POSTHARVEST STORAGE AND HANDLING OF RANUNCULUS ASIATICUS DRIED TUBEROUS ROOTS

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POSTHARVEST STORAGE AND HANDLING OF RANUNCULUS ASIATICUS DRIED TUBEROUS ROOTS

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Ranunculus asiaticus is an ornamental flowering plant with potential to be more widely used by the floriculture industry. Unfortunately, growers are faced with many challenges when producing these plants from their dry tuberous roots following storage; including poor sprouting, non-uniform growth, disease issues upon planting, as well as inconsistent cultural recommendations and lack of proper storage and handling protocols. Several experiments were conducted to determine the influence of temperature and relative humidity during storage on growth and quality of *R. asiaticus* plants. From our experiments it can be concluded that *R. asiaticus* tubers store best under low relative humidity and cool temperatures (above freezing). Also important from a storage perspective, unlike other flower bulbs, we show that *R. asiaticus* tuberous roots are not susceptible to ethylene damage while in the dry state. Prior to planting, tubers should be submerged in room-temperature water at around 20 ^{o}C, for 24 h, and then provided a fungicide treatment. We have shown that proper hydration temperature for *R. asiaticus* tuberous roots is critical for optimal growth. By following the protocol generated from our experiments, many of the production challenges associated with *R. asiaticus* tuberous roots may be avoided.

BIOGRAPHICAL SKETCH

Chris achieved his B.S. in horticulture from Michigan State University in 2002, his M.S. in environmental horticulture from University of Florida in 2006, and his Ph.D. from Cornell University in 2011. During his two years between his B.S. and M.S., Chris was a professional grower and propagation manager. From this experience, Chris developed an appreciation for applied horticulture research that continues to this day. He enjoys using his broad horticulture knowledge to diagnose and solve production challenges in the green industry and hopes to impact the art and science of horticulture through inspiration and leadership of others in the field.

Over the course of his academic and professional career, Chris has developed a strong appreciation for teaching, research, and extension, and recognizes the importance of scholarship in each area. He has taken coursework in curriculum development and leadership, and has designed and administered learning activities for many levels of study including elementary education, college undergraduate and graduate students, master gardeners, and growers. He finds his passion in learning and communicating horticulture findings with others. This is evident from his scholarly achievements including written works, oral presentations, extension and scholastic contributions, and to this point, co-authoring nearly a dozen grants with close to $70,000 secured for his research and the university.

This dissertation is dedicated to my wife, Meghan.

Thank you for believing in me. I love you.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

LITERATURE REVIEW

Introduction

Ranunculus asiaticus is an ornamental flowering plant with potential to be more widely used by the floriculture industry. Unfortunately, growers are faced with many challenges when producing these plants from their dry tuberous roots following storage; including poor sprouting, non-uniform growth, disease issues upon planting, as well as inconsistent cultural recommendations and lack of proper storage and handling protocols. The following literature review chronicles the available information on *R. asiaticus* production and addresses several hypotheses for the causes of these reported difficulties. Additional relevant supporting literature is also included.

Background

Ranunculus asiaticus (L.) is an ornamental geophyte found natively along hillsides in the eastern Mediterranean and southwest Asia. In this habitat, the active growth cycle occurs while the seasonal climate is cool and wet, with blooming from February to early May. During the hot and dry period of summer, the species enters a period of purported dormancy for approximately 5-6 months before growth resumes with regular rain during the fall (Meynet, 1993). Summer dormancy has been documented in several perennial species originating in the Mediterranean region and has been associated with desiccation tolerance, although the two phenomena appear to have evolved independently (Volaire and Norton, 2006).

R. asiaticus is the only commercially cultivated species of the genus, but is a member of the family Ranunculaceae (butter cup family), which contains many other important floriculture genera; such as *Aquilegia* L., *Anemone* L., *Consolida* Gray., *Delphinium* L., *Helleborus* L., and *Clematis* L. (Bailey Hortorium, 1976). *R. asiaticus* is a lesser-known, traditional cut flower and flowering pot or bedding plant which has shown increased use in spring gardening and landscape designs (Hamrick, 2003). The plants are commonly produced from seed, but can also be forced into flower from field-grown tuberous roots (for simplicity, hereafter referred to as "tubers").

Tissue culture has also been used successfully for propagation or *R. asiaticus* although it can be costly (Beruto and Debergh, 2004; Beruto et al., 1996). Growing *R. asiaticus* from dry tubers produces a more prolific and faster flowering crop than from seed, but micropropagated *R. asiaticus* plants produce more tubers per plant (Beruto and Debergh, 2004; Meynet, 1993). Light intensity influences *R. asiaticus* vegetative growth, flowering time, and tuber quality (size and weight) which were shown to improve when plants grown under full sun as compared shade (Hassan et al, 1984). Flower quality (stem diameter, length, and weight) was higher with shade-grown plants than full sun (Hassan et al., 1984). In these studies increasing photoperiod improved all measured variables regardless of light intensity, but 11 h was the longest day length tested. Farina et al. (1985) investigated *R. asiaticus* photoperiod and found long days (14 h) to give both earlier flowering and dormancy onset than short days (11 h); short days gave a longer period of vegetative growth and opportunity for second set of flowering, but plants were only receptive to photoperiod when grown at 9-16 ^{o}C. Ohkawa (1986) found floral initiation to be more advanced under long days, but the accelerated elongation of the inflorescence produced shorter, lighter, and fewer double flowers. Meynet (1993) suggested an antagonistic relationship between shoot growth

and tuberization, and that long days (13 h) induces tuberization when soil temperature is above 18 ^{o}C; young plants will only form tubers at 25 ^{o}C, or higher (Meynet, 1993).

R. asiaticus tubers are well adapted to lengthy dry storage due to their desiccation tolerance and have been labeled "resurrection geophytes" (Kamenetsky et al., 2005; Beruto et al., 2009). Antipov and Romanyak (1983) suggest *R. asiaticus* tubers may be stored indefinitely in the air-dry state. Unfortunately, this does not seem to be the reality in a commercial setting. Growers have reported a number of problems when forcing *R. asiaticus* tubers following storage. Some of the difficulties reported to us include poor sprouting, non-uniform growth and subsequently poor stand establishment, and early flowering on weak stems (M.A. Mellano; Y. Liberman, personal communication). Most of the cultural recommendations for *R. asiaticus* are limited to growth following tuber planting, with almost no research available on tuber storage protocols (Hamrick, 2003; De Hertogh, 1996; Meynet, 1993).

The storage environment

Ethylene

It has been suggested that ethylene exposure could inhibit tuber rooting and lead to plant decay (Meynet, 1993). It is therefore possible that some of the observed problems might be partially explained by ethylene exposure to *R. asiaticus* roots during storage. Kenza et al. (2000) found that *R. asiaticus* cut flowers produce ethylene naturally when harvested but are essentially insensitive to exogenous ethylene applications. The flowers of other plants in the Ranunculaceae family have been shown to have high ethylene sensitivity, including *Aconitum napellus* L., *Anemone* X *hybrida*, *Delphinium ajacis*, and *Nigella damascena* L. (Woltering and Van Doorn, 1988). While tubers and flowers may exhibit different ethylene sensitivity, clear information on the influence of ethylene during dry *R. asiaticus* tuber

storage, or post-hydration, is lacking. It is also possible that hydration state of tubers may play a role in ethylene sensitivity. For example, lettuce seeds (*Lactuca sativa* L. var. Grand Rapids) have shown enhanced germination in response to ethylene exposure when freshly imbibed, but not when dry (Abeles and Lonski, 1969).

Ethylene sensitivity varies greatly among ornamental geophyte storage organs (Kamerbeek and de Munk, 1976). Easter lily (*Lilium longiflorum* Thunb.) bulbs exposed to 2 $\mu L \cdot L^{-1}$ ethylene during vernalization showed a decrease in flower number (Prince and Cunningham, 1991), but not when bulbs were exposed to ethylene post-vernalization. Tulip (*Tulipa gesneriana* L.) bulbs are well known for their ethylene sensitivity, where concentrations as low as 0.1 $\mu L \cdot L^{-1}$ during the summer storage season can cause a number of physiological problems (Kamerbeek et al., 1971); which may include gummosis (excretion of polysaccharides causing bulbs to stick together), loss of fresh weight during storage, reduced or eliminated roots, shortened leaves or flower stems, deformed anthers, flower bud abortion, and excessive growth of daughter bulbs (splitting) (Kamerbeek and de Munk 1976). The degree of ethylene damage depends on a number of factors including: concentration, duration, temperature during exposure, and cultivar (Cerveny and Miller, 2010; Miller et al. 2004; de Wild et al. 2002; De Munk 1973; De Munk 1972).

Ethylene is often introduced to plants inadvertently from loading equipment or greenhouse heaters, but may come from a variety of sources (Arshad and Frankenberger, 2001). A significant source of ethylene in the flower bulb industry is the fungal pathogen Fusarium (*Fusarium oxysporum Schlecht f. sp. tulipae)*, which produces ethylene when colonizing tulip bulbs (Kamerbeek and de Munk, 1976). These fungal infections therefore create a unique production challenge for tulip growers. De Munk and de Rooy (1971) observed smaller plants, decreased rooting, and aborted flowers when ethylene effects on tulip growth were measured by planting

Fusarium infected bulbs alongside healthy tulips in soil culture. Cerveny and Miller (2010) reported similar findings in tulips grown in a hydroponic system. It is possible the ethylene-related growth problems occur in other substrata as well, including sand or soilless mixes as ethylene concentrations emanating from *Fusarium* infected bulbs increase as the gas becomes "trapped" due to low air movement around soil particles, thick canopy density, or other factors limiting diffusion surrounding individual plants (King and Smith, 1987). These influences could therefore contribute to high ethylene concentrations in isolated locations throughout a commercial greenhouse or in the storage environment. Meynet (1993) reported a *Ranunculus*-specific *Fusarium* (*F. oxysporum* f.sp. *ranunculi*) that colonizes tubers in the field and causes dwarfness and plant decay. It is not known whether this particular *Fusarium* produces ethylene when colonizing *R. asiaticus*.

Because of the high sensitivity of many flower bulbs to ethylene, its exposure should be limited. Some chemical methods have been used to control ethylene synthesis, action, or perception. Potassium permanganate is a powerful oxidant that acts as an ethylene "scrubber", removing it from the storage environment (Hernandez et al, 2007). Silver thiosulfate (STS) has long been used in the cutflower industry to prevent damage in tissues sensitive to ethylene; and has been shown to protect other Ranunculaceae flowers from abscission (Woltering and Van Doorn, 1988). STS is considered a permanent means for preventing ethylene action in plant tissues. 1-Methylcyclopropene (1-MCP) has become widely used in postharvest horticulture, with gaining popularity for ornamental plants (Watkins, 2006; Blankenship and Dole, 2003). With 1-MCP, ethylene perception is inhibited for variable periods which can work to the benefit of the handler (Sisler and Serek, 1997). In postharvest handling of flowerbulbs, temporary relief from potential ethylene problems (such as in shipping) can be provided through application of 1-MCP, but plants can later resume growth

(which is not possible with STS). Gude and Dijkema (2005) demonstrated protection from ethylene damage in tulip storage by 24 h 1-MCP applications (0.2 $\mu L \cdot L^{-1}$) at 12 day intervals. Cerveny and Miller (2010) investigated 24 h 1-MCP applications prior to growing tulip under constant ethylene exposure at 1 $\mu L \cdot L^{-1}$. Under these conditions 1-MCP pretreatment was effective at reducing ethylene injury for at least 1 week during the earliest phases of growth and establishment in the greenhouse. The potential for 1-MCP application in *R. asiaticus* tubers should be investigated if ethylene sensitivity is confirmed.

Temperature and relative humidity

Another possibility for the aforementioned problems with *R. asiaticus* growth could be related to the relative humidity during storage and its interaction with temperature (M.A. Mellano, personal communication). It is hypothesized that "high" humidity levels during storage might result in the decline in tuber quality, as is common in orthodox seeds (Copeland and McDonald, 2001; Bewley and Black, 1994; Priestley, 1986). One dry storage recommendation of 15-25 ^{o}C at 50% relative humidity (RH) has been suggested (Meynet, 1993), however this temperature range is broad and would presumably have differing effects based on RH changes with temperature. Specific data on storage humidity have not been reported; however, Beruto et al. (2009) recently described a decrease in *R. asiaticus* tuber viability when stored one year at room temperature (ca. 20 ^{o}C) under ambient conditions as compared to 2 ^{o}C storage. In that experiment humidity was not controlled but was monitored in the room temperature treatments to be 60-70%; humidity under 2 ^{o}C treatments was not reported. Beruto et al. (2009) also tested the influence of modified storage (2% O_2, 4% CO_2) at 2 ^{o}C on tuber viability over one year but found no differences in survival when compared to ambient storage at 2 ^{o}C. Their hypothesis was that

because tubers are dormant during storage, respiration is probably very low, thus the modified atmosphere had negligible influence on slowing this metabolic activity. Respiration was not measured in their study.

The interaction between water and seeds is usually expressed by moisture sorption isotherms, which are obtained by measuring equilibrium moisture content as a function of relative humidity at constant temperature (Priestley, 1986). These curves have a characteristic sigmoid shape which permits expression of three distinct regions of hydration, or "zones". In zone I, tissue moisture content increases rapidly with rising humidity and then slows in zone II. In zone III, moisture content again rises rapidly with increased humidity. Internal composition of seeds (starch or oil) can affect the equilibrium moisture content, but the inflection points between zones are relatively similar among orthodox seeds (Priestley, 1986).

Priestley (1986) diagramed evidence of cellular activity at various levels of hydration in orthodox seeds. He concluded that seed respiration is only feasible in late zone II or in zone III, but indicated these data are often confounded by various microbes living on the seeds which become active at these moisture levels and above. When active, these microorganisms can contribute significantly to the respiratory gas exchange in the storage environment.

Loss of seed quality during storage is common when efficient management of temperature and moisture is not provided. Most orthodox seeds will maintain viability without significant degradation for many years if held cool and dry (Priestley, 1986). Oftentimes seed vigor is measured through germination studies, though this is not always the most accurate means of predicting viability. Ability of orthodox seeds to germinate over time can usually be expressed using a negative sigmoid curve which indicates a slow decline in germination with years of storage but then enters a period of rapid viability loss before slowing again with extended storage. Accelerated aging

treatments can be used to predict long-term storability of seeds. When seeds are held at 35-45 ^{o}C and up to 100 % relative humidity, viability is lost in a matter of days rather than months or years, as would occur in open storage. The disadvantage of using these treatments to predict storability is the tail-ends of the previously described negative-sigmoid curve tend to be cut off; indicating a proportionally faster decline than might occur naturally (Priestley, 1986).

Control of relative humidity in specialized chambers may be accomplished through a variety of means. One option is to use saturated salts, above which the headspace is known to equilibrate to a given relative humidity. Winston and Bates (1960) provided a list of nearly 100 substances above which the relative humidity is known. For example, the equilibrium relative humidity above a saturated solution of $MgSO_4$ is 89% when held at 25 ^{o}C. The disadvantage of using saturated salts in controlling relative humidity is that many are toxic and require specialized handling and disposal. Another option is to use glycerol and water mixed at specific ratios (Forney and Brandl, 1992). As the specific gravity of the glycerol solution increases (i.e. greater proportion of glycerol), the equilibrium relative humidity in the headspace above the solution decreases. These solutions then may be used to humidify air in both dynamic (flow-through) and closed (sealed) systems; although the lowest level of relative humidity achievable varies by system (A.G. Taylor, personal communication).

Planting

Tuber disinfection

The tuberous roots of *R. asiaticus* often become infected with a variety of soil-borne diseases during field production that are still present after storage (Meynet, 1993). Some of these infections include: *Fusarium tabicinum, F. oxysporum* f. sp. *ranunculi, Pythium sylvaticum, P. debaryanum, Itersonilia spp., Erysiphe polgoni,* and

cucumber mosaic virus, tobacco necrosis virus, tobacco rattle virus, potato virus y.f. ranunculi, and tomato spotted wilt virus (Meynet, 1993). Several of the previously mentioned fungal and bacterial pathogens have been isolated from samples in our lab on *R. asiaticus* tubers originating from field-grown sources (Cornell University, Plant Disease Diagnostics Clinic, personal communication). Disinfection of tubers is therefore an important step in the growing process. Meynet (1993) suggested soaking tubers for 3 h in a benomyl or carbendazim fungicide solution at 1000 $mg \cdot L^{-1}$ prior to planting. Umiel and Hagiladi (1999) recommended a 20 min soak for pre-hydrated tubers (after 24 h hydration) in Captan fungicide (N-trichtoromethylthio cyclohexene-1,2-dicarboximide) mixed at 2600 $mg \cdot L^{-1}$ a.i., followed by a soil drench of half-strength solution. The Cornell University Plant Disease Diagnostics Clinic (personal communication) recommended a biocide, Phyton 27 (copper sulfate) (Phyton-27, Phyton Corp., New Hope, MN). We have had good results when dipping pre-hydrated *R. asiaticus* tubers for 5 min in Phyton 27 mixed at 1375 $mg \cdot L^{-1}$ metallic copper; a recommended concentration for treating Calla lilies (Phyton Corp., 2004). Dilute bleach is often mentioned as a possible disinfectant in other horticultural applications, but is not currently labeled for commercial greenhouse use.

