cluster
	2	12	9.3 a	+++	67	100	Leaf/shoot cluster
	5	12	9.0 a	+++	55	92	Leaf/shoot cluster
'LT'	0.1	10	5.8 a	+/++	50	60	0-2 shoots
	0.2	10	5.6 a	+/++	40	80	0-2 shoots
	0.5	10	6.2 a	+/++	60	70	0-2 shoots
	1	10	5.7 a	+/++	90	90	0-2 shoots
	2	10	6.3 a	+/++	50	60	0-2 shoots
	5	10	6.5 a	+/++	30	60	0-2(4) shoots

Note: '+' – represents the amount of callus induced. Mean differences in explant length were determined by LSD (α = 0.05) within cultivar. No significant difference was found in mean length of explant for 'XSF' (P = 0.683) and 'LT' (P = 0.343) among the treatments with TDZ (0.1–5 mg/L).

Table 3. Effect of GA_3 on explant growth and shoot regeneration of 'Xi Shi Fen' in the medium with BA.

Treatment	BA ($mg\ l^{-1}$)	GA_3 ($mg\ l^{-1}$)	Explant No.	Callus	Explant length 14 d (cm)	Shoot induction rate (%)		
						10 d	18 d	40 d
A	1	0	12	+++/++	0.99 c	16.7	33.3	58.3
B	1	0.1	12	+++/++	1.25 b	33.3	41.7	58.3
C	1	0.5	12	+++/++	1.46 ab	41.7	58.3	67.0
D	1	1	12	+++/++	1.53 ab	41.7	58.3	75.0
E	1	2	12	+++/++	1.71 a	16.7	20.0	33

Note: Mean differences in explant length were determined by LSD ($\alpha = 0.05$), after 14 d of culture. '+' – represents the amount of callus induced.

Table 4. Effect of GA_3 on explant elongation and shoot regeneration in the medium with TDZ.

Cv	Treatment	TDZ (mg l^{-1})	GA3 (mg l^{-1})	Explant No.	Explant elongation (mm, 14 d)	Initiation (%, 14 d)	Shoot primordia/shoots per explant (35 d)
'XSF'	1	1	0	12	8.6 b	83.3	Leaf/shoot cluster, 0-40 mm
	2	1	0.1	14	10.4 ab	78.6	Leaf/shoot cluster, 0-30 mm
	3	1	0.2	16	11.7 ab	100	Leaf/shoot cluster, 0-30 mm
	4	1	0.5	14	13.4 ab	100	Leaf/shoot cluster, 0-30 mm
	5	1	1	14	12.4 ab	92.9	Leaf/shoot cluster, 0-30 mm
	6	1	2	12	17.1 a	91.7	Leaf/shoot cluster, 0-30 mm
'LT'	1	1	0	14	1.7 b	57.1	1 shoot/primordium, 1-10 mm
	2	1	0.1	14	3.4 a	42.9	1 shoot/primordium, 3-8 mm
	3	1	0.2	14	2.9 ab	64.3	1 shoot/primordium, 1-6 mm
	4	1	0.5	14	2.6 ab	57.1	1 shoot/primordium, 1-5 mm
	5	1	1	14	2.7 ab	42.9	1 shoot/primordium, 1-4 mm
	6	1	2	14	2.6 ab	50	1 shoot/primordium, 3-10 mm

Note: 'XSF' – 'Xi Shi Fen'; 'LT' – 'Lian Tai'. Mean differences in explant elongation were separated by LSD ($\alpha = 0.05$) within cultivar, after 14 d of culture.

