GAMETOPHYTIC SELECTION FOR THERMOTOLERANCE IN *PHALAENOPSIS*

By
Leslie A. Blischak

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Richard E. Veilleux, Chairman
Jerzy Nowak
Alex Niemiera

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Abstract

Gametophytic selection was examined as a breeding tool in developing *Phalaenopsis* hybrids that are more cool or warm temperature tolerant. Two hybrid *Phalaenopsis*, *P*. (Taisoco Windian × Sogo Yukidian) by *P*. hybrid unknown, were reciprocally cross-pollinated and exposed to 14°C/9°C for 7 days as a cold pollination treatment. Plants were pollinated again and exposed to 30°C/25°C for 3 days for the warm pollination treatment. Each cultivar was placed in either of two growth chambers during the pollination treatments and exposed to the selected temperatures, an 11-h photoperiod with an irradiance of 180 $Mmol \bullet m^{-2} \bullet s^{-1}$ and a relative humidity of 70%. The plants were returned to the greenhouse after pollination and the green capsules were collected after 150 days. Seeds obtained from these treatments were surface-sterilized and equal volumes were placed on Phytamax® medium. Evaluation of protocorm development was done after 73 days on a thermogradient table ranging from 10 to 30ºC. For the first family for which reciprocal crosses were available, the number of protocorms per plate ranged from 0 in the coldest treatments to 290 at 28°C. For cold pollinated seeds, protocorm development was optimum at 22 and 28°C (means of 290 and 250 protocorms per plate, respectively) whereas the greatest protocorm development for warm pollinated seeds occurred at 20°C (103 protocorms per plate). Of the 1471 total protocorms obtained 1095 were from cold pollinations, whereas 376 were from the warm pollinations. Protocorms were evaluated for leaf and root formation 125 days after initial plating. Transfer to warm or cold incubators occurred as protocorms developed leaves and roots. Seedlings were finally transferred to dried sphagnum and placed in growth chambers set to original pollination temperatures. One year after initial plating seedlings were evaluated on the following criteria: wet weight, number of leaves, leaf area, number of roots, and root length. The pollination treatment significantly affected the number of roots per seedling whereas germination temperature during germination significantly affected the weight (g). Weight of the seedlings, number of roots and the average root

length were significantly affected by the interaction between pollination treatment and germination temperature. The weight, number of leaves, and average root length were significantly affected by the interaction between pollination treatment and incubator/growth chamber. These differences indicated that seedlings derived from warm pollination were more vigorous under warm growing conditions and those derived from cold pollination were more vigorous under cold growing conditions. The significance of the interaction between pollination treatment and incubator/growth chamber indicates that gametophytic selection for thermotolerance in *Phalaenopsis* can be successfully used as a plant breeding tool. Additional replication is required to confirm the greater germinability of seed derived from pollination occurring over a greater range of temperatures.

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Literature Review

The primary objective of any plant breeding program is the improvement of those characteristics that contribute to economic value. These characteristics may range from increased yield, ability to tolerate abiotic stresses, resistance to pathogens and pests or even enhancement of aesthetic appeal (Fehr 1991) Traditional selection has mainly focused on the sporophytic phase of a plant's life cycle. Sporophytic selection can be extremely time consuming, labor intensive and can occupy vast quantities of space. Large population sizes are required in order to obtain the essential combination of attributes needed to improve a crop. Many woody perennial species especially have lagged behind in new cultivar development due to larger physical size and long juvenile phases (Hormaza and Herrero 1996). With the introduction of a multitude of biotechnological advances, there are now many options available to plant scientists interested in improving an array of plant responses and characteristics. With more information available from plant genome sequencing projects, and such analysis techniques as microarrays, serial analysis of gene expression (SAGE), and transgenic reconstruction, a small but growing resurgence of research focused on male gamete selection has developed (McCormick 2004).