Hot water immersion treatments (HWT) and hot water rinsing and brushing (HWRB) have been used to kill pathogens that cause surface decay in sweet potatoes (*Ipomoea batatas* L. Lam.) and other produce, which helps maintain quality during storage (Fallik, 2004). HWT are applied at temperatures 43-53 ^{o}C for up to 2 h, while HWRB are provided for 10-25 s at 48-63 ^{o}C (Fallik, 2004). These treatments may be effective for surface sanitation of *R. asiaticus,* but would need to be tested on pre-hydrated tubers as the rapid influx of hot water with dry tissue may cause imbibition stress.

Tuber hydration

As previously stated, the published literature available for handling *R. asiaticus* tubers is limited. The two key reference texts for bulb crop production, Holland Bulb Forcers Guide (DeHertogh, 1996) and The Physiology of Flower Bulbs (Meynet, 1993) differ in the recommended planting procedures for *R. asiaticus* tubers. Meynet (1993) suggested that non-dormant tubers should be planted directly in a moist medium and grown at 16 ^{o}C (61 ^{o}F). He suggested the dormancy period should first be broken by storing the tubers 2 months at 25 ^{o}C (77 ^{o}F), 10 days at 35 ^{o}C (95 ^{o}F), or 2 days at 40 ^{o}C (104 ^{o}F), but when stored at 2 ^{o}C (36 ^{o}F), dormancy lasted for more than 6 months. DeHertogh (1996) suggested *R. asiaticus* tubers should be soaked in slowly running water, provided a cold treatment, and "pre-sprouted" prior to planting. Okhawa (1986) researched the influence of cold storage on pre-soaked tubers by hydrating in water for 8 h at 6 ^{o}C (43 ^{o}F) and then providing cold treatments (5 ^{o}C; 41 ^{o}F). He found 4-6 weeks of cold hastened flowering and is now a commonly recommended treatment by some industry professionals (Y. Liberman, personal communication). Revisiting some of these differing suggestions through objective research is important for establishing unified recommendations to growers.

An important first step in identifying a protocol for *R. asiaticus* planting is to determine the rate at which the tubers imbibe water when submerged. Since many factors could influence the rate of water uptake, an appropriate model must be selected. Mathematical expressions have been used to model water sorption in food products since hydration is often necessary for cooking. In most cases, these models contain one or more constants designed to predict the rate of hydration and a maximum, or equilibrium moisture content. The result is a characteristic asymptotic curve with rapid initial water uptake that slows as the moisture content reaches equilibrium. Krokida and Marinos-Kouris (2003) used a differential expression for

modeling water sorption in dehydrated fruits and vegetables for use in cooking applications. Singh and Kulshrestha (1987) used a similar expression but employed a linearized version for predicting water uptake in soybean (*Glycine max* L.) and pigeon pea (Cajanus cajan L. Misp.). One of the more popular models for describing moisture sorption curves is the Peleg model (Peleg, 1988). The Peleg model has been used to describe moisture sorption in seeds of kidney bean (*Phaseolus vulgaris* L.), chick pea (*Cicer arietinum* L.), and field pea (*Pisum sativum* L.), along with rice (*Oryza sativa* L.), cereal grains, and other food products including dehydrated milk powders and breakfast cereals (Prasad et al., 2010; Bello et al., 2008; Sarchetti et al., 2003; Turhan et al., 2002; Abu-Ghannam and McKenna, 1997; Hung et al., 1993; Sopade et al., 1992; Peleg, 1988). The advantage of using Peleg's model for estimating moisture uptake is the ability to predict long-range moisture gains from relatively short duration experiments (Peleg, 1988).

One of the challenges in using any empirical model to predict equilibrium moisture content is that the theoretical maximum water uptake is usually not technically feasible. Besides the impracticality of soaking tissue for the duration necessary to achieve maximum saturation (>100 h, depending on tissue) there would presumably be an eventual loss of soluble solids and decay, leading to loss of fresh weight. Alternatively the tissue might eventually commence growth, resume rapid accumulation of fresh weight, and therefore pass the estimated value of equilibrium moisture content. To avoid these pitfalls, one option is to set an hydration threshold as a cut-off for practical applications (i.e. 75% of maximum).

Some researchers have noted that the range of data selected for inclusion in a model affects the estimated values of the derived parameters, as well as the overall model fit (Peleg, 1988; Sopade et al., 1992; Turhan et al. 2002). Turhan (2002) explained how points for inclusion in a model should be selected from the curved-

portion of the imbibition period and that including many values past the linear phase is unnecessary for improving the model fit.

Commonly, tissues that undergo faster initial water uptake, such as with elevated water temperature, will have lower equilibrium moisture content. This trend was reported when modeling water sorption in chickpea and kidney bean at increasing temperatures (Turhan et al., 2002; Abu-Ghannam and McKenna, 1997). It is important to point out that much of the reviewed literature on modeling water sorption was published for the purposes of cooking (i.e. soaking dry beans prior to eating) and those temperatures tested are beyond what would be considered practical for plant growth. Nevertheless, the empirical models still apply to lower hydration temperatures.

Some seeds exhibit viability loss when hydrated at cold temperatures, which causes physiological damage to the cellular membranes (chilling injury). Pollock and Toole (1966) thought chilling injury in lima bean (*Phaseolus lunatus* L.) caused physical damage to cellular membranes resulting in their rupture. Christiansen (1968) hypothesized that cold prevents a metabolic response in cottonseed (*Gossypium hirsutum* L.) rather than inducing direct physical damage because damage was additive with increased cold duration. Powell and Mathews (1978) hypothesized that so-called chilling injury is the result of imbibition damage rather than the effects of low temperature. This hypothesis was supported in pea (*Pisum sativum* L.) that had seed coats sliced open to allow more rapid imbibition, but reducing water absorption rate through osmotic inhibitors lessened the degree of injury (Tully et al., 1981). The current thinking is that the cold temperature slows a membrane phase transition during hydration, thus allowing damaging rates of hydration and/or excessive leakage of vital nutrients for growth (Copeland and McDonald, 2001).

When soybean seeds (*Glycine max* L.) were given "priming" treatments (exposed to periods of brief hydration and re-drying at temperatures above which chilling injury occurs [25 ^{o}C]), the cellular damage was lessened when seeds were later soaked in 4 ^{o}C water (Tilden and West, 1985). In one preliminary experiment dry *R. asiaticus* tubers had improved sprouting when hydrated 24 h at 25 ^{o}C, allowed to re-dry at the same temperature, and then re-hydrated 24 h at 25 ^{o}C, compared to those given a single hydration period (24 h at 25 ^{o}C) (Cerveny and Miller, unpublished data). It is not known if priming treatments would be effective in preventing hydration injury in *R. asiaticus*. It will be important to investigate the roll of this hydration temperature in promoting or inhibiting subsequent growth of *R. asiaticus* dried tubers.

Tuber sprouting and growth

As previously mentioned, several researchers have indicated *R. asiaticus* tubers may be dormant during storage (Meynet, 1993; Beruto et al., 2009). It is possible that some of the observed problems with sprouting could be related to forcing dormant tubers rather than planting non-viable tissue.

Potato tubers will not sprout when freshly harvested because of dormant apical buds (called tuber dormancy) but have been shown to break dormancy when provided ethanol (Claassens, et al., 2005). This treatment has also been effective in Jerusalem artichoke tubers (Petel et al., 1993) and in seeds; such as rice (Cohn et al., 1989) and oat (*Avena sativa* L.) (Corbineau et al., 1991). It has been questioned whether dormancy breaking in potato is under hormonal control or if it's related to carbohydrate changes and enzymatic reactions (Claassens and Vreugdenhil, 2000). Classens et al. (2005), showed that ethanol breaks potato tuber dormancy and is blocked by an inhibitor of alcohol dehydrogenase activity, but application of products derived from alcohol dehydrogenase activity (acetaldehyde and acetic acid) were not

effective at breaking dormancy. Ethanol may have practical applicability in *R. asiaticus* for both breaking tuber dormancy and with surface sanitation.

Seed dormancy has been shown to be broken by treating with gibberellins (GA_3), such as in tomato (Groot and Karssen, 1987) and *Arabidopsis* (Koornneef and Van der Veen, 1980), but is not effective in breaking potato tuber dormancy (Claassens et al., 2005). *R. asiaticus* seeds germinate better if given a cold treatment (10 d at 6 ^{o}C) than without; GA_3 was not effective at replacing cold treatment when temperatures were below 26 ^{o}C; 15 ^{o}C is optimum germination temperature (Plenkers-Schneider et al, 1991). Once sprouted, *R. asiaticus* plants have shown improved vegetative growth, flowering time, and nutrient uptake when GA_3 sprays were provided at 200 $mg \cdot L^{-1}$ shortly after sprouting (Hassan et al., 1984b). Application of GA_3 to "dormant" tubers has not been reported.

Other relevant literature

Desiccation tolerance

When considering the enormous number of plant species on Earth, desiccation tolerance in the leaves, stems, and roots of plants is relatively unusual; however, many species of plants produce seeds which are able to tolerate extended dry periods where they are at or below equilibrium moisture content with the ambient environment, called "orthodox seeds" (Scott, 2000; Priestley, 1986). These orthodox seeds seem to have a particular window for tolerance. Seeds that would otherwise be desiccation tolerant, will not germinate if dried before reaching a specific stage of maturity ("maturation drying")(Adams et al., 1983), nor will they survive drying if germination has progressed too far, and will not continue to develop upon rehydration (Senaratna and McKersie, 1983). It should also be mentioned that "dry" seeds are rarely devoid

of all water, rather maintain 4 to 16% moisture when held in open storage; the range is affected by relative humidity in the storage environment (Priestley, 1986).

It has been widely hypothesized that sucrose, a soluble carbohydrate commonly found in seed embryos, could assume a protective role during desiccation (Amuti and Pollard, 1977; Senaratna and McKersie, 1983; Hoekstra and Van Roekel, 1988). Trehalose has also been discussed as having a water-replacing ability (the suggested method of protection) in artificial membrane systems, but has not been reported to occur in the seeds of flowering plants (Crowe et al, 1984; Crowe et al, 1987). The specific mechanism by which all species are able to tolerate desiccation remains vague, and is more likely a result of protection and repair mechanisms working in tandem (Bewley, 1995).

A correlation between sugar content in seeds and the loss of desiccation tolerance has been investigated in some agronomic plants (Koster and Leopold, 1988). Researchers showed that oligosaccharides (including sucrose) were present during desiccation tolerance and were diminished as this tolerance was lost. Lin et al. (1998), looked into the role of oligosaccharides in desiccation tolerance and found that imbibed seed parts did not show differences in their ability to synthesize sugars during slow dehydration and that the ratio of sucrose/oligosaccharides varied among species of intact seeds at the time of losing desiccation tolerance. They concluded that sucrose and other oligosaccharides are not responsible for loss of desiccation tolerance in hydrated seeds.

Pukaka (2001) looked at the changes in carbohydrate content of Norway Maple (*Acer platanoides* L.) seeds at the time desiccation tolerance was lost. Monosaccharide levels for glucose, fructose, and galactose were shown to increase with loss of desiccation tolerance, but sucrose content diminished significantly in embryonic axes where the desiccation tolerance was lost. Pukaka (2001) has the

shared opinion of Koster and Leopold (1988) that the increase in monosaccharides (reducing sugars) could contribute to cell damage during dehydration, but he concludes that ultimately the ability of the seed to survive desiccation is probably related to the mechanism to prevent damages from free radical action instead of the relationship between sucrose and other oligosaccharides.

When Beruto et al. (2009) investigated the carbohydrate content of *R. asiaticus* tubers they identified glucose, fructose, and arabinose. Following one year cold storage (2 ^{o}C) glucose and fructose levels were unchanged, but arabinose content increased. The authors attributed the arabinose increase to cell wall thickening and hypothesized its involvement in stress adaptation. The degree of arabinose increase varied by cultivar but no correlation was found between arabinose content and subsequent greenhouse performance (Beruto et al, 2009). Kamenetsky et al. (2005) found pectin accumulating in distinct layers to the inside of primary cell walls during *R. asiaticus* tuber development. They reported a loss of starch and protein upon hydration and a decrease in cell wall thickness. The protein and pectin were purported to be protection mechanisms during rehydration (Kamenetsky et al., 2005). Further studies should be conducted to further investigate the mechanisms for desiccation tolerance in *R. asiaticus*, the role of these compounds in purported tuber dormancy, and their influence on growth of tubers.

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CHAPTER 2

ETHYLENE EXPOSURE IS NOT HARMFUL TO RANUNCULUS ASIATICUS TUBEROUS ROOTS

Abstract

We investigated the influence of ethylene exposure on dry or hydrated tuberous roots of the ornamental geophyte *Ranunculus asiaticus*. Ethylene concentrations up to 10 $\mu L \cdot L^{-1}$ for 3 weeks in the dry state or at least 1 week in the hydrated state had negligible influence on post-planting growth in any of the observed parameters. We therefore conclude that *Ranunculus asiaticus* tuberous roots are ethylene insensitive and special precautions to avoid ethylene exposure are unnecessary.

Keywords: C_2H_4, Persian Buttercup, Ornamental geophyte

Introduction

Ranunculus asiaticus L. is an ornamental geophyte known for its showy flowers and desiccation tolerant tuberous roots (hereafter simply referred to as "tubers"). *R. asiaticus* is commonly produced by seed, but can also be forced into flower from field-grown tubers which produce a much faster flowering crop (Meynet, 1993). Growers have expressed concern about inconsistent rooting and non-uniform growth habit when producing *R. asiaticus* flowering plants from dried tubers following storage. It has been suggested (Meynet, 1993) that ethylene exposure could inhibit tuber rooting and lead to plant decay, although no data were presented. Kenza et al. (2000) found that *R. asiaticus* cut flowers are essentially insensitive to ethylene. While tubers and flowers may exhibit different ethylene sensitivity, clear information on the influence of ethylene during dry *R. asiaticus* tuber storage, or post-hydration, is

lacking. Therefore, a series of experiments were conducted to illustrate the effects of ethylene on *R. asiaticus* tubers in several typical commercial situations.

Materials and methods

Dried tubers of *Ranunculus asiaticus* 'Tecolote Pink' were obtained from a commercial grower in California, USA, after a normal spring harvest. Tubers from the 2007/2008 growing season were handled according to common commercial practice and were stored at 15 ^{o}C with low relative humidity until treatments were administered. All ethylene experiments were conducted in the same manner except where noted. All treatments were applied in a growth chamber with a 20 ^{o}C set-point temperature.

R. asiaticus tubers (dry or hydrated) were treated with flow-through ethylene for one week at nominal concentrations (± 0.5%) of 0, 1, 5, or 10 $\mu L \cdot L^{-1}$, in plastic storage containers. Ethylene concentrations were verified by daily measurements using a Buck 310 gas chromatograph (Buck Scientific, East Norwalk, CT, USA) fitted with an alumina column. Tubers were hydrated prior to planting (or ethylene exposure), by submerging in tap water for 24h and then for 5 min in a copper sulfate preparation (Phyton-27, Phyton Corp., New Hope, MN, USA) at a metallic copper concentration of 1.4 $g \cdot L^{-1}$. Tubers received a single hydration and copper sulfate treatment, thus the tubers exposed to ethylene in the hydrated state were not re-hydrated prior to planting. Tubers were planted in 7.5 cm square containers using a commercial potting mix (Sun Gro LC1), with crowns covered by approximately 2 cm mix. Containers were then placed in a cooler for 4 or 6 weeks at 5 ^{o}C prior to growing in a greenhouse with a 17 ^{o}C set-point temperature. Plants were organized in a completely randomized design with 6 replicates of 4 sub-samples (averaged) per

treatment (n=6). All data were subjected to analysis of variance using standard least squares method in JMP (SAS Institute, Cary, NC, USA).

Experiment 1

To observe the influence of ethylene exposure prior to planting on fully hydrated or dried tubers, two separate rounds of treatments were applied. In the first round, treatments were initiated on 23 Sept. Tubers were treated with ethylene for 1 week and planted on 1 Oct. After 4 weeks at 5 ^{o}C, plants were moved to the greenhouse. In the second round, ethylene treatments were initiated on 1 Oct., treated for 1 week and planted on 9 Oct. After 4 weeks at 5 ^{o}C plants were moved to the greenhouse. Percent mortality (no visible growth) was determined after 4 weeks in the greenhouse. After 11 weeks of growth, the following above ground observations were made: plant size (mean of 3 lengths: plant height, and two perpendicular canopy diameter measurements), number of flowers per plant, and dry weight.

Experiment 2

In a second experiment, longer durations of ethylene exposure were provided to dry tubers only. Fully hydrated tubers were not able to be held at 20 ^{o}C in the hydrated state for the duration of this experiment. A single round of treatments was initiated on 16 Oct. Tubers were exposed to ethylene for 2 or 3 weeks with treatments terminating on 30 Oct., and 6 Nov., respectively. Dried tubers exposed to ethylene for 2 weeks were removed from treatment containers, then maintained ventilated for 2 additional weeks, until the 3 week treatments were removed from ethylene exposure and given ventilated storage for one week. After hydration and copper application, all treatments were planted 18 Nov., held 6 weeks at 5 ^{o}C, and placed in the greenhouse on 29 Dec. Data were recorded as in experiment 1, except percent mortality and plant

size were determined after 4 weeks and final observations were made after 8 weeks. Additionally, a visual ranking of plant quality was made, but differences were not significant (data not shown).