Figures

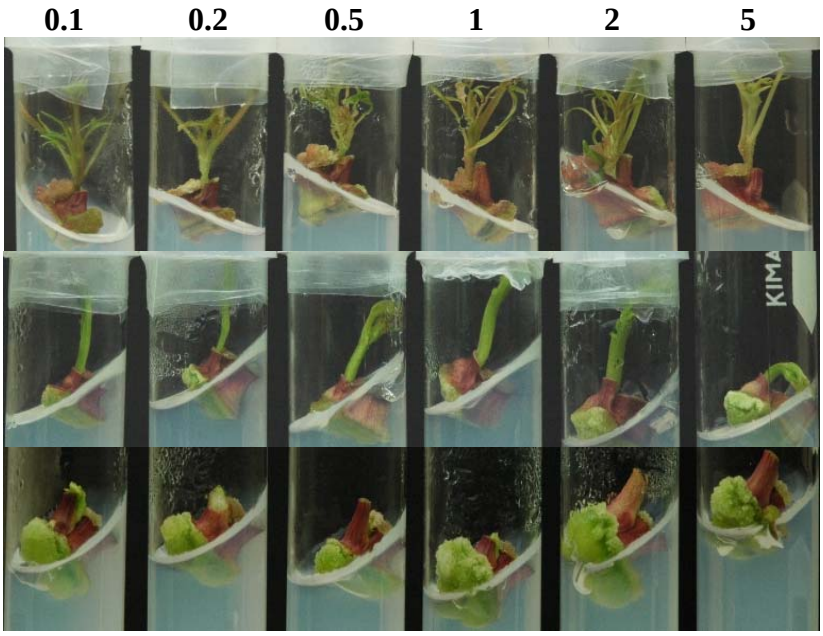

Fig. 1. Callus generated on the sides of nodal stem segments of 'Xi Shi Fen' in the medium contained BA (0.1, 0.2, 0.5, 1, 2, and 5 mg l^{-1} from left to right in 1st row) or TDZ (0.1, 0.2, 0.5, 1, 2, and 5 mg l^{-1}, 2nd and 3rd row) in which more callus produced on middle nodal stem sections (3rd row) than on basal nodal stem sections (2nd) but a single shoot elongated earlier in the latter.

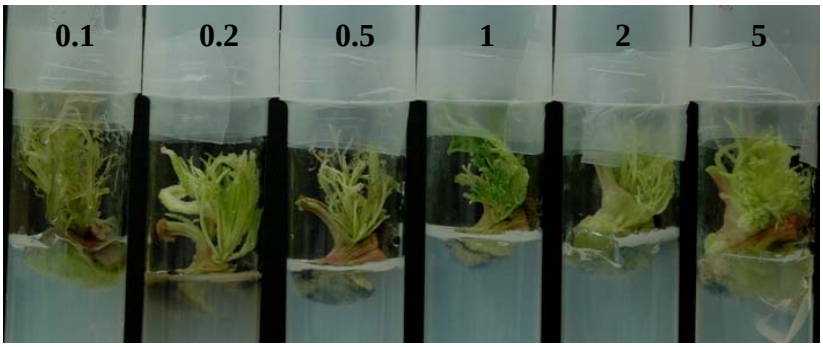

Fig. 2. Leaf clusters generated in middle-position nodal stem segments of 'Xi Shi Fen' on shoot elongation medium: ½ MS + 0.1 mg l^{-1} BA + 1 mg l^{-1} GA_3 after 14 d of culture in initial medium: ½ MS+ TDZ (0.1, 0.2, 0.5, 1, 2, and 5 mg l^{-1}).