The unique life cycle of angiosperms offers the opportunity to introduce early selection into breeding programs via gametphytic selection. The alternation of generations that occurs in angiosperms is a conserved attribute of the plant kingdom. The most prominent phase of the angiosperm life cycle is the sporophytic generation. Sporophytes develop from the zygote following fertilization. The gametophyte generation develops from spores derived from meiosis and so exists in a haploid form. In comparison to the sporophyte, the gametophyte has been reduced in both size and life span (Hormaza and Herrero 1996). Selection pressure can be applied to both male and female gametophytes. However, competition between the large numbers of male gametophytes during development in the anther, pollen germination and tube growth in the style, as well the independent phase that pollen experiences between shedding and stylar deposition, create an opportunity for easier manipulation using the male gametophyte (Ottaviano and Sari Gorla 1993). Due to the large population size and haploid state of the male gametophyte, selection during this phase could be more efficient than selection during the sporophytic phase (Sari-Gorla et al. 1992).

One important reason why selective pressures applied to male gametophytes have an effect on the sporophyte is the overlap of gene expression between the gametophyte and the sporophyte (Mascarenhas 1990). Genes affecting basic functions such as metabolic activities occur in both phases of life and, therefore, variability associated with this overlap can be affected by selective pressure on the gametophyte (Mascarenhas 1990; Ottaviano et al. 1982; Ottaviano and Mulcahy 1986). In addition genes that contribute to pollen success may also confer resistance to various stresses or increased vigor to the sporophyte (Chasan 1992). Comparison of the sporophyte and gametophyte transcriptomes of *Arabidopsis thaliana* using micro-array analysis reveals an extensive overlap of 61% indicating that 1 in 20 genes is preferentially or specifically active in *Arabidopsis* pollen (Honys and Twell 2003; Honys and Twell 2004). Estimates of a 58% overlap in tomato (Tanksley et al. 1981), a 60% overlap in barley (Pedersen et al. 1987) and a 72 % overlap in maize (Sari-Gorla et al. 1986) were calculated using isozyme profiling. A hybridization kinetics study estimated a 54% overlap in *Tradescantia* (Willing and Mascarenhas 1984). Advances in biotechnology such as microarray analysis have lent support to these early estimates of gametophytic and sporophytic transcriptome overlap (Honys and Twell 2003).

Many forms of gamete selection already exist in naturally occurring plant populations. According to Mulcahy (Mulcahy 1979) the "genecological" factor contributing to the rise of angiosperms is the ability to screen "any haploid genome that does not function with a high degree of metabolic vigor". A prominent example of this point is the tendency in most plant towards intense selection against meiotic chromosome deficiencies such as aneuploidy and deleterious alleles in microspore development (Ottaviano and Mulcahy 1986; Ottaviano and Mulcahy 1989). This may allow angiosperms the unique opportunity to benefit from the possible adaptive value of recombinants while avoiding the negative affects of poorly functioning recombinants (Mulcahy 1979). Selection of gametophytes with beneficial characteristics could confer an adaptive flexibility to the sporophyte thus producing plants able to pervade new habitats. If the resulting adaptation becomes "genetically fixed" new ecotypes and ultimately new species could evolve (Sultan 2005). Theoretically these long-term effects would only occur if population sizes were minimally 10 times the reciprocal of rates at which favorable mutations occur. Mutation rates are estimated to occur at 1×10^{-6} per locus. Microgametophyte populations of 10^7 are easily obtained in angiosperms (Honys and Twell 2003; Mulcahy 1979). Plant styles can screen large numbers of pollen grains in a short amount of time allowing selection of

favorable mutations in the microgametophyte population to occur without reduction in sporophytic fitness (Frova and Sari Gorla 1992; Mascarenhas 1990).

Male gametophytic selection can occur in two fashions: selection for pollen competitive ability or selection for tolerance to environmental stresses. The concept of selecting for pollen competitive ability relies on the assumption that the most vigorous pollen grains will confer vigor to the sporophyte (Ottaviano and Mulcahy 1989; Ottaviano *et al.* 1991). This concept is feasible when considering the previously discussed transcriptome overlap between the sporophyte and gametophyte. Two different methods can be used to modulate the intensity of gametophytic competition: varying the pollination intensity or varying the distance that pollen tubes need to grow in order to reach the ovule (Hormaza and Herrero 1996).