Results and discussion

Experiment 1

At the concentrations tested, ethylene exposure to dry or hydrated tubers had little effect on mortality, plant size, tissue dry weight, or number of flowers present at harvest (Table 2.1). In round 1, the number of flowering stems increased when dry ranunculus tubers were exposed to ethylene at any concentration, with marginal significance in hydrated tubers (Table 2.1). These differences were not significant in round 2 or in experiment 2. Additionally, the means presented in Table 2.1 for round 2 dry tubers appear to suggest a slight trend toward increasing plant mortality, plant size, and plant dry weight with increasing ethylene concentration; however these differences were not statistically significant. Ultimately, any differences between treatments were not consistent in repeat experiments and are therefore considered different by happenstance. Regardless, in no case was ethylene shown to cause damage to ranunculus tubers.

Ethylene sensitivity varies greatly among ornamental geophyte storage organs (Kamerbeek and de Munk, 1976). For example, tulip (*Tulipa gesneriana* L.) bulbs are well known for their ethylene sensitivity, where ethylene concentrations as low as 0.1 $\mu L \cdot L^{-1}$ during the summer storage season can cause a number of physiological problems (Kamerbeek et al., 1971). Easter lily (*Lilium longiflorum* Thunb.) bulbs exposed to 2 $\mu L \cdot L^{-1}$ ethylene during vernalization showed a decrease in flower number (Prince, 1991), but not when bulbs were exposed to ethylene post-vernalization.

Table 2.1. Effect of ethylene (0 to 10 $\mu L \cdot L^{-1}$) on dry or hydrated *Ranunculus asiaticus* 'Tecolote Pink' tubers given for 7 days immediately prior to planting. Percent mortality (no visible growth) was determined after 4 weeks in the greenhouse. Size (average of height and two perpendicular canopy diameter measurements), number of flower stems, and plant dry weight (shoot biomass) were determined after 11 weeks in the greenhouse for round 1, or after 8 weeks in round 2.

Round 1	Dry tubers				Hydrated tubers			
Eth. concn. ($\mu L \cdot L^{-1}$)	Mortality[z] (%)	Size (cm)	Flowers	Plant dry weight (g)	Mortality[z] (%)	Size (cm)	Flowers	Plant dry weight (g)
0	25.0	37.4	0.5	3.6	12.5	39.8	2.9	2.9
1	16.7	41.6	2.5	3.7	8.3	40.2	3.7	3.7
5	25.0	38.4	1.8	4.0	16.7	46.5	4.7	4.7
10	25.0	36.1	2.5	3.7	4.2	44.9	4.6	4.6
p-value	0.9004	0.5131	0.0223	0.9861	0.5117	0.1676	0.0928	0.1150
Significance[y]	NS	NS	*	NS	NS	NS	NS	NS

Round 2	Dry tubers				Hydrated tubers			
Eth. concn. ($\mu L \cdot L^{-1}$)	Mortality[z] (%)	Size (cm)	Flowers	Plant dry weight (g)	Mortality[z] (%)	Size (cm)	Flowers	Plant dry weight (g)
0	25.0	33.7	2.6	3.2	0	35.2	2.0	3.6
1	25.0	38.7	3.0	4.4	0	37.5	1.8	3.6
5	12.5	40.8	1.9	4.1	0	44.4	2.2	4.8
10	8.3	41.1	1.7	3.1	4.2	39.2	2.2	4.0
p-value	0.4331	0.1710	0.1526	0.3047	0.4133	0.1010	0.9352	0.2430
Significance[y]	NS	NS	NS	NS	NS	NS	NS	NS

[z]Indicates data collected after 4 weeks in greenhouse

[y]Indicates not significant (NS) or significant (*) at $\alpha = 0.05$.

Hydration state would logically be expected to play a role in ethylene sensitivity. For example, lettuce seeds (*Lactuca sativa* L. var. Grand Rapids) are perceptive to ethylene when freshly imbibed, but not when dry (Abeles and Lonski, 1969). In our experiment, differences in response to ethylene among dry and hydrated tubers were not significant at the concentrations tested.

Experiment 2

In the second experiment, the duration of ethylene exposure was increased to 2 or 3 weeks. In this experiment, only dry tubers were used, owing to excessive microbial growth observed on hydrated tubers in preliminary experiments. There were no significant differences among ethylene concentrations or duration of exposure for mortality, plant size, number of flowers, or plant dry weight, which averaged 12%, 28.6 cm, 2, or 4.1 g, respectively.

Commercial geophyte producers are often concerned with ethylene levels in the post-harvest environment. Under the conditions of our experiments, *Ranunculus asiaticus* 'Tecolote Pink' tuberous roots did not exhibit any deleterious effects associated with as much as 10 $\mu L \cdot L^{-1}$ ethylene when exposed for up to 3 weeks in a dry state or after 1 week in the hydrated state. Our data contradict statements by Meynet (1993) suggesting that ranunculus tuberous roots are ethylene-sensitive. We conclude that *Ranunculus asiaticus* tuberous roots are ethylene insensitive, and therefore special precautions to avoid ethylene exposure prior to planting of dry or hydrated tubers are unnecessary.

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CHAPTER 3

RESPIRATION AND SUBSEQUENT VIABILITY OF RANUNCULUS ASIATICUS DRY TUBEROUS ROOTS ARE AFFECTED BY STORAGE TEMPERATURE AND MOISTURE CONTENT

Abstract

The ornamental geophyte *Ranunculus asiaticus* was investigated to determine the influence of temperature and relative humidity during storage on viability of its desiccation tolerant dry tuberous roots. Tubers were stored in specialized chambers with glycerol-water solutions mixed to specific ratios for controlling relative humidity under both flow-through and closed storage. In the flow-through system, air was bubbled through glycerol-water solutions, which then was passed through storage chambers held at 5, 20, or 35 ^{o}C for 20 weeks. In closed storage, tubers were equilibrated to a given relative humidity treatment at 15 ^{o}C by suspending tubers over the glycerol-water solutions with fans used to circulate air; these containers were closed for 4 weeks and then tissue was transferred to sealed jars for the duration of temperature treatments (5 or 25 ^{o}C) for 16 or 17 weeks, depending on experiment. Tubers stored under elevated temperature and relative humidity showed the largest decrease in percent survival in both systems. Results from flow-through storage were more variable with regard to tuber moisture content (fresh basis) than closed storage. When respiration rate was measured in tubers held under closed storage, those stored at 25 ^{o}C had higher respiration rates than at 5 ^{o}C; elevated moisture content also led to increased respiration. When tubers were stored at 15 ^{o}C over desiccant (anhydrous calcium sulfate), there were no differences in plant survival or other quality parameters as compared to the control. From these results it can be concluded that for long-term viability, *R. asiaticus* tubers should be stored cool and dry.

Introduction

Ranunculus asiaticus (L.) is a desiccation tolerant ornamental geophyte known for its showy flowers and dry tuberous roots (for simplicity, hereafter referred to as "tubers"). *R. asiaticus* is native to the eastern Mediterranean, in areas with a cool, wet winter and hot, dry summer. In their native habitat or similar climate, the plants are commonly grown in the field for one or more seasons before the tubers are harvested and sold to flower growers worldwide. This process allows for tuber size to be increased, before commercial distribution. Growing *R. asiaticus* from dry tubers reportedly has the advantage of faster and more profuse flowering than when grown from seed (Meynet, 1993). Traditionally, *R. asiaticus* tubers are handled by distributors of other ornamental geophytes who typically have limited storage conditions at their disposal; cool and moist, along with their tulip (*Tulipa gesneriana* L.) bulbs, cool and dry, such as with Gladiolus (*Gladiolus spp.* L.), or room-temperature and dry, along with other dry-packed bulbs such as Calla lilies (*Zantedeschia sp.* Koch.). Dry storage is common with orthodox seeds, which are known for their desiccation tolerance (Copeland and McDonald, 2001).

Investigations of the influence of temperature and relative humidity on the aging effects of seeds have been well documented (Justice and Bass, 1978). The general recommendation is that the drier and cooler the storage environment, the longer orthodox seeds retain the ability to germinate (Priestley, 1986). Artificial aging of orthodox seeds has been recognized as a useful predictor of storability; those fairing poorly under accelerated aging treatments usually perform poorly under long-term open storage (Priestley, 1986). In these experiments seeds are stored much warmer than is typical, at 35-45 ^{o}C, and up to 100% relative humidity which lowers viability in orthodox seeds in a matter of days compared to weeks or years in naturally aging tissues (Bewley and Black, 1994). Meynet (1993) suggested that *R. asiaticus* tubers

should be stored at 15-25 ^{o}C and 50% relative humidity, which is common in the industry (M.A. Mellano and Y. Liberman personal communication), but this range has not been scientifically tested. It is not clear which of the commonly implemented storage conditions is most appropriate for long-term viability of *R. asiaticus.* Therefore the purpose of this research was to develop a storage protocol for *R. asiaticus* tuber viability by testing a range of storage moistures and temperatures and observing the influence on respiration and subsequent growth.

Materials and methods

Experiment 1: dynamic storage

To control relative humidity and temperature during storage, a dynamic (flow-through) system was used. Dried tubers of *Ranunculus asiaticus* 'Tecolote Merlot' (M07) were obtained in Apr. 2008 from a commercial grower (California Flowerbulb Co., Carlsbad, CA). Tubers originated from commercial plantings in the 2006-07 winter growing season. On 10 Apr. 2008, tubers were placed into modified storage at 5, 20, or 35 ^{o}C for 5, 10, or 20 weeks. Storage chambers consisted of standard 5-gallon plastic buckets into which humidified air introduced. Humidity treatments were generated by pumping 500 mL·min^{-1} air through glycerol-water solutions mixed to desired ratios as outlined by Forney and Brandl (1992). Two, 2 L treatment jars, each containing 1500 mL of glycerol-water solution, were plumbed in tandem to provide the desired relative humidity to the storage buckets. The specific gravity in the treatment jars averaged 1.221, 1.218, 1.178, 1.094, or 1.000 over the course of the experiment, which provided relative humidity of ca. 20%, 40%, 60%, 80%, or 100%, respectively. The chamber humidity was verified using HOBO Pro v2 data loggers (model# U23-002, Onset Computer Corp., Bourne, Mass.) and was determined to be accurate within ± 5% across all treatments. A schematic of the flow-through system is

shown in Fig. 3.1. After 5, 10, or 20 weeks storage, buckets were opened and 20 tubers from each humidity treatment were removed for moisture content determination (dried at 70 ^{o}C until constant weight was achieved, calculated on a fresh weight basis), and the buckets resealed. No tubers were planted after 5 weeks, but after 10 weeks storage, 48 tubers from each temperature and humidity treatment were removed for planting. These tubers were placed in mesh bags suspended ca. 2.5 cm over saturated potting media for 3 d to prepare samples for hydration. This treatment should allow the very low moisture content tubers to receive some equilibration to higher moisture prior to submerging. It is not known if this treatment is necessary in *R. asiaticus* but was included as a safeguard to prevent possible hydration injury from biasing treatment effect (Copeland and McDonald, 2001). Tubers were then submerged in tap water for 24 h and provided a 20 min soak in a commercial fungicide, (Captan, N-trichloromethylthio cyclohexene-1,2-dicarboximide, Southern Agricultural Insecticides Inc., Hendersonville, NC) at 2.6 $g \cdot L^{-1}$ a.i. On 24 June, tubers were planted in 7.5 cm square pots using a commercial potting mix (Sun Gro LC1, Sun Gro Horticulture Canada Ltd. Vancouver, British Columbia) with crowns covered approximately 2 cm. A 5 week cooling period was provided at 5 ^{o}C, which allowed some root establishment prior to moving to the greenhouse on 1 Aug. (De Hertogh, 1996). After 3 weeks growth (on 18 Aug.) plants were evaluated for percent survival (any visible growth).

Tubers stored for 20 weeks were handled in the same manner as those stored 10 weeks, except were planted on 2 Sept., given 6 weeks storage at 5 ^{o}C, and were moved to the greenhouse on 3 Oct. Percent survival was calculated after 4 weeks on 30 Oct. In both 10 and 20 week plantings, pots were arranged in a completely randomized design with two 24 sub-sample replicates (averaged) per treatment. Data were analyzed using standard least squares in JMP (SAS Institute, Cary, NC).

Experiment 2: closed storage

In this second experiment *R. asiaticus* tubers were stored under a closed system as opposed to the flow-through system described in experiment one. Because moisture content of stored tubers rose over the entire 20 weeks of experiment one (Fig 3.3), this experiment involved equilibrating tubers to a desired moisture content over 4 weeks before randomly assigning tubers to temperature treatments. This created uniformity in tuber moisture content at the various temperatures tested and allowed us to observe a more controlled interaction between temperature and tuber moisture level (Priestley, 1986). Dried tubers of *Ranunculus asiaticus* 'Labelle Cream' (L08) were obtained in Dec. 2008 from a commercial producer in France (unknown origin) grown during the 2007-08 season. 'Tecolote Pink' (P09) and 'Tecolote White' (W09) were obtained in Aug. 2009 from the 2008-09 growing season in southern California (California Flowerbulb Col, Carlsbad, CA). Tubers were stored at 15 ^{o}C and ca. 50% relative humidity until treatments were initiated.

Humidity equilibration chambers were constructed using two standard 5-gallon buckets stacked inside one another (Fig. 3.2). The first bucket, A, was a standard bucket containing 2 L glycerol-water solution mixed to the desired specific gravity (see below). The second bucket, B, had the bottom removed and replaced with a plastic grid, along with a hard-wired 12 V DC micro fan (model 273-240, Radio Shack Corp. Ft. Worth, TX) on each end. Bucket B was inserted into bucket A with a rubber gasket around the perimeter (X-TREME^{TM} rubber weather seal, Thermwell Products Co., Mahwah, NJ), which created an air-tight seal between the buckets. *R. asiaticus* tubers and two data loggers were then placed into bucket B and the top was sealed using a tightly fitting lid reinforced with duct tape. The fans were powered using a 15 amp 13.8 V DC power supply (model# 22-508, Radio Shack Corp.) with wires extending out of a gasket-sealed 2 mm port in the bucket lid. In this experiment tubers

were stored in a closed system, suspended for 4 weeks (at 15 ^{o}C) over a glycerol-water solution with head-space equilibrium relative humidity of 35%, 60%, or 85%. These humidity treatments were measured using HOBO Pro v2 data loggers and were determined to be accurate within ± 5% across all treatments. The specific gravity of the glycerol water solutions (the same in both rounds of the experiment) was 1.240, 1.190, or 1.126 in the 35%, 60%, or 85% relative humidity chambers, respectively. After 4 weeks equilibration, tubers were removed from the humidity chambers and were randomly assigned to one of two temperature treatments, 5 ^{o}C or 25 ^{o}C, for 16 weeks storage. Four subsample tubers were sealed in 0.5 L jars, with 6 replicate jars per treatment (temperature and moisture content) per cultivar. Additionally, 10 tubers from each humidity chamber per cultivar were sacrificed for determination of moisture content on a fresh weight basis ([water weight / fresh weight] x 100). These tubers were dried in a 70 ^{o}C oven until constant weight was achieved. The moisture content of tubers stored at 35%, 60%, or 85% relative humidity was 6.9%, 10.1%, or 18.4%, respectively, and was not different between rounds of the experiment.

Upon removal from 16-week storage treatments (not including 4 weeks equilibration), tubers were placed in mesh bags over saturated potting media for 3 d to prepare samples for hydration as in experiment 1. Tubers were then submerged in tap water for 24 h at 25 ^{o}C and a provided a 5 min soak in a copper sulfate biocide (Phyton-27, Phyton Corp., New Hope, MN) at 1375 mg$\cdot$L^{-1} metallic copper. They were planted, four per pot, in 15 cm diameter azalea pots using a commercial potting mix (Sun Gro LC1, Sun Gro Horticulture Canada Ltd. Vancouver, British Columbia) with crowns covered approximately 2 cm, on 6 or 21 Jan., for rounds one or two, respectively. Planted tubers were moistened with tap water and then provided a 4 week cooling period at 5 ^{o}C to allow some root establishment prior to growing in a 15 ^{o}C set-point temperature greenhouse starting on 4 or 16 Feb., for rounds one or two,

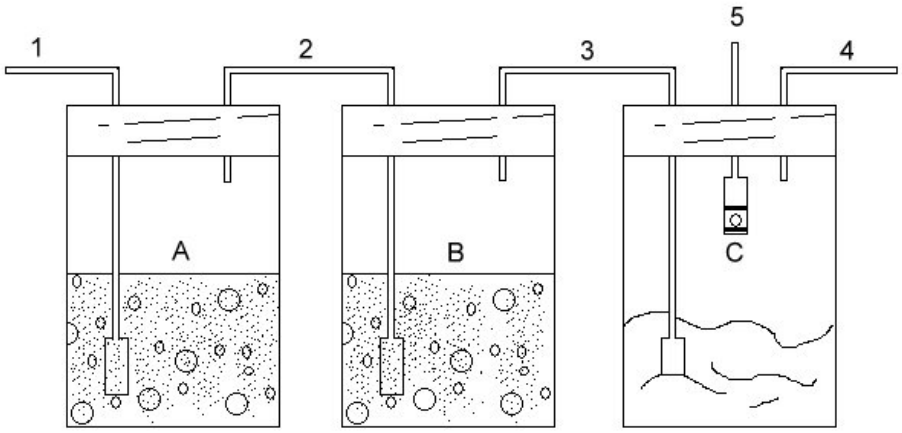

Figure 3.1. Flow-through humidity chambers used in experiment 1. Air was pumped through line 1 into one of two jars containing glycerol and water mixed to the desired specific gravity, A and B, respectively. Humidified air was then pumped through line 2, then line 3, and finally line 4, leading into the storage vessels. Jar C contained a paper towel to absorb any condensation/liquid and a RH/Temp monitoring probe (5). (Adapted from Forney and Brandl, 1992).