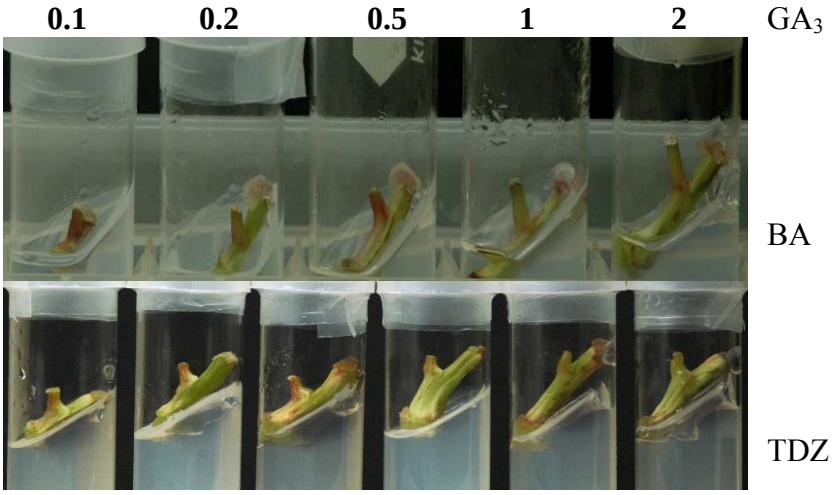

Fig. 3. Elongation of nodal stem explants in 'Xi Shi Fen' increased with increased GA_3 (0, 0.1, 0.2, 0.5, 1, 2 mg l^{-1}, from left to right) in the medium containing 1 mg l^{-1} BA (1st row) or 1 mg l^{-1} TDZ (2nd row).

Fig. 4. The angle between stem and petiole base at bud eye region of explants was often enlarged to 90º–180º in treatment with TDZ medium. It provided plenty space for development of adventitious shoots on bud eye.

Appendix Short Reports on Other Studies of In Vitro Culture of Herbaceous Peony

1. Effect of Gelling Agents on Tissue Culture of Herbaceous Peony

Four types of agar products were used to compare responses of nodal stem explants of 'Yang Fei Chu Yu' ('YFCY') and 'Xi Shi Fen' ('XSF') in ½ MS + 1 mg l^{-1} TDZ medium with (1) A111 (Phytotechlab) 5 g l^{-1}; (2) A296 (Phytotechlab) 6 g l^{-1}; (3) A133 Agagellam™ (Phytotechlab) 4 g l^{-1}; and (4) A20020 (high gel strength) 5 g l^{-1} according to the recommended rates. Explants responded to the types of agar gelling agents differently. Generally A111 (5 g l^{-1}) and A133 (4 g l^{-1}) were much better than A296 (6 g l^{-1}) and A20020 (5 g l^{-1}) for all indexes (Table 1). Response was also genotype dependent. A133 and A111 significantly increased explant growth of 'YFCY' as compared to A296 and A20020 but this effect did not occur in 'XSF'. Similar results were noted on induction of callus and shoots. The highest rates of shoot initiation and callus production were seen on explants with treatments of A111 and A133, respectively, in 'YFCY', but no significant difference was observed in 'XSF'. For both cultivars, more phenolic compounds exuded into media with A296 and A20020, and explant color looked abnormal. Agar has long been used to solidify media for plant tissue culture. There are lots of brands of agar gelling agents. The type of agar or other gelling agents significantly influences plant growth in tissue culture. Differences of results are even seen in the same product made at different times or locations (Torres et al., 2007).

2. Comparison of Basal Medium in Tissue Culture of Herbaceous Peony

The choice of type of basal medium is important for tissue culture. MS, ½ MS and WPM have been mostly used in peony tissue culture for shoot induction (Chapter 1, Table 1). The comparative effects of basal media in peony have only been studied by Lang and Luo (2007) for callus induction and by An (2005) for root induction of tree peony. A similar study has not been reported in herbaceous peony. In this experiment, effects of MS (1/4, half, full strength) and WPM (half, full strength) medium with 1 mg l^{-1} TDZ + 0.1 mg l^{-1} BA + agar (A111, 4 g l^{-1}) was evaluated on callus and shoot induction of nodal stems from three herbaceous cultivars: 'Bin Shan' ('BS'), 'Fen Ling Hong Zhu' ('FLHZ') and 'Yang Fei Chu Yu' ('YFCY').

Explants generally performed best in full strength medium MS followed by half strength MS, and worst in half strength WPM. Nodal stems of all three cultivars remained green in full strength MS medium (Table 2), while explants turned pink and showed slight abnormalities in other MS media. Explants cultured in WPM turned pink, then red over time. MS was the most favorable medium for explant growth of 'BS'. Explants cultured in ½ WPM had the least increase in growth for all three varieties. Browning was more visual in WPM than MS. MS medium was more favorable than WPM for callus induction but results of shoot induction were complicated. Explants cultured on full strength WPM produced the highest shoot initiation rates for 'BS' and 'FLHZ' but the lowest rate for 'YFCY'. Explants of 'BS' generated the lowest shooting rate on half strength WPM while explants of 'FLHZ' produced the lowest shooting rate in ½ MS. Compared with the data collected on 24 and 40 days after inoculation, the difference in the initiation rate among basal medium types was consistent.