The most comprehensive research on pollen competitive ability has occurred in maize. In perhaps the first study linking gametophytic performance to a resulting sporophytic trait in maize was conducted in 1971 by Mulcahy. Heavier seeds resulted from fertilization by gametes from faster growing pollen tubes. In a later study, the main components of pollen competitive ability, faster pollen germination time and pollen tube growth rates, were shown to give rise to positive responses in kernel weight, seedling mean weight and root tip growth in the resulting seedlings of hybrid maize (Ottaviano et al. 1982). In a study focused on the genetic dissection of pollen competitive ability, germinability and pollen tube growth rate were studied through molecular marker analysis (RFLP). Both traits were found to be highly variable and heritable (Sari-Gorla et al. 1992). Germinability was linked with the early stages of pollen function and was determined to be largely controlled by the sporophyte during pollen development. In contrast, pollen tube growth rate was confirmed to be controlled by the gametophytic genome. This occurs because of the transition of the gametophyte from auxotrophic development to an independent state following the release of the pollen from the anther (Ottaviano et al. 1988; Sari-Gorla et al. 1992). In many species, including maize, pollen deposited on the style is composed of multiple genotypes. Intergametophytic effects were tested in maize by comparing mixtures of pollen from different genetic sources. The competitive ability of each line was significantly affected by the genotype of the competing pollen (Sari-Gorla and Rovida 1980).

Other species have also been used to investigate pollen competitive ability as a means of gametophytic selection as well. In naturally occurring populations of *Hibiscus moscheutos* (Malvaceae) commonly referred to as the rose mallow, individual pollen donors with the fastest growing pollen tubes consistently sired a larger proportion of seeds across multiple maternal

plants (Snow and Spira 1991; Snow and Spira 1996). Faster growing pollen tubes outcompeted slower tubes when applied at the same time, but that "faster" pollen did not sire more seeds when both types of pollen were applied after delays of the same duration (Snow et al. 2000). In *Erythronium grandiflorum* (Liliaceae) the composition of the pollen pool was the significant factor determining growth rate and pollen tube attrition.

Anemophilous plant species often have much lower pollination intensities, but that does not necessarily prevent pollen competition from occurring. A study on *Betula pendula* (Betulaceae) revealed that the response of the ovules contributes to pollen competition. Ovules do not develop until after male anthesis is complete, thus allowing all pollen tubes a "fair start". The first ovule of a pair penetrated by a pollen tube outgrows the other ovule leading to the fixed abortion of the second ovule (Dahl and Fredrikson 1996).

Pollen competition in *Silene latifolia* var Poiret (Caryophyllaceae), a dioecious, insect pollinated species, significantly affected the progeny sex ratio but did not affect several seed and seedling performance measures. Previous studies had indicated pollen competition affected seedling emergence time. That was not proven in the following study thus demonstrating the inconsistent effects observed in multiple experiments (Lassere et al. 1996).

These findings indicate the complex nature of pollen competition. Direct intergametophytic or pollen-style interactions could be responsible for these results. Advantageous maternal allocation of resources to seeds sired by the first pollen grains to arrive in *Cucurbita pepo* (Cucurbiatceae) may exacerbate the effects of pollen competition (Johannsson and Stephenson 1998a). This would provide zygotes derived from the "faster" pollen an advantage in resources such as endosperm over "slower" pollen. Pollen competitive ability would then manifest as a stronger, faster sporophyte due to maternal provisioning. Pollen-style interactions could account for much of the variability in pollen tube growth. According to one hypothesis "gametes from pollen tubes that function well in a particular stylar environment may give rise to heterotic progeny" (Mulcahy 1971). Johannsson and Stephenson were able to show that hybrid vigor extends to the gametophyte generation in a cross between a cultivated and wild *C. pepo,* although ultimately this was regarded as due to an environmental effect related to developmental provisioning of the pollen grains (Johannsson and Stephenson 1998b). Despite their sessile nature, a remarkable amount of control is exerted by plants over their "reproductive fates" through "elaborate, multilayered interactions" between pollen and stylar tissues as well as male gametophytic competition (Chasan 1992).