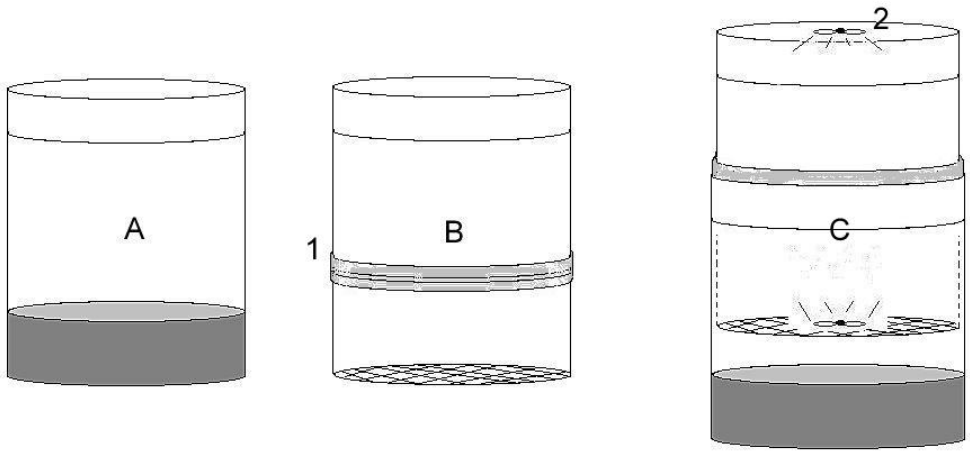

Figure 3.2. Closed humidity chambers used in experiment 2. Bucket A contained glycerol and water solutions mixed to the desired specific gravity. Bucket B had a tightly fitting lid, a micro fan at each end (2) and the bottom replaced with a plastic screen. Bucket B was inserted into bucket A and a rubberized gasket (1) created an air-tight fit between buckets, the lid of bucket B was also reinforced with duct tape (not pictured).

6 replicate pots (one per block) of 4 sub-samples (pooled) per treatment.

After 4 weeks in the greenhouse (on 11 or 17 March, for rounds 1 or 2, respectively), percent survival (any visible growth), plant size (mean of height and two cross canopy diameter measurements), and foliar dry weight (severed at soil line and dried 3 d at 70 ^{o}C) were calculated. A visual ranking of plant quality was also made using a 1-5 scale: 1= poor quality, one to two leaves, diseased and/or dying; 2= slightly more growth, three to six leaves, but still unacceptable quality; 3= acceptable level of quality, seven to 10 leaves, non-uniform growth; 4= moderate quality, uniform growth, greater than ten leaves; 5=best quality, ideal size and shape, greater than ten leaves.

For both rounds, any experimental unit which returned a zero value for survival was excluded from further calculations; therefore all remaining measurements and subsequent analyses were conducted only on plants showing visible growth. All data were analyzed using standard least squares in JMP (SAS Institute, Cary, NC).

Experiment 3: Respiration under closed storage

In the third experiment, CO_2 was measured in the headspace of tubers under similar conditions as in experiment 2. Closed humidity chambers were set up as above with tubers equilibrated 4 weeks at 15 ^{o}C starting on 22 Oct. An additional cultivar 'Tecolote Pink' (P08) (from the 2007-08 growing season) was also included, along with the cultivars mentioned in experiment 2 (L08, P09, and W09). On 17 Nov., buckets were opened and tubers were randomly assigned to 5 or 25 ^{o}C storage. Tubers were placed in 100 mL jars with air-tight lids fitted with rubber septa. There were 2 subsample tubers per jar and 6 replicate jars per treatment per cultivar. In this second experiment, moisture content was determined with 5 tubers per humidity chamber per cultivar, and then at each sampling date (see below). Dry weights were collected after

freeze-drying samples 5 days at -28 ^{o}C. The moisture content (calculated on a fresh weight basis: [{fw-dw}/dw x 100]) of tubers in this round (not significantly different between cultivars or sampling dates) was 1.9%, 4.9%, or 14.1% from the 35%, 60%, or 85% relative humidity treatments, respectively.

Gas samples were collected and measured for CO_2 (see below) after 4, 12, and 17 weeks of storage in jars, on 16 Dec., 10 Feb. and 17 Mar., respectively. At each sampling date, two jars from each treatment and cultivar were opened, flushed with air, and resealed. These jars were sampled again 3 days later for CO_2 evolution and then tubers were analyzed for moisture content (see previous paragraph). Carbon dioxide measurements were made by sampling the headspace in each jar using plastic syringes through rubber septa. Samples were injected (1 mL) into a CA-10 carbon dioxide analyzer (Sable Systems Intl. Las Vegas, NV) and referenced against a 50,000 $\mu L \cdot L^{-1}$ CO_2 standard (balanced with nitrogen). A calibration curve was created using increasing volumes of the CO_2 standard (0.2, 0.5, or 1.0 mL) to establish a conversion factor for peak areas generated by the CA-10 software. Atmospheric CO_2 concentration was obtained by including four empty jars per sampling period with their mean value subtracted from total CO_2 evolved. To calculate respiration rate, the fresh weight of tissue in each jar was subtracted from the headspace volume (assuming 1g tissue fresh weight is equal to 1 mL headspace) and then data were expressed as mL CO_2 evolved per kg of tuber fresh weight per day. Data were analyzed using standard least squares in JMP (SAS Institute, Cary, NC).

Experiment 4: Closed storage with desiccant

To determine efficacy of desiccant for short-term *R. asiaticus* storage, P09 tubers were placed in either empty 0.5 L jars or in those containing 100g of expired or non-expired anhydrous calcium sulfate. Tubers were sealed in jars for 10 weeks at 15

°C beginning 2 Sept. On 11 Nov. jars were opened and tubers were placed in mesh bags suspended ca. 2.5 cm over saturated soilless potting media for 3 d moisture equilibration and then handled and planted as previously described. Tubers were potted on 18 Nov., moved to the greenhouse on 17 Dec., and evaluated for the previously mentioned parameters after 7 weeks growth on 1 Feb. Data were again analyzed using standard least squares in JMP. (SAS Institute, Cary, NC)

Results

Experiment 1: dynamic storage

The moisture content of tubers under modified storage (Fig. 3.3) had a significant temperature by relative humidity by time interaction (Table 3.1). Generally tuber moisture content increased with relative humidity; however, after 5 weeks storage this was only evident with storage at 35 °C. This trend at 5 weeks with 35 °C storage was subsequently the same trend after 10 weeks at 20 °C. Tubers stored at 5 °C developed this characteristic increase in moisture content with humidity after 20 weeks storage. Tubers stored 20 weeks at 35 °C and 100% relative humidity showed excessive decay; therefore these data were not collected.

The temperature by relative humidity by time interaction was significant for percent survival (Table 3.1). After 10 weeks storage, a significant reduction in percent survival was observed after storage at 35 °C, especially when relative humidity was 80% or higher (Fig. 3.4). Percent survival was generally similar across humidity treatments at cooler storage temperatures, but was slightly reduced when stored at 20 °C and 100% relative humidity (Fig. 3.4). After 20 weeks storage, tuber survival decreased as relative humidity increased at both 20 and 35 °C, and those stored

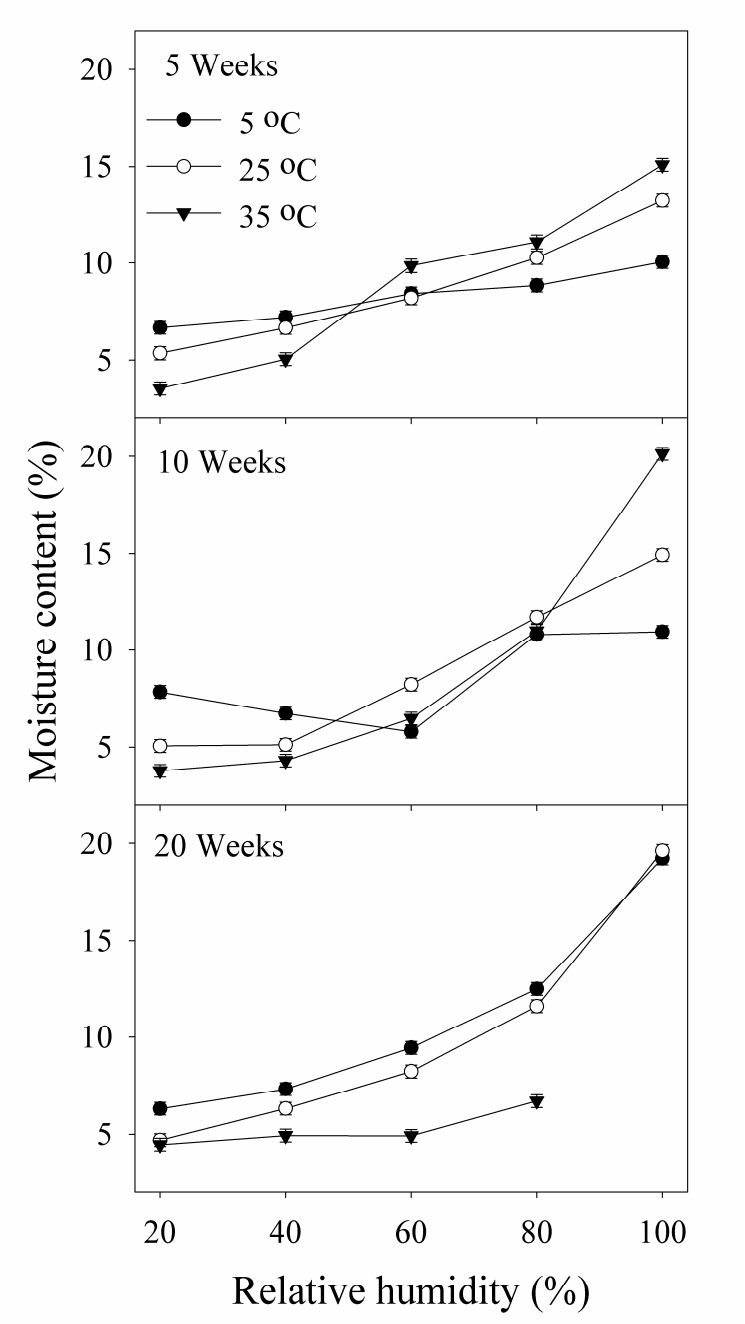

Figure 3.3. Experiment 1: Dynamic storage. Fresh basis moisture content of *R. asiaticus* dried tubers stored 5, 10, or 20 weeks under several temperature and relative humidity conditions. Data are means ± standard error (bars not visible are within range of data marker).

Table 3.1. Experiment 1. Significance, indicated by p-value, of temperature, relative humidity during storage, time, and their interaction on tuber moisture content of *Ranunculus asiaticus* dried tubers; percent survival is any visible growth (after 3 weeks in the greenhouse) following modified storage.

Source	MC[z]	Percent Survival
Temp (T)	<0.001	<0.001
RH	<0.001	<0.001
T x RH	<0.001	<0.001
Time (W)	<0.001	0.001
T x W	<0.001	ns
RH x W	<0.001	0.013
T x RH x W	<0.001	0.042

[ns]Not significat at $\alpha = 0.05$

Table 3.2. Experiment 2. Significance, indicated by p-value, of cultivar, moisture content, or temperature, an their interaction during storage on the percent survival, visual ranking of quality, size, and foliar dry weight of *R. asiaticus* tubers following 20 weeks specialized storage and 4 weeks growth in the greenhouse.

Source	Percent survival	quality ranking	Plant size	Foliar dry weight
Cultivar (C)	ns	ns	0.001	<0.001
Moisture content (MC)	<0.001	ns	ns	ns
C x MC	ns	ns	ns	ns
Temperature (T)	<0.001	ns	ns	ns
C x T	ns	ns	ns	ns
MC x T	<0.001	ns	ns	ns
C x MC x T	ns	ns	ns	ns

[ns]Not significat at $\alpha = 0.05$

warmer, showed greater survival loss at each humidity level. Tubers stored 20 weeks at 5 ^{o}C had similar survival across humidity treatments.

Experiment 2: Closed storage

Results from both rounds of the experiment were similar; therefore, only results from round two are presented. There were no significant differences among cultivars for percent survival or ranking of visual quality (Table 3.3). The moisture content by temperature during storage interaction was significant for percent survival (Table 3.3). Tubers with 22.6% moisture content had zero plants survive when stored at 25 ^{o}C, while all other treatments had similar survival (92.3%). Once percent survival was calculated, non-sprouted tubers were treated as missing data for subsequent analysis. No differences in visual quality, plant size, or foliar dry weight (averaged 4, 11.3 cm, or 0.66 g, respectively) were shown with regard to storage moisture content or temperature (Table 3.2). Plant size and foliar dry weight were different among cultivars (Table 3.2). With regard to plant size, L08 was significantly smaller than W09 (11.3 vs 10.7cm, respectively), however both were similar to P09 which averaged 11.0 cm per plant. Foliar dry weight followed a similar trend except all three cultivars were significantly different at 0.82, 0.67, and 0.50 g for W09, P09, and, L08, respectively.

Experiment 3: Respiration during closed storage

Respiration rates were similar for tubers with 1.9% or 4.9% moisture, regardless of storage temperature (Fig. 3.5). Increasing tuber moisture content to 14.1% increased respiration rate, which was at least 12.5 times higher with tubers held at 25 ^{o}C than at 5 ^{o}C. Respiration rates were not different among cultivars or sampling dates (Table 3.3).

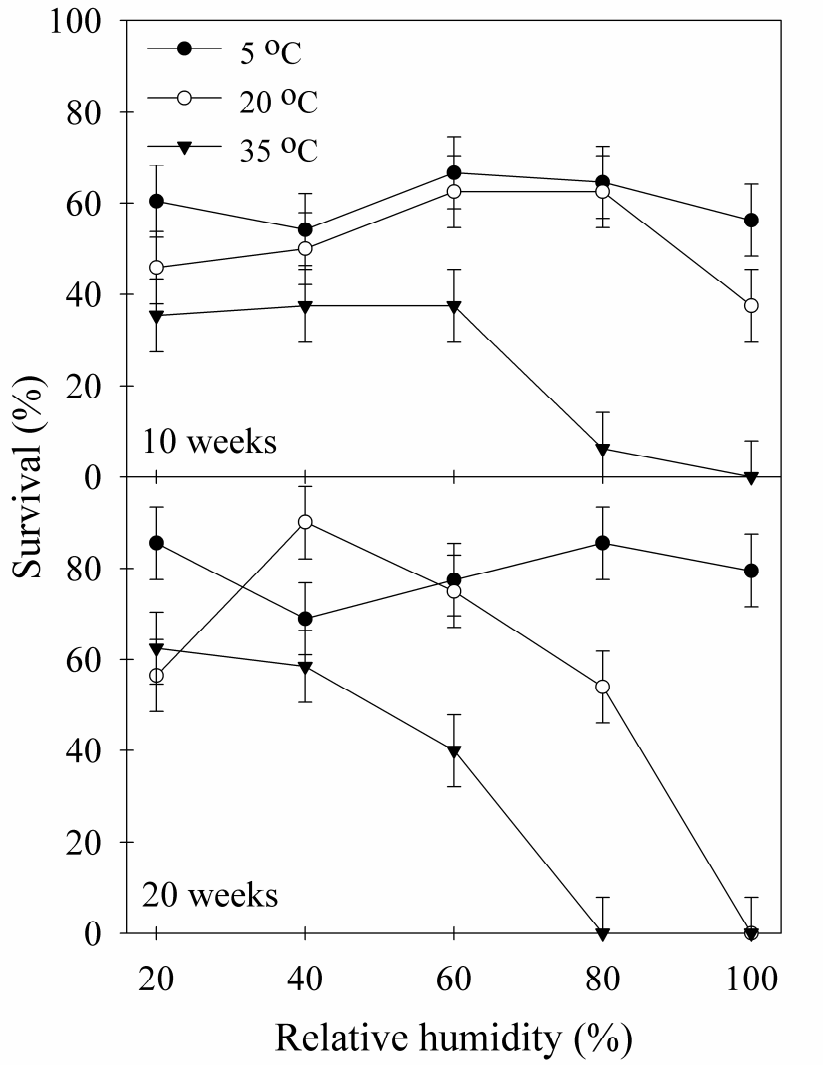

Figure 3.4. Experiment 1: Dynamic storage. Influence of time, temperature, and storage humidity on percent survival of *Ranunculus asiaticus* 'Tecolote Pink'. Data are means ± standard error.

Table 3.3. Experiment 3. Significance, indicated by p-value, of time (weeks), cultivar, temperature, tuber moisture content, and their interaction on respiration rate (CO_2 evolution) of *R. asiaticus* tubers during specialized storage.

Source	Respiration rate
Weeks (W)	ns
Cultivar (C)	ns
W x C	ns
Temp (T)	0.005
W x T	ns
C x T	ns
W x C x T	ns
Moisture Content (MC)	<0.001
W x MC	ns
C x MC	ns
W x C x MC	ns
T x MC	<0.001
W x T x MC	ns
C x T x MC	ns
W x C x T x MC	ns

[ns]Not significant at $\alpha = 0.05$

Experiment 4: Closed storage over desiccant

When considering tubers stored over desiccant, there were no differences in percent survival, visual rank, plant size, or foliar dry weight among any of the storage methods which averaged 96%, 4, 14.2 cm, or 0.65 g, respectively. That is, tubers stored over desiccant showed no differences from those stored over expired desiccant and those stored alone.