The highest shoot length was observed in explants of 'BS' cultured on 1/2 MS and 'FLHZ' on MS. For these two cultivars, shoot growth of explants performed worst on ½ WPM. Shoot growth generally increased from ¼, ½, to 1 MS. Quality of generated shoots was better in MS than WPM medium.

3. Effect of Pretreatment of Underground Buds on Tissue Culture of Herbaceous Peony

Responses of underground buds to three pretreatments were evaluated in tissue culture of two varieties of herbaceous peony. Underground buds (Fig. 1) from containerized stock plants of 'Da Fu Gui' ('DFG') were treated in three ways: (1) plants remained untreated; (2) plants were treated in cooler for 20 d beginning Nov. 20, 2006; and (3) plants from outside were washed by tap water and repotted in container with perlite, then directly moved to greenhouse to break dormancy and force growth. In this treatment, the stock plants were also rinsed with 1% Zerotol for two times, once per week, to decrease contamination. After surface sterilization of 20 sec in ethanol (75%) following by 25 min in 10% Chlorox bleach, the buds (tips) were inoculated in test tubes with medium ½ MS + 1 mg l^{-1} BA + 0.1 mg l^{-1} GA_3 or ½ MS + 0.1 mg l^{-1} BA + 0.1mg l^{-1} GA_3 on Dec 10, 2007. Shoot multiplication medium was ½ MS + 0.1 mg l^{-1} BA + 0.1 mg l^{-1} TDZ + 0.1 mg l^{-1} GA_3.

A previous experiment produced 100% contamination of 'DFG' and 'Xi Shi Fen' ('XSF') dormant underground buds with a sterilization treatment of 10 sec in ethanol (75%) following by 10–20 min in 10% Chlorox bleach. After 15 d of culture, no callus formed and buds did not grow. In this experiment, contamination and phenolic exudation

were visual within one day of culture and increased over the time of the 15-d experiment. All explants (100%) treated in greenhouse were contaminated within 3 d of culture (Table 3). The cooler treated explants performed better. Only 2 of 16 explants were contaminated within three days although contamination rate went up to 75% after 15 d culture. More explants were contaminated by bacteria than fungi. Contamination with bacteria also occurred earlier. There was significant difference in fungi caused infection among treatments. At the end of experiment, only one bud from the outside treatment and 4 buds from the cooler treatment were sterile for use of shoot induction after transfer.

Before culture, buds treated in the cooler for 20 d were already slightly elongated (dormant release) and looked better in quality than both outside and greenhouse treated buds. After inoculation these buds grew very fast and main shoots as well as some lateral shoots elongated (Fig. 2). These elongated shoots could be cut into nodal sections and transferred to fresh medium for shoot multiplication. Buds from the outside also elongated but did not grow as fast as the cooler treated buds. The shoots were much shorter than those generated from the cooler treated buds (Fig. 2). The greenhouse perlite medium treated buds performed worst and all nearly stopped growing. These buds finally died and no axillary shoots generated. For all treated buds, no callus formed in the early stages. Only very little green callus was induced at the cutting side of buds after more than 15 d of culture.

It has been widely reported that underground buds of herbaceous peony are difficult to disinfect compared with aerial tissues. Response of underground buds was different in tissue culture between stages of growth or when different pretreatments are used. Contamination was a big problem with the use of underground buds as explants in tissue

culture of herbaceous peony. A more effective sterilization approach must be developed. Dormant buds were not effective for shoot induction. If the buds were treated in cooler, contamination decreased significantly. Shoot or bud explant could readily grow and axillary shoots were easily induced after bud dormancy was broken.