Analysis of a multi-locus selection model used to determine the effects of selection in the gametophyte stage on mutational load led to a major debate as to the ultimate effects of gametophyte selection on plant populations. A major point of contention was "If pollen tubes with the fastest growth rates tend to produce progeny with high fitness, this would lead to fixation of alleles for the best competing phenotype, and thus to loss of genetic variation" (Charlesworth and Charlesworth 1992b). In addition the model used in their analysis determined that subjecting pollen to strong selection would only increase sporophytic fitness marginally (Charlesworth and Charlesworth 1992a). Although mutation is considered the primary source of variation, recombination is the "immediate" source of variation. Gametophytes ranging from highly functional to nonviable can be generated through recombination in "well adapted and segregating" genomes (Mulcahy et al. 1996). Variation in pollen performance may be maintained by a combination of mutations, genotype × environment interactions, gene flow from populations without pollen competition, intergametophytic interactions, or negative genetic correlations (Pasonen *et al.* 2001; Snow and Spira 1996). Perhaps the most important point is that genotype × environment interactions can have a strong influence on population genetics. Different characteristics or conditions within the style or environment limit different pollen genotypes. Genotypes that confer fitness in one population may not confer the same level of fitness in another population, which would maintain the genetic variability (Delph et al. 1997; Mulcahy et al. 1996). The microenvironment in which some species exist may be more important in determining mating success than its genotype (Pasonen et al. 2000). Many studies confirm the findings that "regardless of other internal barriers, pollen competition strongly increases assortative mating" (Williams et al. 1999).

A transgenic reconstructive approach using tobacco pollen provides direct proof that selection acting on male gametophyte development can alter the allele frequency of the sporophyte (Touraev et al. 1995). Determining what characteristics the alteration of gene frequency confers to the sporophyte is the complicated aspect of studying male gametophytic selection. Applying environmental stresses to developing gametophytes is a possible means to alter allele frequency in order to select for resistance (Ottaviano et al. 1991). Once pollen is released from the anther it enters a quiescent stage until germination. Selective pressure can be most effectively applied at two times: during pollen development or during pollen germination and tube growth (Hormaza and Herrero 1996). Selection for such traits as herbicide resistance is effective during the quiescent stage, although for many environmental stresses it is important to

apply the selection pressure when genetic differences are fully expressed. Complex traits such as thermo-tolerance and disease resistance are generally controlled by multiple interactions acting on all levels of plant organization and are not often constitutively expressed (Ottaviano and Sari Gorla 1993). Selection operating on complexly and simply inherited traits as well as inducible and constitutive responses has been studied in many plant species (Hormaza and Herrero 1996).

Resistance to the herbicides alachlor and chlorsulfuron in maize was conferred using gametophytic selection during pollen storage and microgametophyte development respectively (Frascaroli and Songstad 2001; Sari-Gorla *et al.* 1994). Studies of gametophytic selection favoring pollen with disease resistant characteristics were successful in two separate species. By applying fusaric acid, a toxin produced by *Fusarium oxysporum*, to developing microgametophytes, (Ravikumar and Patil 2004), were able to increase the number of chickpea (*Cicer arietinum* L.) progeny that were resistant to wilt. The resistance to leaf blight of progeny was increased in *Helianthus annuus* (Asteraceae) through gametophytic selection by applying pathogen culture filtrate to the stigma and style. Gametes conferring resistance to the progeny were able to germinate and traverse the stylar tissues whereas non-resistant gametes were not able to germinate and therefore fertilize any ovules (Chikkodi and Ravikumar 2000; Ravikumar and Chikkodi 1998). Plants resistant to drought stress were produced using gametophytic selection on sorghum (Ravikumar et al. 2003). Polyethylene glycol was applied to stigma and stylar tissue before pollination to produce moisture stress conditions for the germinating pollen. Tolerance to multiple diseases, heavy metals, salinity and herbicides have all been achieved through gametophytic selection (Hormaza and Herrero 1996). In addition, male gamete selection has been used to select for early maturity in tomato, and linolenic acid content in rapeseed (Crispi and Peirce 1992; Jourdren *et al.* 1996).

Heat stress resistance is a complex trait that gametophytic selection appears to be effective in selecting. Increased heat stress tolerance of sporophytic progeny in tobacco was generated through treatments to developing microgametophytes. In addition seedling vigor was higher in progeny derived from selection even under treatments without undue stress (Mandhu et al. 1992). Petolino et al. (Petolino et al. 1990) also selected for heat stress tolerant maize progeny after applying heat to germinating pollen. Selected progeny were superior in seedling vigor, grain yield, and exhibited reduced stalk and root lodging. Heat tolerant seedlings were selected in *Lilium longiflorum* using heat treatments on pollen tube growth (Chi et al. 1999). Studies involving the heat shock response of maize have shown that pollen selection can improve