Discussion

To establish proper storage protocols for *R. asiaticus* dried tubers, modified temperature and relative humidity treatments were imposed under dynamic or closed storage systems. In both systems, tuber moisture content (humidity) interacted with time to indicate elevated moisture and temperature to be detrimental to long-term tuber viability. When comparing these results to data from the third experiment, we see that tubers stored with the highest moisture content also had the highest respiration rates (Fig. 3.5).

In an effort to establish a paradigm for long-term open storage of orthodox seed, seeds are often exposed to accelerated aging treatments (35-45 ^{o}C and up to 100% relative humidity) which provide viability loss in a much shorter duration than under natural aging (Copeland and McDonald, 2001). Priestley (1986) cautioned against using accelerated aging studies to replace the need for long-term verification of results, but suggested these treatments give good approximations to what will happen with natural aging. When pea seeds (*Pisum sativum* L.) were given accelerated aging treatments (37 ^{o}C and 100% relative humidity), respiration rate increased and vigor decreased compared to those stored cooler (20 ^{o}C) (Ozga et al., 2004). Storage of *R. asiaticus* tubers at 5 to 35 ^{o}C and up to 100% relative humidity was effectively "accelerated aging" of the dried tubers. When stored at 35 ^{o}C,

complete viability was lost within 10 weeks at 100% relative humidity or 20 weeks at 80% relative humidity. Tubers stored at 5 ^{o}C showed mildly elevated respiration rates with higher moisture content, yet did not exhibit significant survival loss in the greenhouse (experiments 2 and 3). It stands to reason that tubers stored at 5 ^{o}C under elevated moisture would eventually have had reduced viability if the experiment duration were longer, especially when considering the change in moisture content with time, temperature, and relative humidity during storage (Fig. 3.3).

Loss of seed quality during storage is common when efficient management of temperature and moisture is not provided. Most orthodox seeds will maintain viability without significant degradation for many years if held cool and dry (Priestley, 1986). Long term viability of orthodox seeds is greatly influenced by temperature and moisture content during storage, for example, it has been noted that the storage life doubles for each 1% decrease in seed moisture content and/or 5.6 ^{o}C decrease in storage temperature (Bewley and Black, 1994). The range for acceptable humidity and temperatures would certainly depend on the tolerance of the species to both extreme drying and low temperature. The relationship between relative humidity and seed moisture content is usually expressed by moisture sorption isotherms, which are obtained by measuring equilibrium moisture content as a function of relative humidity at constant temperature (Priestley, 1986). These curves have a characteristic sigmoid shape which permits expression of three distinct regions of hydration, or "zones". In zone I, tissue moisture content increases rapidly with rising humidity and then slows in zone II. In zone III, moisture content again rises rapidly with increased humidity. Internal composition of seeds (starch or oil) can affect the equilibrium moisture content, but the inflection points between zones are relatively similar among seeds (Priestley, 1986).

In our experiments, a similar sigmoid shaped curve emerged when tubers were stored under increasing relative humidity (Fig. 3.3), yet "zone I" was virtually indistinguishable. Moisture content of tubers under dynamic storage generally rose with increasing humidity and temperature; however, after 20 weeks at 35 ^{o}C, moisture content was similar among humidity treatments. Since respiration was shown to be greater as temperature and humidity increased in the closed system (Fig. 3.5), this probably resulted in a loss of tuber weight as the carbon substrate in the roots was consumed (Hopkins, 1999), which appeared as a relative decline in moisture content at higher humidity over time (10 vs 20 weeks). Although relative humidity is the chief contributing factor to decreased longevity in stored orthodox seeds, this effect is largely explained through its influence on seed moisture content (Priestley, 1986). Priestley (1986) diagramed evidence of cellular activity at various levels of hydration in orthodox seeds. He concluded that seed respiration is only feasible in upper zone II or in zone III, but indicated these data are often confounded by various microorganisms living on the seeds which become more active as moisture level increases. When active, these pathogens can contribute significantly to the respiratory gas exchange in the storage environment. *R. asiaticus* is known for having infections of various root-rot pathogens during storage, thus inclusion of biocide treatments prior to planting (Meynet, 1993). In our studies we were not able to distinguish the source of respiration gas. It is possible that CO_2 produced during storage of *R. asiaticus* tubers was the result of respiring microorganisms colonizing the tubers rather than from the tissue itself. If further investigations warrant revisiting respiration rates of *R. asiaticus*, it may be necessary to disinfect tubers prior to storage. This may isolate the source of respiration gas to the tissue. It should be noted that the observed tuber respiration rates are quite low, even in treatments showing comparatively "high" respiration (Fig. 3.5). In our experiments, the highest respiration rate observed was

0.25 $mL \cdot kg^{-1} \cdot d^{-1}$ (14.06% moisture, 25 ^{o}C) as compared to the lowest rate reported in stored pea seeds, 4,800 $mL \cdot kg^{-1} \cdot d^{-1}$ (Ozga et al., 2004).

In a recent study, when *R. asiaticus* tubers were stored for one year at 2 ^{o}C under either open or modified atmosphere storage (2% O_2 and 4% CO_2), percent survival was maintained at 90%, which was significantly greater than those under open storage at room temperature (ranged ca.15 to 23 ^{o}C) (Beruto et al., 2009). It was proposed that the non-significant influence of atmosphere modification on viability was due to low metabolism during storage. This hypothesis is further upheld by the low respiration rates of our tubers stored at 5 ^{o}C. The long-term results under natural aging presented by Beruto et al. (2009) support our findings over the relative short-term.

Under dynamic storage, the moisture content of tubers did not appear to reach equilibrium moisture levels across temperature treatments over the 20 weeks of storage, rather increased with increasing temperature (Fig. 3.3). Thus, when redesigning the experiment for closed storage, tubers were first equilibrated to a moisture content that was predicted to cause damage (or not), when stored 4 weeks over 85% relative humidity at 15 ^{o}C. This treatment allowed much more uniform response to relative humidity at the temperatures tested and did not change from the start of temperature treatments to completion. Under commercial conditions, it is difficult to speculate exactly how episodes of high or low humidity would impact the overall storability of *R. asiaticus*, but the general tenet emerges that dry storage is better than humid.

It should be noted that in Expt. 1, average plant survival across all treatments was lower after 10 weeks than 20 weeks storage. This was due in part to the need to grow *R. asiaticus* plants in late summer, when greenhouse temperatures were too warm for proper growth (Meynet, 1993). Subsequent experiments were scheduled to

avoid storage treatments ending during an unfavorable season. In Ithaca, NY, *R. asiaticus* plants grow best in the greenhouse from late fall through early spring, when temperatures can more reliably be kept below ca. 20 ^{o}C. This observation is consistent with common cultural recommendations for growth (Meynet, 1993).

In experiment four, *R. asiaticus* tubers stored over desiccant for 10 weeks at 15 ^{o}C had similar survival to those stored alone or without expired desiccant (no longer absorbing moisture). Storing tubers with desiccant may have practical applications for commercial handlers. Tilden and West (1985) showed accelerated aging studies predispose soybean (*Glycine max* L.) seeds to hydration injury unless slowly hydrated following storage. These researchers "primed" seeds by slowly hydrating them at 20 ^{o}C and then re-drying, which repaired cellular damage from the aging treatments. It is not known if omitting our "partial priming" treatment, storing *R. asiaticus* tubers suspended ca. 2.5 cm over saturated media for 3 d prior to submersion, would result in hydration injury of tubers stored under extremely dry conditions (i.e. with desiccant).

Since *R. asiaticus* is native to a dessert climate, it is not surprising that relatively dry handling is superior to higher moisture content. In our experiments, tubers stored at 60% relative humidity or below maintained tuber viability over 16 to 20 weeks when stored at 5 or 20 ^{o}C. For commercial handlers, low temperature should be the first priority when storing *R. asiaticus* tubers, but attention should be given to keep temperatures above freezing. *R. asiaticus* tubers have been shown to withstand submersion in liquid nitrogen for 24 h in the dry state, but minimal hydration resulted in viability loss at -5 ^{o}C (Sakai, 1960). A combination of low temperature and low humidity provides the highest tuber viability and should therefore be considered optimum for long-term storage.

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CHAPTER 4

SOAKING TEMPERATURE INFLUENCES HYDRATION KINETICS AND GROWTH OF RANUNCULUS ASIATICUS DRIED TUBEROUS ROOTS

Abstract

The published literature is inconsistent with recommendations for hydrating *Ranunculus asiaticus* dried tuberous roots, a common practice in commercial production systems for this ornamental geophyte. A simple two parameter model was used to express water uptake in *R. asiaticus* roots. As hydration temperature increased, so did imbibition rate, but to lower equilibrium moisture content as compared to cooler hydration temperatures. In the greenhouse, percent survival was optimum when tubers were hydrated around 20 ^{o}C. Plant height, visual quality, and foliar dry weight followed a similar trend 4 weeks after planting. These results suggest that handlers should carefully monitor hydration temperature when growing *R. asiaticus* from its dried tuberous roots.

Introduction

Ranunculus asiaticus is a traditional cut flower and flowering potted plant that is becoming more popular in early spring gardening and landscape designs (Hamrick, 2003). *R. asiaticus* is commercially grown from seed or from its tuberous roots (hereafter simply referred to as "tubers") which flower faster and more profusely than from seeds (Meynet, 1993). The tubers of *R. asiaticus* are well adapted to lengthy dry storage and have therefore been identified as "resurrection geophytes" (Kamenetsky et al., 2005; Beruto et al., 2009). It is common commercial practice to hydrate tubers prior to planting, as this provides handling uniformity and facilitates fungicide application (Y. Liberman, personal communication); however, the published

information on hydration duration and temperature is inconsistent. Meynet (1993) suggested direct planting without a hydration treatment, while De Hertogh (1996) recommended soaking tubers in slowly running water for 24 h prior to planting; specific hydration temperature recommendations were not provided. Other published recommendations for tuber handling prior to planting are limited. In an information leaflet provided from an Israeli *R. asiaticus* grower, tuber hydration was recommended via "cool tap water" for 24 h (Umiel and Hagiladi, 1999). While investigating the effects of tuber stratification on *R. asiaticus* flowering, Okhawa (1986) soaked tubers 8 h at 6 ^{o}C prior to planting. Soaking time and temperature were not considered as treatments, but is mentioned here to highlight the inconsistency with which *R. asiaticus* tubers are handled prior to planting. It is important to establish a unified protocol of *R. asiaticus* tuber hydration, for future scientific work and to commercial handlers. In order to accomplish these goals, we determined how long it took for submerged tubers to hydrate, and how sensitive the imbibition rate and subsequent plant quality are to varying hydration temperature.

Because several processes could limit the rate of hydration in *R. asiaticus* tubers, an appropriate hydration kinetics model must be selected. An empirical model for describing moisture sorption curves, the Peleg model, has been used to model mass transfer in a number of dehydrated and rehydrated products including seeds of kidney bean (*Phaseolus vulgaris* L.), chick pea (*Cicer arietinum* L.), and field pea (*Pisum sativum* L.), along with rice (*Oryza sativa* L.) and other cereal grains, and other food products (Peleg, 1988; Sopade et al., 1992; Hung et al., 1993; Abu-Ghannam and McKenna, 1997; Turhan et al., 2002; Sarchetti et al., 2003; Bello et al., 2008; Prasad et al., 2010). The advantage of using Peleg's model for estimating moisture uptake is the ability to predict long-range moisture gains from relatively short duration

experiments (Peleg, 1988). In this research, the Peleg model was investigated for applicability in modeling *R. asiaticus* hydration.

Materials and methods

Hydration Kinetics

Dried tubers of *Ranunculus asiaticus* 'Tecolote Pink' were obtained from a commercial producer in California (California Flowerbulb Co, Carlsbad, CA) and randomly assigned to one of three distilled water baths at 5, 20, or 35 ^{o}C. There were five replications per treatment with five sub-sample tubers per replicate. Tubers were submerged for 1 h, removed, blotted dry, weighed, and then re-submerged. Data were collected hourly for 12 h, then after 24 h and 30 h. After 30 h, tubers were placed in a 70 ^{o}C oven and dried until constant weight was achieved. The experiment was conducted three times with similar results, therefore only data from the third experiment are presented.

Model Fitting

Turhan et al. (2002) used the Peleg model to describe moisture sorption in chickpea; much of their work is adapted for interpretation of our results. The two-parameter sorption equation proposed by Peleg (1988) is considered for modeling *R. asiaticus* hydration:

$$M(t) = M_0 \pm \frac{t}{K_1 + K_2 t} \qquad \text{Eq[1]}$$

Where $M(t)$ is the dry basis moisture content of tubers at time t (%), M_0 is the initial moisture content (%), K_1 is the Peleg rate constant (%·h^{-1}), and K_2 is the Peleg capacity constant (%$^{-1}$). Since we are modeling an adsorption/absorption process, the "±" in Eq [1] becomes positive (+).

The momentary sorption rate (S), is given by the first derivative of the Peleg equation:

$$S = \frac{dM(t)}{dt} = + \frac{K_1}{(K_1 + K_2 t)^2} \quad \text{Eq[2]}$$

The Peleg rate constant, K_1, relates to the initial sorption rate, i.e. S at $t = t_0$.

$$S_0 = \frac{1}{K_1} \quad \text{Eq [3]}$$

The Peleg capacity constant, K_2, relates to the equilibrium moisture content, M_E, as $t \rightarrow t_{\infty}$.

$$M_E = M_0 + \frac{1}{K_2} \quad \text{Eq [4]}$$

The model was fit to our data using Eq [1] and non-linear model fitting procedures in JMP (SAS Institute, Cary, NC) to generate values for K_1 and K_2. M_0 was calculated directly from our data. To verify the accuracy of the Peleg model for predicting water uptake in *R. asiaticus*, Eq [1] was rearranged to its linear form, Eq[5], with linear regression was used to asses adequacy of fit (coefficient of variance, R^2).

$$\frac{t}{M(t) - M_0} = K_1 + K_2 t \quad \text{Eq [5]}$$

If R is the hydration ratio at a given value of M(t), it may be calculated via Eq [6].

$$R_t = \frac{M(t) - M_0}{M_E - M_0} \quad \text{Eq [6]}$$

Once a value for a desired hydration ratio is determined, R_{t1}, it may be subsequently used to estimate the time necessary to achieve other hydration ratios, R_{t2}.

$$\frac{R_{t1} \cdot (1 - R_{t1})^{-1}}{t_{R1}} = \frac{R_{t2} \cdot (1 - R_{t2})^{-1}}{t_{R2}} \quad \text{Eq [7]}$$

Greenhouse experiment 1

To determine the influence of hydration temperature on growth, dried tubers of *Ranunculus asiaticus* 'Tecolote Pink' from the 2006/2007 growing season (here after referred to as 'P07') were obtained in Sept. 2008 from a commercial producer in California (California Flowerbulb Co., Carlsbad, CA), and were stored at 15 ^{o}C and ca. 50% relative humidity, as per common commercial practice, until treatments were initiated on 6 Oct 2008. Tubers were submerged in tap water at 5, 17, 23, or 35 ^{o}C for 24 h, then provided a 5 min soak in a copper sulfate biocide (Phyton-27, Phyton Corp., New Hope, MN) at 1375 mg·L^{-1} metallic copper. Tubers were planted four per pot, on 7 Oct in 15 cm diameter azalea pots using a commercial potting mix (Sun Gro LC1, Sun Gro Horticulture Canada Ltd. Vancouver, British Columbia) with crowns covered approximately 2 cm. Planted tubers were moistened with tap water and then held at 5 ^{o}C for 4 weeks to allow some root establishment prior to growing in a 15 ^{o}C set-point temperature greenhouse starting on 4 Nov. The plants were organized in a completely randomized design with 6 replicate pots of 4 sub-samples (pooled) per treatment.

After 4 weeks in the greenhouse plants were evaluated for percent survival (any visible growth), plant height (soil line to tallest leaf), and foliar dry weight (severed at soil level and dried 3d at 70 ^{o}C).

Greenhouse experiment 2

Dried tubers of P07 were handled in a similar manner as Expt. 1, except 24 h hydration temperatures were 5, 10, 17, 20, 25, 30, or 35 ^{o}C. These treatments were initiated on 8 Dec., planted on 10 Dec., cooled 5 weeks, and then moved to the greenhouse on 14 Jan. 2009. Plants were arranged in a completely randomized design with 9 replicate pots of 4 sub-samples (pooled) per treatment. Percent survival and plant height were evaluated after four weeks in the greenhouse. After 11 weeks,

plants were evaluated for number of flowering stems (stems with at least one flower) and foliar dry weight (as in Expt. 1).