4. Investigation of Callus and Shoot Induction Ability of Flower Tissue

Anther culture of peony has been much studied and plantlets have been successfully developed from embryos which were induced from anthers (Chapter 1, Table 1). However, the information on culture of petals or other tissue of flowers is very limited. In two cases reported on petal culture, shoots either failed to develop or generated at a very limited number after more than one year of culture (Orlikowska et al 1998; Beruto et al 2004). Callus and shoot induction ability of 'Da Fu Gui' ('DFG') and 'Paula Fay' ('PF') were studied in this experiment using petals, anthers, filaments, and pistils as explants from the tight flower buds (TFB), one week before blooming, newly opening flowers (OF) with a small mouth or the fully open flowers (FOF). The petals of TFB, OF and FOF were from 'DFG', and the petals, anthers and filaments of TFB were from 'PF'. Three media were tested: A: ½ MS (PGR-free); B: ½ MS + 1 mg l^{-1} TDZ; and C: ½ MS + 1 mg l^{-1} TDZ + 0.1 mg l^{-1} GA_3.

After 14 d of culture, contamination occurred on the petals but not on either anthers or filaments (Table 4). Petals from FOF had higher contamination rate than those from TFD and OF in 'DFG'. Browning was obvious in all treatments and it was more severe on the petals of FOF followed by OF in 'DFG'. Petals from TFB had the least browning problem. However, in 'PF' nearly all of the petals from TFB turned brown. The least

browning rate was seen on anther culture of this cultivar. No difference in browning was found among PGR treatments.

There were large differences in growth of explants among types of petals. Petal sections from both TFD and OF grew fast, whereas those from FOF grew slow and lost their original color within two days of culture. Callus generated very slowly and no callus formed within 15 d. Following 30 d of culture, very little callus was induced from the base cutting side of the petals from TFB and OF in both B and C media (Table 4). No callus was seen in A (PGR-free) medium for petals. Anthers and filaments also did not produce callus. Results indicated 1 mg l^{-1} TDZ could induce callus from petal culture. However the induction rate as well as the amount of callus was very low. There was no difference in callus induction between B and C media. It showed that GA_3 at 0.1 mg l^{-1} concentration had no effect on induction and production of callus compared with the control. No adventitious shoots developed.

5. Effect of PPM on Decontamination and Rescue of Explants

High contamination of peony in tissue culture is possibly caused by endogenous bacteria. Plant preservative mixture (PPM™) is a broad-spectrum preservative and biocide, which kills bacteria and fungi cells, prevents germination of spores, and in higher concentrations, can eliminate endogenous contamination of explants. PPM has been widely used in other species for tissue culture purposes. It is also reported that PPM is effective for rescuing lightly contaminated explants in tissue culture. However, little information is available on its efficiency on disinfection of peony. Effect of PPM on decontamination of nodal stem segments from ‘Da Fu Gui’ (treated 3 months in the

cooler) and rescue of contaminated explants was investigated in media with ½ MS + 1 mg l^{-1} BA + 0.2 mg l^{-1} GA_3 with supplement of 0, 0.05, 0.1, and 0.2% (v/v) of PPM, respectively. The explants were surface sterilized with a 12–15 sec quick soak in 70% Ethanol followed by 20 min in 10% Bleach. For rescue of contaminated explants, 12 of bacteria contaminated explants were cleaned by a soft brush under running tap water following by a 12 min soak in 50% PPM and then were inoculated on fresh medium.

PPM had effect on minimizing contamination of explants and the contamination rate decreased in 0.2% PPM-treated medium (Table 5). However, lower concentrations (<0.2%, v/v) of PPM only delayed occurrence of contamination. No difference was observed in browning, the callus and shoot induction rate, and shoot growth of post-transferred explants between PPM treatment and the control explants. This indicated application of PPM at low level in medium had no side effect on tissue culture of peony. Contamination of the rescued contaminated explants significantly decreased following PPM treatment but black phenolics exuded quickly to medium from explants. Browning was a big problem which quickly caused death of plant material. Therefore, it was not a viable option to rescue contaminated peony explants using PPM even though successful in decontamination.