Greenhouse experiment 3

In the third experiment the number of cultivars and tuber sources were increased. *R. asiaticus* 'Tecolote Pink' (P08) and 'Labelle Cream' (L08) were obtained from 2007/2008 growing seasons in southern California (California Flowerbulb Co., Carlsbad, CA) and France (unknown origin), respectively. Additionally 'Tecolote Red' and 'Tecolote White' from the 2008/2009 growing season (R09 and W09, respectively) were also obtained from southern California. Tubers were held at 15 ^{o}C and ca. 50% relative humidity until treatments were initiated on 28 Sept. 2009. Tubers were hydrated at 5, 15, 25, or 35 ^{o}C for 24 h, given copper biocide, and planted as above. After 6 weeks cooling, plants were moved to the greenhouse on 9 Nov. Percent survival, plant size (mean of height and two cross canopy diameter measurements), and foliar dry weight (as above) were calculated after 4 weeks in the greenhouse (7 Dec. 2009). A visual ranking of plant quality was also included on a 1-5 scale: 1= poor quality, one to two leaves, diseased and/or dying; 2= slightly more growth, three to six leaves, and unacceptable quality; 3= acceptable quality, seven to 10 leaves, non-uniform growth; 4= moderate quality, uniform growth, greater than ten leaves; 5=best quality, ideal size and shape, greater than ten leaves.

For experiments 1-3, any experimental unit which returned a zero value for survival was excluded from further calculations; therefore all remaining measurements and subsequent analyses were conducted on plants showing visible growth. All data were analyzed using standard least squares in JMP (SAS Institute, Cary, NC, USA).

Table 4.1. Water sorption parameters calculated from hydration of *R. asiaticus* in distilled water.

Temp. (°C)	Initial moisture (%)	Moisture content[x] (%) After (h)	Calc[z]	Obs	Calc M_E[y] (%)
5	23.0	12	149.4	153.3	267.5
		24	189.3	195.4	
20	22.4	12	176.3	177.8	249.5
		24	205.7	208.6	
35	23.2	12	185.1	188.6	230.3
		24	204.7	205.0	

[z]See Eq[1]
[y]See Eq[4] M_E is the equilibrium moisture content
[x]Calculated on a dry weight basis

Table 4.2. Water sorption parameters calculated from hydration of *R. asiaticus* in distilled water.

Temp. (C)	Calculated sorption rate (%·h^{-1})[z] Initial	After 12 h	After 24 h	t_R (h)[y] $R_{0.5}$	$R_{0.75}$	$R_{0.85}$	$R_{0.95}$
5	22.3	5.1	2.2	9.4	30.0	57.5	195.0
20	41.0	4.1	1.5	4.6	15.0	28.7	97.4
35	63.0	3.0	0.9	2.6	9.6	16.5	56.1

[z]See Eq[3]
[y]See Eq[6], Eq[7], t_R is the time (h) to reach a given hydration level

Results and discussion

Assessment of the model

R. asiaticus exhibited typical absorption behavior at all temperatures and was more rapid as temperature increased (Fig. 4.1). The hydration rate slowed as moisture content approached equilibrium, which was lower and occurred sooner as temperature increased. The linear form of the Peleg model (Eq[5]) is shown in Fig. 4.2 with R^2 values from 0.96 to 0.99. It should be noted that Eq[5] was only used to verify the adequacy of the model; therefore, values for K_1 and K_2 used for further analyses were generated by our statistical software package using the standard form of the Peleg model (Eq[1]).

When comparing calculated to observed moisture content in hydrating *R. asiaticus* tubers, the model's predicted values were reasonably consistent with our data at both 12 and 24 h hydration (Table 4.1). The deviation was slightly less as hydration temperature increased but in all cases was within 97% of the observed values.

As mentioned previously, one advantage of using Peleg's model for estimating moisture uptake is the ability to predict long range moisture gains from relatively short duration experiments (Peleg, 1988). It has been noted that the range of data selected for inclusion in the model affects the estimated values of the derived parameters, as well as the overall model fit (Peleg, 1988; Sopade et al., 1992; Turhan et al. 2002). We determined the best fit for *R. asiaticus* hydration data (via R^2 increase) was achieved by inclusion of all points measured between 0 and 30 h soaking.

The Peleg rate constant, K_1, is related to the mass transfer rate in that a lower value of K_1 indicates a faster initial water absorption rate. In our experiment, as temperature increased, K_1 decreased, corresponding to faster initial water absorption at higher temperatures (Table 4.1). The influence of temperature on the Peleg rate constant is shown in Fig. 4.3 via the linearized Arrhenius equation with an R^2 of 0.91:

$$\ln K_1 = \ln K_0 - \frac{E_a}{R_g T} \qquad \text{Eq[8]}$$

Where, K_0 is a constant, the pre-exponential factor, or prefactor (h $\%^{-1}$), E_a is the activation energy (kJ mol^{-1}), R_g is the universal gas constant (8.314 $kJ \cdot mol^{-1} \cdot K^{-1}$), and T is the absolute temperature (K). To determine the activation energy, the slope of the line generated in Fig. 4.3 is multiplied by $-R_g$. The resultant activation energy for *R. asiaticus* hydration is 24.8 $kJ \cdot mol^{-1}$. In chickpea (*Cicer arietinum* L.) and other starchy grains, there is a structural change at around 55 ^oC associated with internal gelatinization, that affects the initial water absorption rate (Sayar et al., 2001; Turhan et al, 2002). Since the temperatures we tested were well below gelatinization temperature, it is impossible to speculate on a similar phase transition in *R. asiaticus*; however, since we are primarily concerned with hydration kinetics as they pertain to growth, rather than cooking (as in chickpea), verification of this structural change is unnecessary for our purposes.

The Peleg capacity constant, K_2, is inversely related to maximum water absorption; therefore, the lower the K_2 the higher the absorption capacity. As hydration temperature increased, K_2 for *R. asiaticus* also increased coordinating with lower equilibrium moisture content at 30 ^oC, than at 5 ^oC (Fig. 4.4 and Table 4.1). Similar trends in K_2 values were observed when chickpea and kidney bean (*Phaseolus vulgaris* L.) were soaked at increasing temperatures (Turhan et al., 2002; Abu-Ghannam and McKenna, 1997b).

One of the challenges in using an empirical model such as this to predict equilibrium moisture content is that the theoretical maximum water uptake is not technically feasible. Besides the impracticality of soaking tubers for the duration necessary to achieve maximum saturation, there would presumably be an eventual loss of soluble solids and decay, leading to loss of fresh weight. Alternatively the tubers

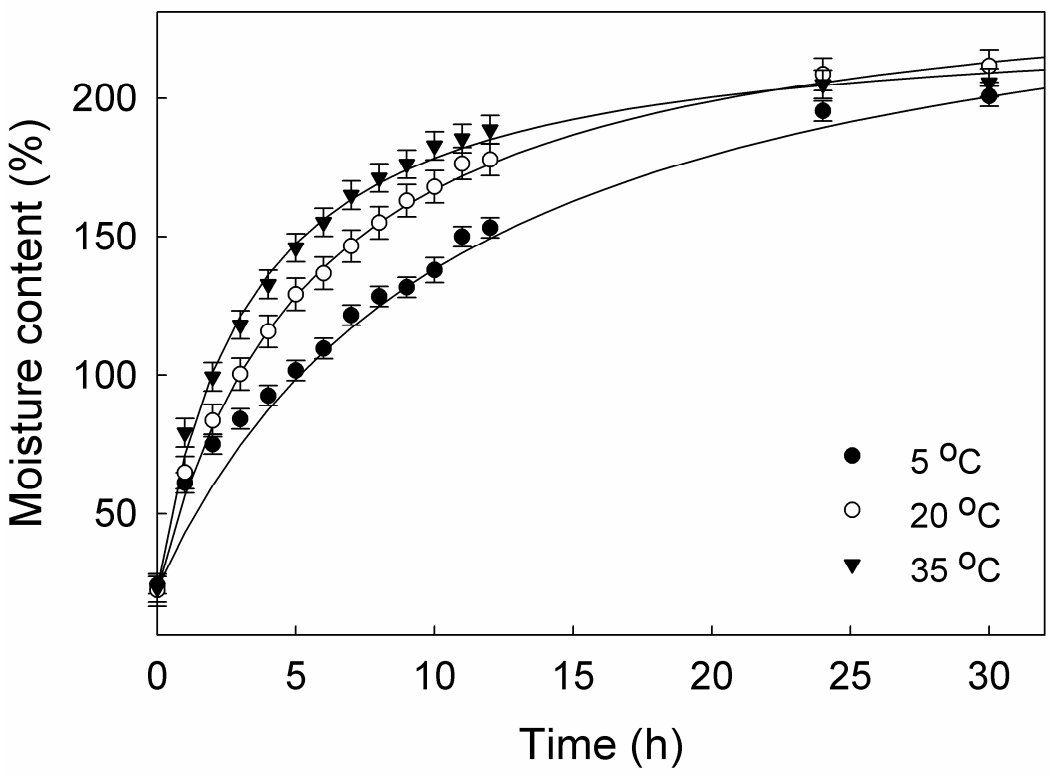

Figure 4.1. Hydration kinetics of *R. asiaticus* tubers submerged in distilled water. Data are means ± standard error.

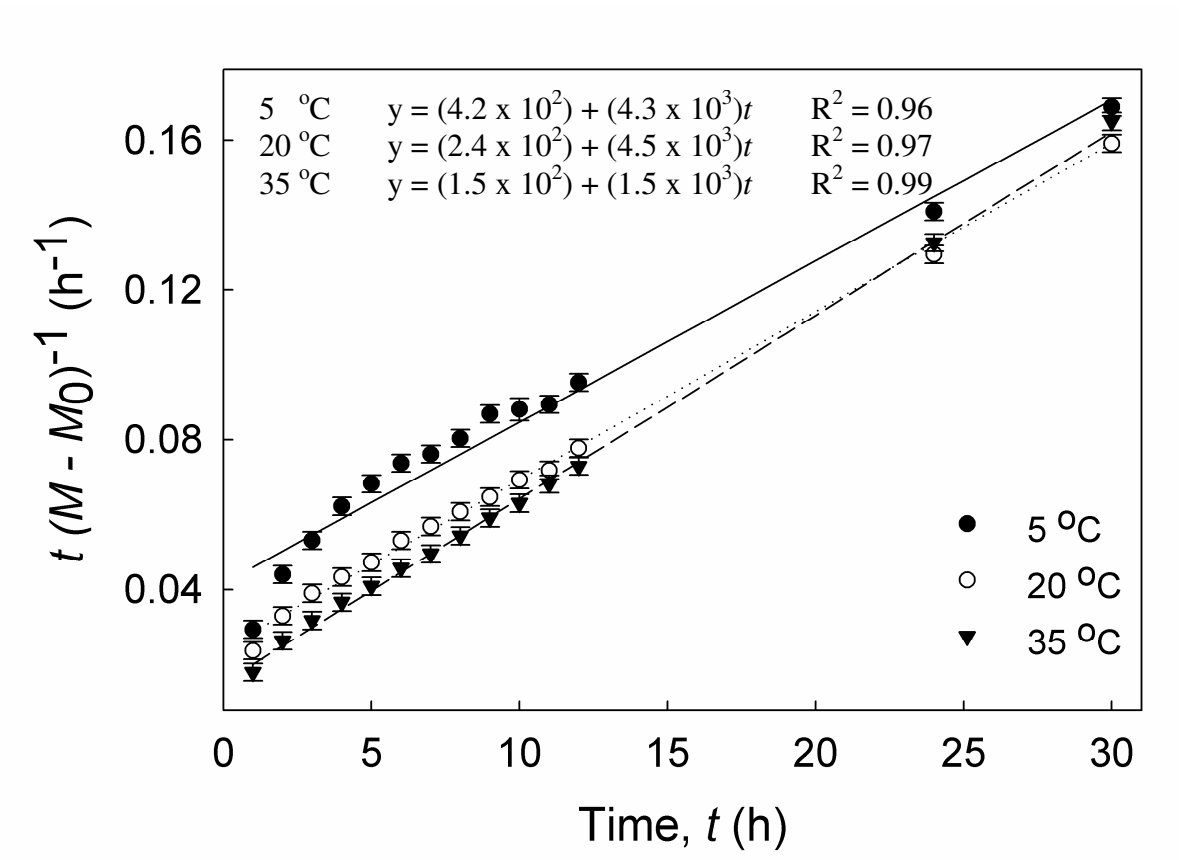

Figure 4.2. Linearization of moisture sorption curves of *R. asiaticus* tubers submerged in distilled water. *M(t)* and M_0 are the moisture contents (% dry basis) at time t and 0, respectively. Data are means ± standard error.

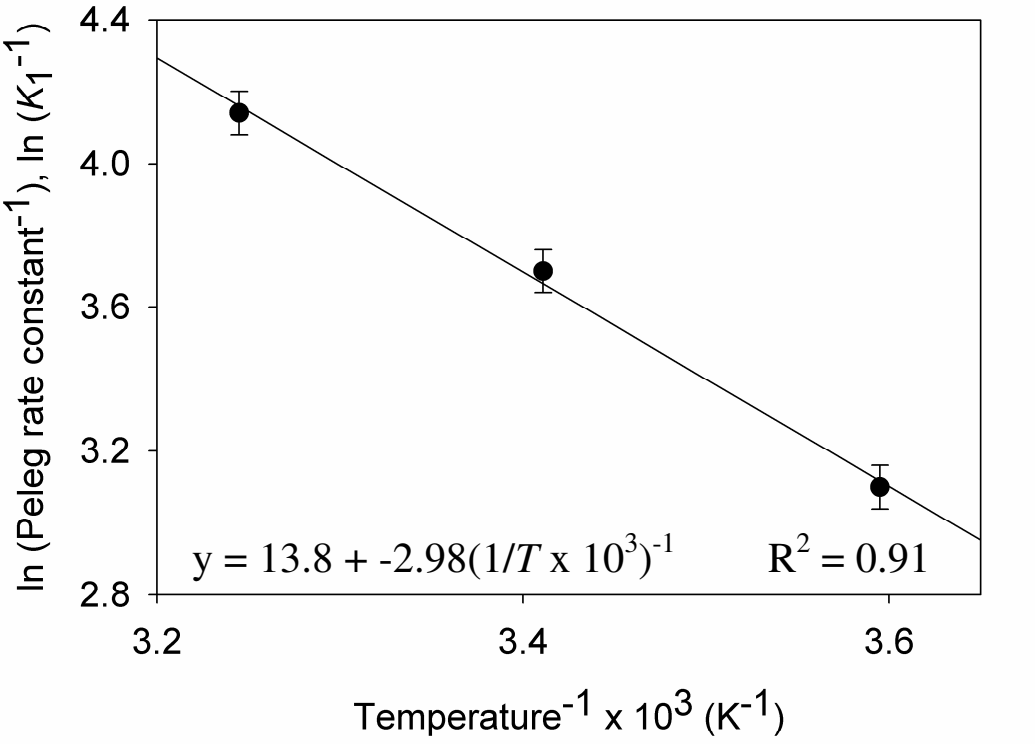

Figure. 4.3. Arrhenius plot for the Peleg rate constant, K_1 during hydration of *R. asiaticus* dried tubers. Symbols represent means ± standard error.

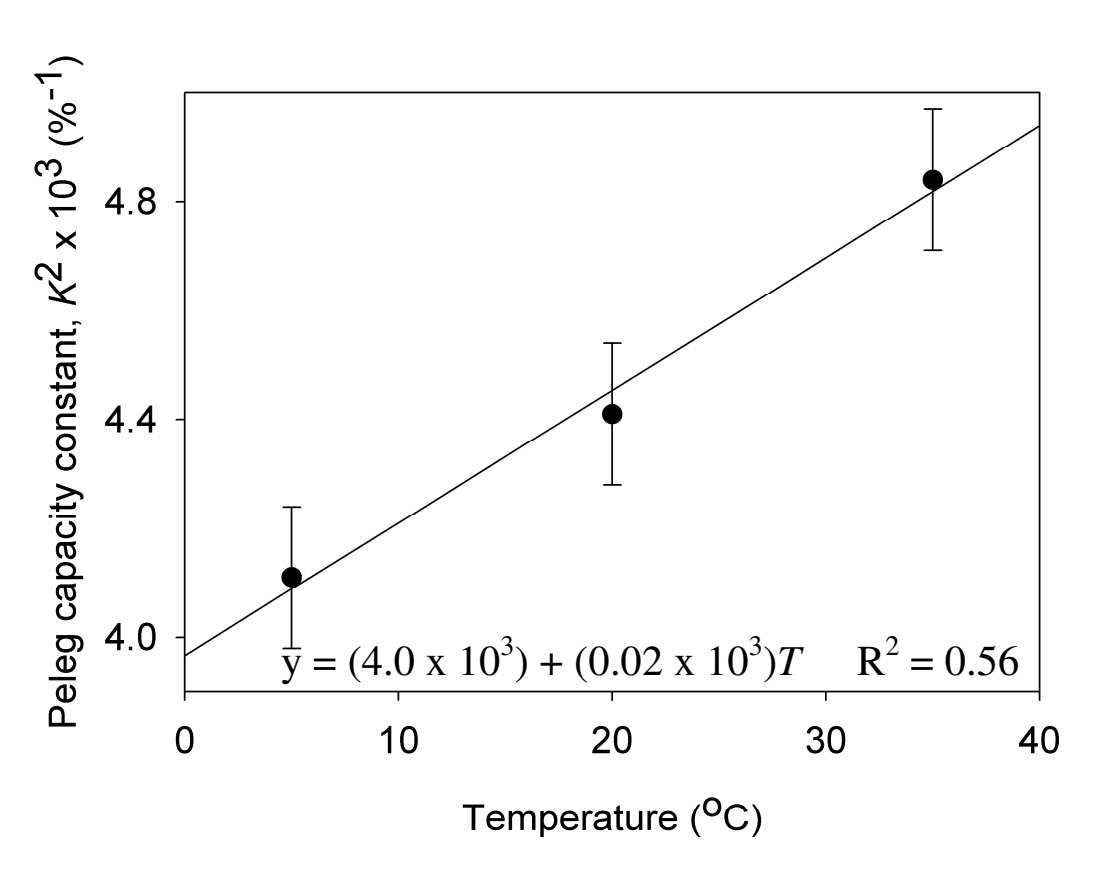

Figure 4.4. Influence of temperature on the Peleg capacity constant, K_2 during hydration of *R. asiaticus* dried tubers. Symbols represent means ± standard error.

might eventually commence growth, resume rapid accumulation of fresh weight, and therefore pass the estimated value of M_E. To avoid these pitfalls, one option is to set an hydration threshold as a cut-off for practical applications. In our experiment the time necessary to achieve 75% of M_E was 30 h at 5 ^oC and decreased to 15.0 or 9.6 h as temperature increased from 20 or 35 ^oC, respectively (Table 4.2). Increasing the cut-off to 85% might remain practical at higher temperatures, depending on the application, but not at 95% of M_E (Table 4.2). Hydrating tubers for 12 h at 5, 20, or 35 ^oC resulted in moisture contents within 57%, 71%, or 82% of M_E, respectively (Table 4.1 and 4.2). Increasing the soaking time to 24 h increased moisture content another 16%, 13%, or 7% to 73%, 84%, or 89% of M_E when hydrated at 5, 20, or 35 ^oC, respectively (Table 4.1). So, depending on the hydration temperature and desired cut-off threshold, 12 to 24 h appears to be sufficient. The influence of soaking duration on subsequent plant performance of *R. asiaticus* is not known.