6. Root Induction and Transplanting of Herbaceous Peony In Vitro

Several trials on root induction were conducted on vitroshoots from the varieties: ‘Xi Shi Fen’, ‘Da Fu Gui’, and ‘Cytherea’. The treatments included following variables: light and darkness; liquid (paper bridge method, Hosoki et al 1989), half solid and solid ½ MS

medium; activated charcoal (AC); temperature; and IBA with different concentrations or a quick soak in high concentration of IBA (10 mg l^{-1}).

Following 7–10 d of culture in rooting medium, all vitroshoots generated callus. Callus even formed sometimes on the petioles. Roots were successfully induced from plantlets in solid medium (Fig. 4) or using paper bridge method (Fig.5) in some cases. Plantlets rooted following about 20 d of culture and up to 20 roots developed on a shoot. Roots grew up to 3 cm after 45 d of shoot inoculation. The vitroshoots grew very fast in semi-solid rooting medium possibly benefiting from an easy absorption of nutrients (Fig. 3).

AC treatment increased the shoot length but not significantly. IBA shock treatment (10 mg l^{-1}) did not influence growth of in vitro shoots on rooting medium. IBA at 1 mg l^{-1} in a continuous treatment was too strong for small younger vitroshoots and caused their petioles to calluse. It remains not clear how long vitroshoots should be treated in medium with high concentration IBA before transfer to root growth medium with either lower concentration of IBA or no IBA. High-quality shoots are the basic requirement to obtain high rate of rooting. Several studies have reported treatment of darkness and chilling was beneficial for rooting of peony in vitro (Kunneman and Albers 1989; Habib and Donnelly 2001; Beruto et al 2004; Chen 2005). The present experiment showed that the shoots with a 40-d darkness treatment at 25ºC nearly stopped growth and completely died later. The shoots with a 40-d darkness treatment at 10ºC survived with chlorotic tissue. However, no roots were obtained in both treatments. For both of the rooted and non-rooted shoots, the earlier developed leaves showed obvious necrosis after more than two months culture

but the later formed young leaves remained healthy. It was not clear if necrosis problem could be avoided or minimized by an increase of transfer frequency.

Limited number of shoots with roots or root primordia was transplanted to jars with non-sterile peat moss. These vitroplants grew in the first week but quickly died because of infection with fungi (Fig. 6). Treatment with a spray of 1% Hydrogen peroxide (H_2O_2) did kill fungi but could not save the plantlets. Four plantlets transplanted to sterile peat-moss mix died quickly with rot of roots and stem base.

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Tables

Table 1. Response of nodal stem explants of herbaceous peony to agar in vitro culture.

Cultivar	Agar Treatment	Explant Elongation (mm)	Browning	Calli	Shoot Initiation (%)
'YFCY'	(1)	3.5 b	+/-	+	100
	(2)	2.0 c	++/+++	+/-	21
	(3)	5.3 a	+/-	+	84
	(4)	2.5 c	++	+/-	35
'XSF'	(1)	2.5 c	+/-	++/+++	60
	(2)	2.2 c	++	++/+++	60
	(3)	2.4 c	+	++/+++	75
	(4)	1.9 c	+/++	++/+++	50

Agar treatments: (1) A111 (Phytotechlab) 5 g l^{-1} (w/v); (2) A296 (Phytotechlab) 6 g l^{-1}; (3) A133 Agagellam™ (Phytotechlab) 4 g l^{-1}; and (4) A20020 (high gel strength) 5 g l^{-1}. Means of explant elongation were compared by LSD ($\alpha = 0.05$) and callus induction and shoot initiation were valuated based on after 14 d of culture. 'YFCY' – 'Yang Fei Chu Yu'; 'XSF' – 'Xi Shi Fen'; '+ or -' – degree of browning or amount of callus.

Table 2. Effect of basal media on performance of nodal stem explants of herbaceous peony.

Cultivar (stage)	Medium	Explant Color (8 d)	Browning (3 d)	Callus (24 d)	Shoot Initiation (%)		Shoot Length (mm)
					24 d	40 d	40 d
'BS'	1/4 MS	Green with pink	+	+/-	42.6	78.9	16.4 ab
(II-III)	1/2 MS	Green with pink	+	+/-	83.3	88.9	22.7 a
	1 MS	Green, largest size	+/++	+	60.0	85.0	17.8 ab
	1/2 WPM	Pink, smallest size	+/++	-/+	45.0	60.0	6.0 c
	1 WPM	Green with pink	+/++	-/+	89.5	100	13.1 b
'FLHZ'	1/4 MS	Green with pink	+	-/+	90.0	90.0	18.4 bc
(III)	1/2 MS	Green	+	-	61.5	76.9	22.8 bc
	1 MS	Green	+	-/+	63.6	81.8	43.1 a
	1/2 WPM	Pink with green	+	-/+	75.0	87.5	16.4 c
	1 WPM	Pink with green	+	-/+	100	100	31.3 ab
'YFCY'	1/4 MS	Green with pink	++/+	-	16.7	16.7	–
(I-II)	1/2 MS	Green with pink	++/+	-	18.2	18.2	–
	1 MS	Deep green	++/+	-	25.0	25.0	–
	1/2 WPM	Pink with green	++/+	-	18.2	18.2	–
	1 WPM	Pink-red	++/+	-	7.7	7.7	–
'YFCY'	1/4 MS	Green with pink	+/++	+/-	5.6	5.6	–
(II-III)	1/2 MS	Green	+/++	+/-	5.3	5.3	–
	1 MS	More green	+/++	+/-	0	0	–
	1/2 WPM	Green with pink	++/+	-/+	0	0	–
	1 WPM	Pink-red	++	-/+	0	0	–

Note: 'BS' – 'Bin Shan', 'FLHZ' – 'Fen Ling Hong Zhu', 'YFCY' – 'Yang Fei Chu Yu'. Plant stage: II – the buds already grow out of soil and shoots are visible with elongated stem; III – the stems have much elongated but the young leaves are still closed. '+ or -' in columns of browning and callus indicates degree of browning or amount of callus. '–' in shoot length column indicates data unavailable. Length means of the longest shoots were compared by LSD ($\alpha = 0.05$) within cultivar after 40 d of culture.

Table 3. Comparison of pretreated underground buds of 'Da Fu Gui' in tissue culture.

Treatment	Sample Size	Contamination				Callus		Bro.	Shoot Growth
		3 d	7 d	15 d	% (15 d)	3 d	15 d	3 d	
Cooler	16	2b	9b+1f	11b+1bf	75	-	+/-	+	Much elongation
Outside	12	8b	8b+1f	10b+1bf	91.7	-	-/+	+	Elongation
Greenhouse	16	16b	8bf+8b	8bf+8b	100	-	-	+	Less elongation

Note: b – bacteria; f – fungi; Bro. – browning of medium caused by phenolics exuded from explants. '+ or –' – degree of browning or amount of callus.

Table 4. Callus and shoot induction ability of flower tissue of 'Da Fu Gui' (DFG) and 'Paula Fay' (PF).

Cultivar	Flower stage	Explant Type	Medium	Contamination (%, 14 d)	Browning (%, 24 d)	Callus Induction (%, 30 d)
'DFG'	Tight bud	Petal	A			0
			B	10	21.5	26.7
			C			26.7
	Just opening	Petal	A			0
			B	8	45	22.5
			C			22.5
	Fully open	Petal	A			0
			B	26.3	75	0
			C			0
'PF'	Tight bud	Petal	A			0
			B	3.8	92.5	13.3
			C			10
		Anther	A			0
			B	0	27.5	0
			C			0
		Filament	A			0
			B	0	53.1	0
			C			0

Medium: A: ½ MS (PGR-free); B: ½ MS + 1 mg l^{-1} TDZ; C: ½ MS + 1 mg l^{-1} TDZ + 0.1 mg l^{-1} GA_3.

Table 5. Effect of PPM on contamination of nodal stem segments from ‘Da Fu Gui’.