Greenhouse experiments

In all three experiments an optimum hydration temperature was shown in plant survival, indicated by significance of the quadratic relationship (Temp x Temp interaction) (Fig. 4.5, Table 4.3 and 4.4). As hydration temperature increased the percent survival also increased to a maximum at ca. 20 or 15 ^oC, for experiments one and two, or three, respectively, then decreased as hydration temperature increased (Fig. 4.5, Table 4.3 and 4.4). In the first experiment, plant height and foliar dry weight followed a similar trend, although were less affected by temperature when hydrated at 5 ^oC than at 35 ^oC (Fig. 4.5). In the second experiment, plant height after 4 weeks was optimum when tubers were hydrated at ca. 25 ^oC. There were no statistically significant differences in plant height and number of flowers after 11 weeks growth, averaging 22.2 g and 2 per plant, respectively. Foliar dry weight did not indicate an

optimum hydration temperature, but increased linearly with hydration temperature (Table 4.3).

When the number of tested cultivars was increased in Expt. 3, the overall quadratic nature for plant survival was unchanged; although R09 had the highest survival, followed by W09, L08, and P08 with the lowest (86%, 81%, 64%, and 34%, respectively) (Table 4.4). The visual quality, size, and foliar dry weights of plants after 4 weeks growth all indicated an optimum hydration temperature between 15-25 ^{o}C. Visual plant quality was similar among cultivars (Table 4.4). The overall trend toward optimum plant size in response to tuber hydration temperature was not influenced by cultivar, however certain cultivars responded differently to specific hydration temperatures (Table 4.5). For example, P08 plants were at least 50% smaller than the other cultivars when hydrated at 5 ^{o}C, yet when hydrated at 25 ^{o}C all cultivars had similar size after 4 weeks growth. Overall, growth of R09 plants was least affected by hydration temperature.

Tuber age appeared to have some influence on response to hydration temperature. Since P08 and L08 tubers were at least one year older than the 2009 cultivars (R09 and W09), we are able to make some assumptions in this regard. P08 was the most susceptible to temperature influence among the cultivars tested (Table 4.4 and 4.5), while young tubers, R09 and W09, were less affected by temperature. There is a similar phenomenon in seeds, where some older seeds are more susceptible to hydration injury than fresh due to weaknesses in membrane integrity attributed to the natural aging process (Tilden and West, 1985; Priestley, 1986).

Results were initially surprising. We expected decreased plant survival at higher temperature due to the rapid influx of water, a condition commonly observed in large seeds and legumes (Priestley, 1986; Copeland and McDonald, 2001). This trend

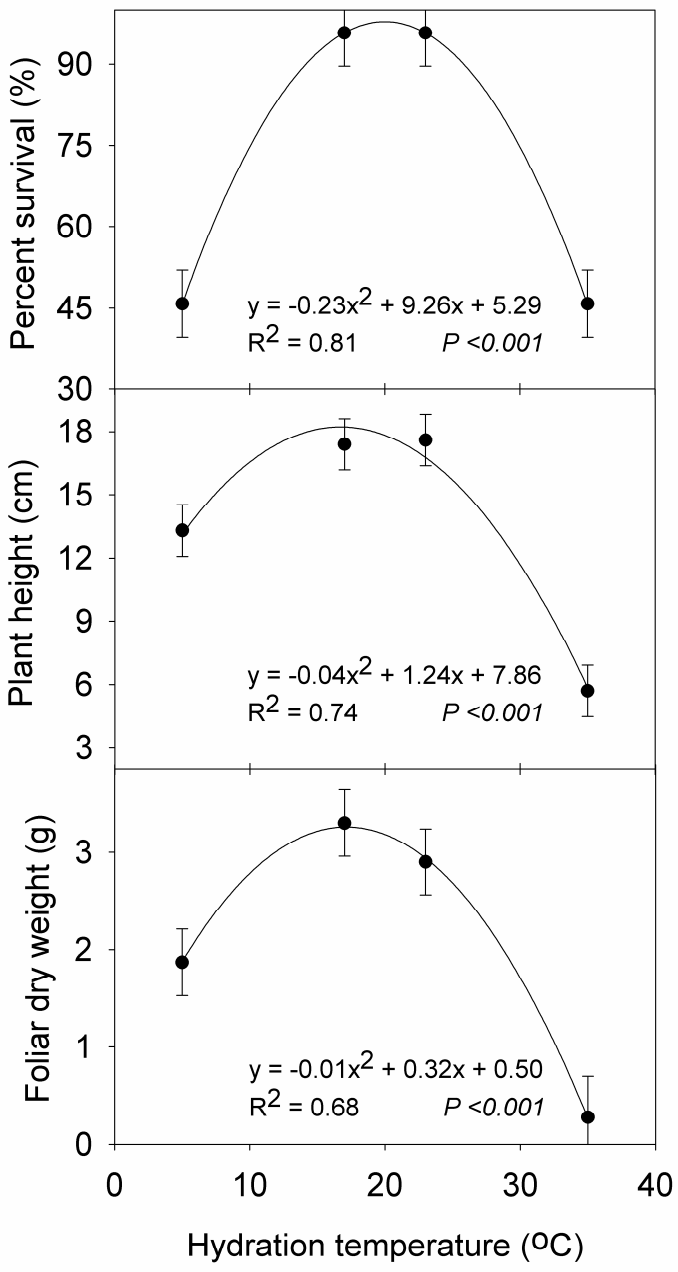

Figure 4.5. Expt. 1. Influence of tuber hydration temperature on percent survival (any visible growth after 4 weeks), plant height (pot rim to tallest leaf), and foliar dry weight (severed at soil line and dried 3d at 70 ^{o}C) of *Ranunculus asiaticus*. Data are means ± standard error.

Table 4.3. Expt. 2. Influence of tuber hydration temperature on percent survival (any visible growth after 4 weeks), plant height (pot rim to tallest leaf after 4 or 11 weeks in greenhouse), and foliar dry weight (severed at soil line and dried 3d at 70 ^{o}C) of *Ranunculus asiaticus*.

Hydration temp. (^{o}C)	Survival (%)	Height 4 wk (cm)	Height 11 wk (cm)	Foliar dry weight (g)
5	36	4.7	20.7	2.99
10	50	6.1	21.7	3.72
17	69	8.4	24.4	4.03
20	89	8.0	21.9	4.77
25	56	9.7	18.8	3.48
30	83	9.2	26.2	5.62
35	56	5.9	21.5	4.36
Temp. (T)	***	**	ns	*
T x T	***	***	ns	ns

ns, *, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively

Table 4.4. Expt. 3. Influence of tuber hydration temperature on percent survival (any visible growth) and plant visual quality (1=poor; 5=best) of *Ranunculus asiaticus* after 4 weeks in the greenhouse. P08, L08, R08, and W09 represent the following cultivars from 2008 and 2009 harvest seasons, respectively: Tecolote Pink, Labelle Cream, Tecolote Red, and Tecolote White.

Hydration	Cultivar					Cultivar				
temp. (^{o}C)	P08	L08	R09	W09	avg	P08	L08	R09	W09	avg
	Survival (%)					*Visual quality*				
5	25	70	90	100	71	3	3	3	4	3
15	60	85	95	95	84	4	4	3	4	4
25	35	70	100	90	74	3	4	4	4	4
35	15	30	60	40	36	3	2	3	4	3
avg	34	64	86	81	66	3	3	3	4	3
Cultivar (C)			***					ns		
Temp. (T)			***					**		
C x T			ns					ns		
T x T			***					**		
T x T x C			ns					ns		

ns, *, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively

Table 4.5. Expt. 3. Influence of tuber hydration temperature on plant size (mean of height and two cross canopy diameter measurements), and foliar dry weight (after 3d at 70 ^{o}C) of *Ranunculus asiaticus* after 4 weeks in the greenhouse. P08, L08, R08, and W09 represent the following cultivars from 2008 and 2009 harvest seasons, respectively: Tecolote Pink, Labelle Cream, Tecolote Red, and Tecolote White.

Hydration	Cultivar					Cultivar				
temp. (^{o}C)	P08	L08	R09	W09	avg	P08	L08	R09	W09	avg
	Size (cm)					*Foliar dry weight (g)*				
5	7.5	15.3	14.9	17.2	13.7	0.10	0.32	0.38	0.54	0.34
15	11.7	16.2	14.7	18.3	15.2	0.40	0.43	0.37	0.65	0.46
25	13.8	15.9	17.0	16.0	15.7	0.47	0.48	0.45	0.46	0.47
35	14.0	9.3	13.8	14.5	12.9	0.28	0.17	0.28	0.46	0.30
avg	11.8	14.2	15.1	16.5	14.4	0.31	0.35	0.37	0.53	0.39
Cultivar (C)			***					**		
Temp. (T)			**					**		
C x T			**					ns		
T x T			**					***		
T x T x C			ns					ns		

ns, *, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively

was clearly evident at 30 ^{o}C and above; however, we did not expect to see reduced survival at low hydration temperatures.

It is possible that moderate temperature water helps break dormancy in *R. asiaticus* tubers that is not achieved when hydrated cooler. Meynet (1993) speculated on a water-soluble thermo-labile compound that may be responsible for tuber dormancy; however this has not been identified. He suggested storing tubers for two months at 25 ^{o}C, ten days at 35 ^{o}C, or two days at 40 ^{o}C to break dormancy, with those stored at 2 ^{o}C maintaining dormancy for more than six months. In our experiments the hydration period was 24h, which does not support Meynet's hypothesis.

An alternative hypothesis is that cooler temperatures cause physiological damage to the cellular membranes (chilling injury). Pollock and Toole (1966) thought chilling injury in lima bean (*Phaseolus lunatus* L.) caused physical damage to cellular membranes resulting in their rupture. Christiansen (1968) hypothesized that cold prevents a metabolic response in cottonseed (*Gossypium hirsutum* L.) rather than inducing direct physical damage because damage was additive with increased cold duration. Powell and Mathews (1978) hypothesized that so-called chilling injury is the result of imbibition damage rather than the effects of low temperature. This hypothesis was supported in pea (*Pisum sativum* L.) that had seed coats "nicked" to allow more rapid imbibition, but reducing the water absorption rate through osmotic inhibitors lessened the degree of injury (Tully et al., 1981). The current thinking is that the cold temperature slows a membrane phase transition during hydration, thus allowing damaging rates of hydration and/or excessive leakage of vital nutrients for growth (Copeland and McDonald, 2001). It is therefore possible that the same phenomenon, rapid imbibition, is responsible for decreased survival in *R. asiaticus* at both high and low temperatures. Further investigations are necessary to determine if mixing an osmotic or matric inhibitor, such as polyethylene glycol (PEG) into the *R.*

asiaticus hydration solution will alleviate symptoms observed at potentially damaging temperatures. Since the PEG solution would reduce the rate of water uptake, it may serve to further determine a mechanism for damage outside of the optimum range for growth.

When soybean seeds (*Glycine max* L.) were given "priming" treatments (exposed to periods of brief hydration and re-drying at temperatures above which chilling injury occurs [25 ^{o}C]), the cellular damage was lessened when seeds were later soaked in 4 ^{o}C water (Tilden and West, 1985). In one preliminary experiment dry *R. asiaticus* tubers had improved sprouting when hydrated 24 h at 25 ^{o}C, allowed to re-dry one week at the same temperature, and then re-hydrated 24 h at 25 ^{o}C, compared to those given a single hydration period (24 h at 25 ^{o}C) (Cerveny and Miller, unpublished data). It is not known if priming *R. asiaticus* tubers at moderate temperature (20 ^{o}C) will later alleviate the observed problems when hydrating at low temperatures.

Considering non-sprouted tubers were treated as missing data after percent survival calculations, it is interesting that the trend for plant quality parameters were generally consistent with trends in plant survival for the first 4 weeks of growth (Tables 4.3-4.5). This effect appears transient however, since those observed parameters were not significantly different at 11 weeks growth. Number of flowers was not affected by hydration temperature presumably because initiation occurs after sprouting in the *R. asiaticus* growth cycle (Kamenetsky et al., 2005).

Due to noticeable growth habit variations between individual *R. asiaticus* plants, we were unsure if our measured parameters were adequate to describe plant growth in experiment one, thus the increase in measured parameters with subsequent experiments; however, since height, size, and plant visual quality were consistently

supported by foliar dry weight values it seems the measured parameters were appropriate quantifiers for *R. asiaticus* growth (Fig. 4.5, Tables 4.3-4.5).

Commercial producers are obviously interested in growing plants for profit, so ideally one plant would be produced from each tuber planted. Therefore, plant survival is probably the most important variable for determining commercial treatment success. Once survival is optimized, quality parameters become increasingly important. Although many plant quality parameters appeared to peak at around 20 ^{o}C, a range of temperatures between 15 and 25 ^{o}C should be considered acceptable (Fig. 4.5, Tables 4.3-4.5).

It is important to point out the common cultural recommendation to "hydrate tubers via slowly running water" (De Hertogh, 1996) may be flawed. In Ithaca, NY, tap water temperature varies with season. We have observed tap water temperatures between 5 and 15 ^{o}C, which may be too cold for optimum hydration of *R. asiaticus*. Under normal commercial conditions it would be unlikely that *R. asiaticus* tubers would be hydrated at a temperate high enough to cause damage. Obviously, careful attention should be given if it becomes necessary to heat hydration water. Nevertheless, water temperature is an important factor when hydrating *R. asiaticus* and should be monitored.

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APPENDICES

Introduction to appendices

The following appendices chronicle several exploratory experiments that may warrant future investigation. Much of the work listed below helped shape the focus of the dissertation and provided useful experience working with *R. asiaticus*. Because these were intended to be exploratory experiments, a few of the details normally provided in the materials and methods sections are missing; admittedly, this was partly due to mismanagement of records. While some of the results are debatable, I've tried to highlight them where appropriate. Nevertheless, the information is recorded here for future researchers of *Ranunculus asiaticus* who may come across this dissertation. Hopefully it will save them some time from having to repeat my experiments.

Appendix I

Fungicide Experiment

A fungicide experiment was conducted to determine if timing of application influences the efficacy of Phyton 27. *R. asiaticus* tubers 'W09' and 'R09' were obtained from the 2008/2009 growing season in southern California (California Flowerbulb Co., Carlsbad, CA) and were treated with Phyton 27 (Phyton Corp., New Hope, MN) a copper sulfate biocide, at 1375 $mg \cdot L^{-1}$ metallic copper. We have found this concentration to be effective at reducing damage associated with root-rot pathogens during growth of *R. asiaticus*. Hydration of tubers was conducted by submersion in tap water for 24 h at 25 ^{o}C. Treatments consisted of soaking tubers in biocide for 5 minutes before or after hydration, or before direct planting in moist potting media. A full hydration (24 h) in biocide and two controls (5 min soak in water post or without hydration) were also included. At the conclusion of all

treatments, tubers were planted four per pot in standard 15 cm containers using a commercial substrate (Sun Gro LC1, Sun Gro Horticulture Canada Ltd. Vancouver, British Columbia) with crowns covered approximately 2 cm. After holding potted tubers for 4 weeks at 5 ^{o}C, plants were moved to a 15 ^{o}C set-point temperature greenhouse. After 4 weeks growth, plants were evaluated for percent survival (any visible growth) and foliar dry weight (after 3 d at 70 ^{o}C). A visual ranking of plant quality was also made using a 1-5 scale: 1= poor quality, one to two leaves, diseased and/or dying; 2= slightly more growth, three to six leaves, but still unacceptable quality; 3= acceptable level of quality, seven to 10 leaves, non-uniform growth; 4= moderate quality, uniform growth, greater than ten leaves; 5=best quality, ideal size and shape, greater than ten leaves.

Results

There were no differences between cultivar, therefore all results are averages of the two investigated. Applying fungicide during hydration was apparently phytotoxic to *R. asiaticus* tubers since all measured parameters (survival, rank, dry weight) were lowest following this treatment. It is possible that a lower concentration of fungicide may have prevented damage. The initial concern was that tubers would need to be hydrated prior to applying this fungicide because rapid uptake of active ingredient (copper sulfate) may cause problems. This treatment was designed to determine if damage would occur under a "worst case" scenario; and it did (Fig. A.1).