Treat	PPM (v/v, %)	Explant Number	Contamination (%)			Browning (%, 15 d)	Calli (%, 15 d)	Shoot Induction (%, 15 d)
			3 d	7 d	15 d			
1	0	20	0	30	30	0	75	30
2	0.05	20	0	20	30	0	70	35
3	0.1	20	0	10	30	0	45	45
4	0.2	20	0	10	20	0	70	35

Note: medium was added with PPM (Plant Preservative Mixture) at 0, 0.05, 0.1, or 0.2% (v/v).

Figures

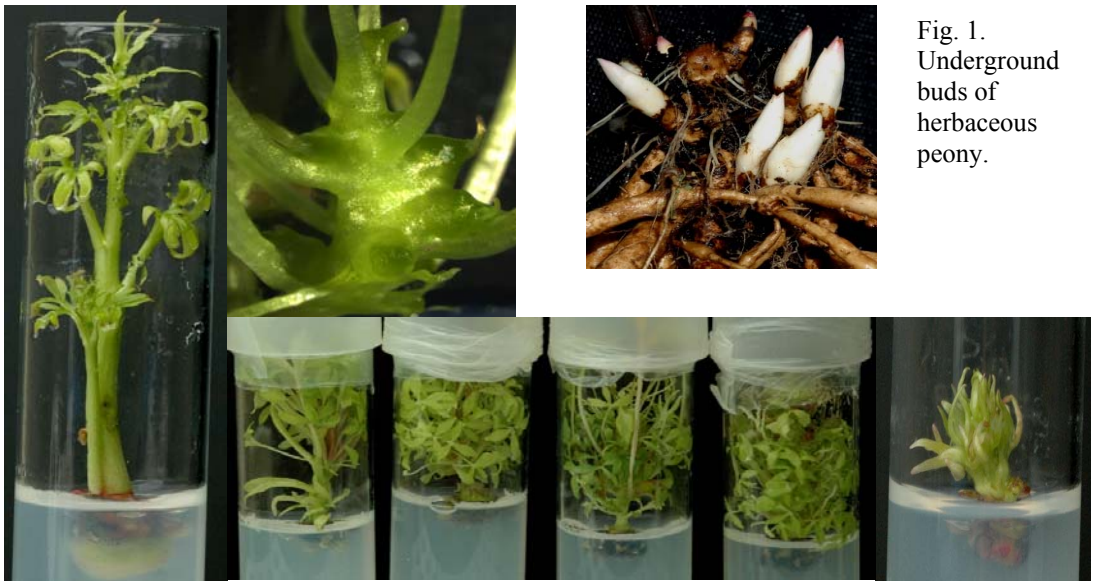

Fig. 1. Underground buds of herbaceous peony.

Fig. 2. After culture in ½ MS + 1 mg l^{-1} BA + 0.1 mg l^{-1} GA_3, the cooler treated buds (left) grew fast and produced elongated shoots (main and lateral). After cutting of shoots and subculture in ½ MS + 0.1 mg l^{-1} BA + 0.1 mg l^{-1} TDZ + 0.1 mg l^{-1} GA_3, the number of shoots was multiplied, each bud eye region formed one to several new shoots (middle). While bud explants from outside stock plants (right) grew slow and shoot stem did not elongated much.

Fig. 3. Shoots grew fast in half-solid medium and there was no difference in shoot length between treatments (with or without IBA shock, AC).

Fig. 4. Roots were only induced on the shoots with a 10-min pretreatment in high concentration of IBA (10 mg l^{-1}) before inoculation on medium with ½ MS + 1 mg l^{-1} IBA ± 5% AC (w/v).

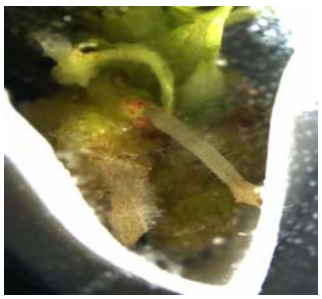

Fig. 5. Roots were induced from vitroshoots of 'Da Fu Gui' by paper bridge (white material) method with liquid medium: ½ MS + 1 mg l^{-1} IBA.

Fig. 6. After transplanting, rooted shoots grew in the first week but then died with contamination of fungi or rot of roots and stem.