Treating tubers with fungicide before or after hydration worked equally well as long as tubers were given a hydration treatment. When dry *R. asiaticus* tubers were soaked in fungicide and then planted without a 24 h submersion treatment, these plants had lower survival, visual quality, and foliar dry weight than those given hydration. It is possible that the fungicide had a phytotoxic effect, as with the longer soak, but the

decline with this treatment may also be due to other factors. In other experiments, we have shown that *R. asiaticus* tubers perform poorly when hydration temperature is below 15 ^{o}C. In this experiment the dry-planted tubers were immediately stored at 5 ^{o}C, which ultimately imposed a 5 ^{o}C hydration treatment; this could have contributed to the decline in measured parameters. The dry control, tubers given a 5 min soak in tap water before direct planting, substantiates this hypothesis for visual quality and foliar dry weight, but not with percent survival. Percent survival was similar among the dry control and those given a hydration treatment. From this experiment we were unable to tell if a copper sulfate fungicide treatment prior to direct planting is phytotoxic to *R. asiaticus.*

In nearly all cases fungicide application was not necessary; as the hydrated control performed equally well to those not treated. However, from past experience with *R. asiaticus*, the biocide is more of an "insurance policy" for prevention of disease. The non-effect of its application in this experiment may speak more to the lack of disease pressure with tubers planted than its true necessity. If this experiment is to be repeated, it may be necessary to inoculate tubers with known pathogens prior to the copper sulfate treatment. This treatment might determine the true efficacy of Phyton 27 on surface sterilization *R. asiaticus* tubers. Nevertheless, tubers treated with Phyton 27 at 1375 mg·L^{-1} were consistently among the highest performing plants with all measured parameters. Although its clear influence was not clearly isolated, in no case did copper sulfate application contribute to a decline in measured parameters, as long as tubers were given a hydration treatment.

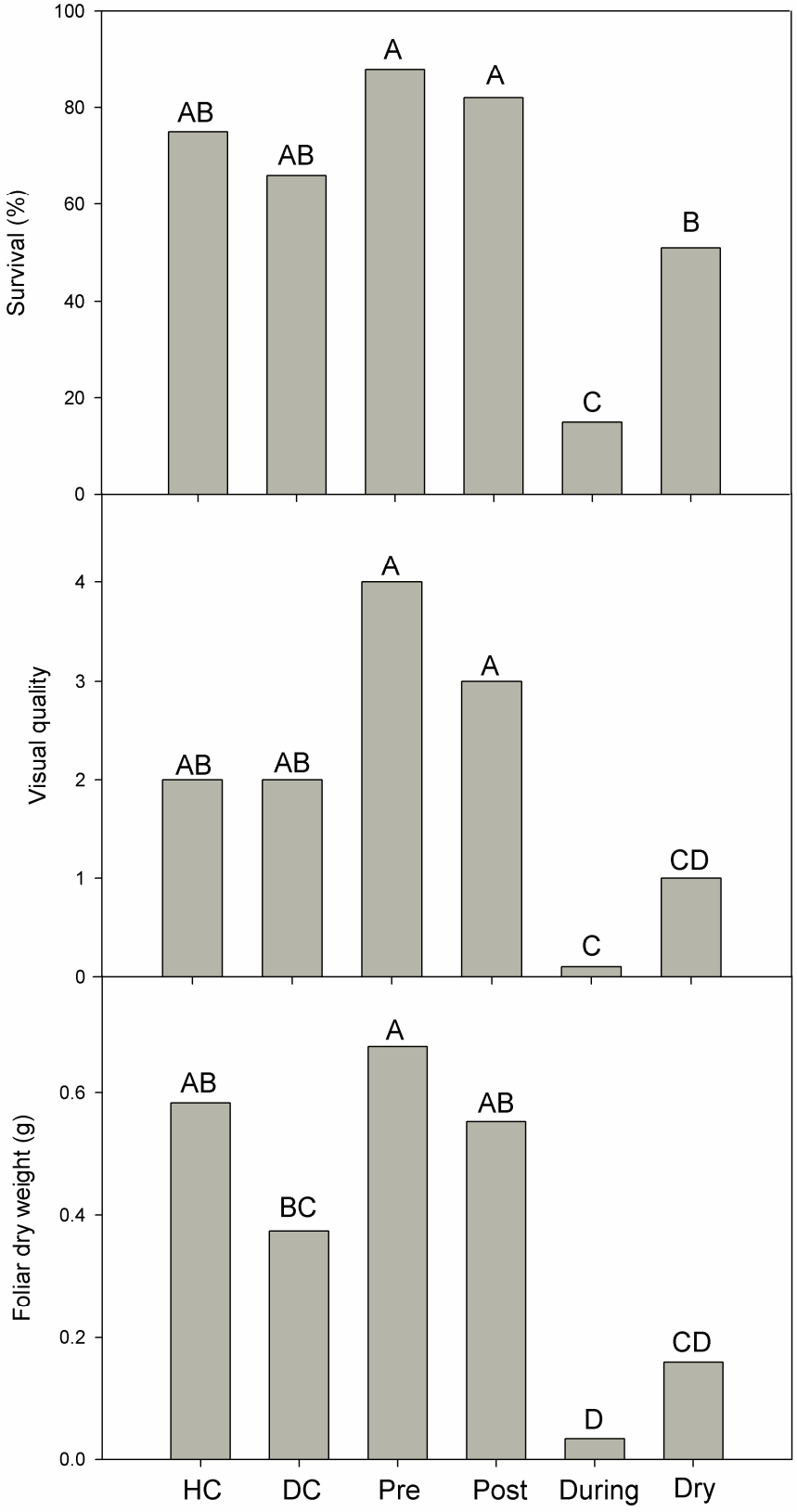

Figure A.1. Influence of fungicide (Phyton 27) application technique on percent survival (any visible growth), visual quality (1-5; 1=poor, 5=best), and foliar dry weight (biomass severed at soil level, dried 3 d at 70 ^{o}C) of *R. asiaticus* dried tuberous roots following planting. DC or HC signify dry or hydrated control, respectively, and were dipped in water for 5 min before planting or after hydration (24 h at 25 ^{o}C). Pre, Post, or During are soaks in Phyton 27 biocide for 5 minutes before, after, or during hydration. Dry is a fungicide treatment to dry tissue that was planted without a hydration treatment.

Results

Appendix II

Hydration Method

There is a cultural recommendation "to hydrate tubers in slowly running water" that persists in the literature (De Hertogh, 1996; Umiel and Hagiladi, 1999). It is unclear the origin of this recommendation or what in particular about "running water" is important for *R. asiaticus* tuber hydration.

It is possible that *R. asiaticus* tubers are sensitive to anaerobic conditions and therefore become damaged when hydrated in stagnant tap water. In this case, slowly running water may serve to aerate the hydration solution. Kamenetsky et al. (2004) described a loss of protein and starch compounds during tuber hydration. It is possible that some inhibitory compound for sprouting must first be leached away before tubers will commence growth. Lastly, it is also possible that the recommendation persists without value. That is, running water is not essential to sprouting. Before an experiment was designed to elucidate the reasoning behind running water, a simpler test was conducted to determine if running water is necessary for tuber sprouting or growth.

Experiment 1

In a preliminary study tubers were hydrated in stagnant tap water, tap water with air bubbled through it, or slowly running water. In this experiment tubers were hydrated 24 h in 5-gallon plastic buckets filled with approximately 6 L water. They were then provided a fungicide treatment, 20 min soak in Captan (N-trichloromethylthio cyclohexene-1,2-dicarboximide) mixed at 2600 $mg \cdot L^{-1}$ a.i., followed by a soil drench of half-strength solution. The tubers were planted in 7.5 cm diameter plastic pots using a commercial substrate (Sun Gro LC1, Sun Gro

Horticulture Canada Ltd. Vancouver, British Columbia). Potted tubers were held at 5 ^{o}C for 4 weeks to allow some root establishment prior to growing in a 15 ^{o}C set-point temperature greenhouse.

After 4 weeks growth, there were no differences among hydration treatments. It was then proposed that the lack of difference may be due to a large tuber to volume ratio. That is, the volume may have been large enough to leach away inhibitory compounds in all treatments, and that dissolved oxygen was high enough in all treatments to avoid damage. Therefore a second experiment was designed in which smaller volume vessels were utilized.

Experiment 2

In a second study, tubers were hydrated in 100 mL plastic vials containing 4 tubers per container. This amount of tissue filled the bulk of the container and minimized the tuber to volume ratio, as compared to the same vessel with less tissue. Tubers were hydrated 24 h at room temperature (ca. 22 ^{o}C), provided fungicide, and planted as above, except were planted in 15 cm diameter pots with the 4 hydration sub-samples planted in the same container. There were 4 repeat hydration vessels per treatment. This experiment was conducted twice.

After 4 weeks growth plants were measured for percent survival; results are presented in Fig. A.2. In both rounds of the experiment, bubbling water gave the highest percent survival, but was similar to flowing water in round 1 or stagnant water in round 2. It is unclear why flowing water was equal to the superior treatment in round 1 but not round 2; however, it may be due to the temperature at which tubers were hydrated. In another experiment (see chapter 2), we determined that hydration temperature below 15 ^{o}C may inhibit *R. asiaticus* sprouting and subsequently affect plant quality. Although tubers were hydrated at room temperature, the running water

temperature was probably much colder. Since the time of this experiment, we have observed tap water temperatures to be 5-10 ^{o}C, which may be too cold for optimum growth. This does not explain why running water was among the best treatments in the first round, but it may serve to highlight the variability encountered when hydrating below 15 ^{o}C.

From these experiments we were unable to determine the best means for hydration of *R. asiaticus* tubers prior to planting, but were able to show that it may not matter if the tuber surface area to hydration volume is sufficiently high. Further studies should be conducted to further develop a hydration protocol for *R. asiaticus*.

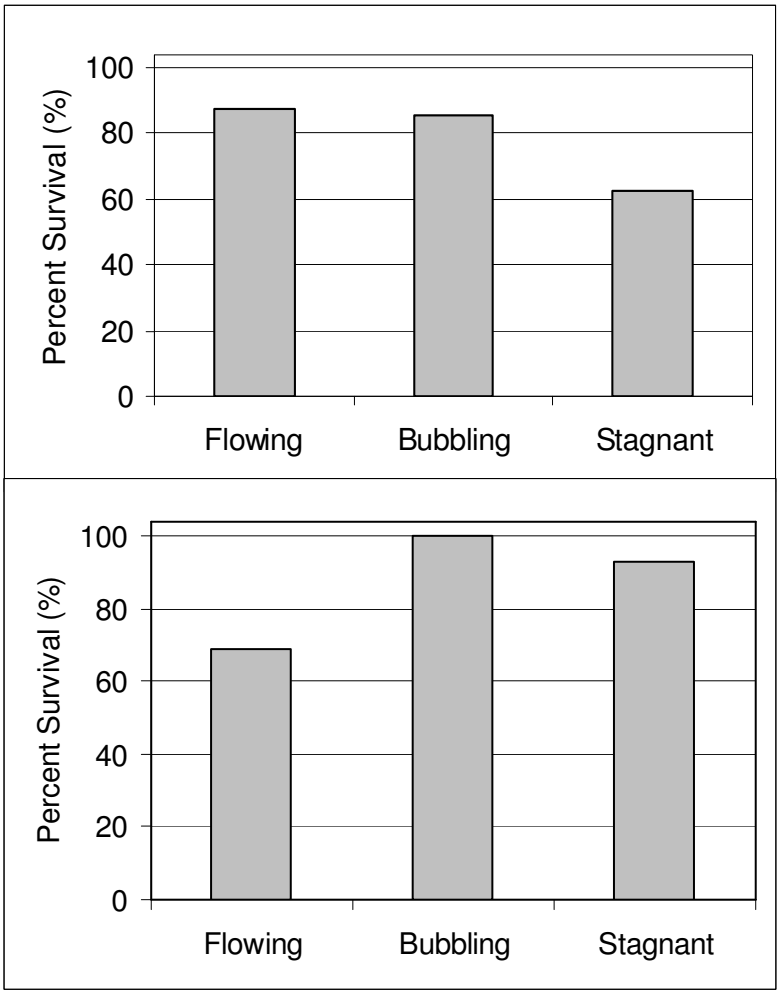

Figure A.2. Influence of hydration method on percent survival of *R. asiaticus* after 4 weeks in the greenhouse from two repeat experiments. (Round 1, top; Round 2, bottom)

Appendix III

Planting depth

To investigate the influence of planting depth on *R. asiaticus* tuber growth, an experiment was initiated on 24 Sept. 2008 using 'Tecolote Pink' tubers from the 2007 harvest season in southern California (California Flowerbulb Co., Carlsbad, CA). Tubers were submerged in tap water for 24 h at 18 ^{o}C, then provided a 20 min soak in Captan fungicide (N-trichtoromethylthio cyclohexene-1,2-dicarboximide) mixed at 2600 mg$\cdot$L^{-1} a.i., followed by a soil drench of half-strength solution. They were planted using a commercial potting mix (Sun Gro LC1, Sun Gro Horticulture Canada Ltd. Vancouver, British Columbia) in 7.5 cm square pots. Treatments consisted of planting tubers at the top of the pot (with crowns covered approximately 2 cm), at the middle of the soil column (ca. 6 cm deep), or placed in the bottom of the pot (ca. 12 cm deep). Potted tubers were then held at 5 ^{o}C for 4 weeks to allow some root establishment prior to forcing in a 15 ^{o}C set-point temperature greenhouse. Plants were evaluated for percent survival (any visual growth) and height (soil line to tallest leaf) after 4 weeks growth.

Results

After 4 weeks growth in the greenhouse, there were no differences among treatments for percent survival or plant height, averaging 94.4% or 18.1 cm, respectively. Although a totally exposed (uncovered) treatment was not specifically tested, in previous experiments this caused some challenges in the greenhouse. When crowns are left uncovered in the dark rooting room (5 ^{o}C), roots grow along the soil surface before penetrating into the pot. Once these plants are brought into the bright greenhouse, exposed roots become burned in the sun, desiccate, and the benefit of pre-sprouting is lost.

Since there were no differences in percent survival or plant height among treatments, it can be concluded that as long as crowns are at least minimally covered, planting depth is not an important factor when producing *R. asiaticus* plants from dried tubers,

Appendix IV

Extreme handling of R. asiaticus

Liquid nitrogen

The following observational experiment was conducted to verify claims by Meynet (1993) and Sakai (1960) that *R. asiaticus* tubers can survive being immersed in liquid N_2. Dried tubers from the 2008 harvest season in California (California Flowerbulb Co., Carlsbad, CA) (cultivar not recorded) and France (unknown origin) ('Labelle Cream') were submerged in liquid nitrogen for 5 minutes. The tissue was removed from N_2 treatment and then allowed to thaw 24 h at room temperature (ca. 22 ^{o}C). Tubers were then submerged in tap water for 24 h at 25 ^{o}C, and then given a 5 min soak in a copper sulfate biocide (Phyton-27, Phyton Corp., New Hope, MN) at 1375 $mg \cdot L^{-1}$ metallic copper. Tubers were planted four per pot, in 15 cm diameter azalea pots using a commercial potting mix (Sun Gro LC1, Sun Gro Horticulture Canada Ltd. Vancouver, British Columbia) with crowns covered approximately 2 cm. The specific quantity of tubers planted was not recorded but was approximately 16 of each cultivar (4 pots). Planted tubers were moistened with tap water and then held at 5 ^{o}C for 4 weeks to allow some root establishment prior to growing in a 15 ^{o}C set-point temperature greenhouse. Data were not formally collected.

Approximately 50% of tubers submerged in liquid nitrogen sprouted. The plant quality was poor, but this may have been related to the original quality of these tubers at treatment, than submerging in liquid nitrogen. It is impossible to make a

direct comparison though, as an appropriate control was not included in this experiment. Approximately 25% of the sprouted tubers flowered. Results from this casual experiment are consistent with suggestions by Meynet (1993) and Sakai (1960) that dried tubers of *R. asiaticus* are able to withstand submersion in liquid N_2 while in their fully dried state.

Drying oven

To investigate extreme drying of *R. asiaticus,* tubers collected from moisture content analysis in another experiment (storage under modified temperature and relative humidity) were planted following 3 weeks in a drying oven at 70 ^{o}C. Randomly selected tubers of unknown cultivars were prepared for planting as previously described (submerged 24 h at 25 ^{o}C and provided copper sulfate biocide before planting in 15 cm pots). After 4 weeks rooting at 5 ^{o}C, potted *R. asiaticus* were grown in a 15 ^{o}C set-point temperature greenhouse. Formal data collection did not occur.

Approximately 25% of the planted tubers sprouted after 3 weeks extreme drying at 70 ^{o}C. The majority of these sprouted plants had moderate to good visual quality and eventually flowered. These results were surprising as this treatment was designed to remove all internal moisture for determining dry weight; that is, the extreme drying "should have" killed all tissue. It is worth mentioning that tubers planted from these extreme drying conditions originated from an experiment designed to reduce plant vigor. Since tissue was randomly collected from a pool of tubers possibly containing non-viable tissue, sprouting may have been biased toward the low-end of what would be achievable with otherwise healthy tissue subjected to 3 weeks at 70 ^{o}C. This casual study serves to highlight some extreme conditions in which these (obviously) desiccation tolerant tuberous roots survive.

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