some gametophytic and sporophytic components of heat stress. Neither cellular membrane stability nor pollen tube growth itself responded to pressures applied through male gametophytic selection. Gametophytic selection for heat stress tolerance in maize increased the ability of progeny to synthesize heat shock proteins (Frova et al. 1995; Frova et al. 1992). Several HSP genes are already active in pollen development while other HSPs are inducible under heat stress conditions (Crone *et al.* 2001; Mascarenhas and Crone 1996). Induced HSP expression has been determined as critical to tolerance of high temperature stress in plants (Katiyar-Agarwal et al. 2001). Because of the overlap of transcriptomes, selecting for gametophytes with greater or more efficient HSP response to heat stress can positively affect the heat stress response of the resulting sporophyte. Without the additional response of the sporophyte, selecting for pollen that is able to germinate under high heat stress conditions is important in those summer flowering crops where seeds are the main yield component (Frova et al. 1995). Using male gametophtyic selection in the improvement of thermotolerance can be an efficient way to augment plant breeding programs (Frova et al. 1991).

Resistance to cold temperature stress is another complex trait considered to be controlled on multiple levels of plant organization. Many annual crops originated in the tropics or subtropics and are susceptible to chilling injury at temperatures of just 15^{o}C. In maize, an annual crop where gametophytic selection was studied intensely for high temperature tolerance, a correlation between the pollen germination characteristics and low temperature germination of kernels was observed. Cold storage conditions of pollen were determined to apply a strong selective pressure on different pollen genotypes. Cold tolerant lines produced pollen with a much higher germination rate after cold storage than those lines considered sensitive to cold. Only cold tolerant seeds were obtained after maize pollen was stored at 4^{o}C for one week. Using both gametophytic and sporophytic selection (Kovács and Barnabás 1992) were able to obtain S_6 lines of maize with stronger cold tolerance than the original parents. Pollen selection was investigated as a means to enhance chickpea (*Cicer arietinum*) breeding for chilling tolerance (Clarke and Siddique 2004). In a previous study, pollen germination was determined to be highly sensitive to cold temperatures leading to a decreased yield. By placing pollinated plants under cooler temperatures during pollen germination and tube growth, researchers were able to select for pollen that was resistant to cold temperatures. A lower threshold temperature for podding as well as pod-setting 2 to 4 weeks earlier were two significant advantages of chickpea plants produced through gametophytic selection (Clarke et al. 2004). Unintentional selection for decreased frost

hardiness has been studied in coniferous seedlings from seed orchards (Johnsen et al. 1995) as well as *Betula pendula* 'Roth' seedlings from plastic greenhouses(Pasonen et al. 2000). Gametophytic selection has also been investigated in gymnosperms (Johnsen et al. 1995). In one study, coniferous seedlings produced in a seed orchard were less resistant to frost when transplanted to harsher growing conditions. Deviations from the expected genetic composition of the seed crop produced in plastic greenhouses were observed in *Betula pendula* 'Roth' seed orchards. The number of pollen grains deposited on the stigma of trees in the plastic greenhouses consistently exceeded the number of ovaries. In addition, the daily mean temperature during pollen germination was 13^{o}C higher in plastic houses. A Correlation, due to gametophytic selection, between reduced frost hardiness and the prevailing environmental conditions during pollen germination and tube growth has been hypothesized (Pasonen et al. 2000). Specific sporophytic traits have been linked to gametophytic selection for cool temperature tolerance in both tomato and cucumber(Johannsson and Stephenson 1998a; Zamir and Gadish 1987). Root growth of tomato was found to be influenced by pollen selection during germination and tube growth. Pollen of an interspecific hybrid between *Lycoperiscon hirsutum* and *L. esculentum*, was applied to male sterile *L. esculentum* plants under normal and cold conditions. The rate of root elongation of seedlings produced from cold temperature crosses was higher than seedlings from normal temperature crosses when each was placed in cold growing conditions. Under normal growing conditions progeny derived from both temperature conditions exhibited no significant differences in rate of root elongation (Zamir and Gadish 1987). Pollen generated under cool conditions was found to produce seedlings with significantly faster root growth, greater seedling mass, and larger leaf area than pollen generated under warm conditions in *Cucurbita pepo* (cucumber). In addition pollen developed under cool conditions grew longer pollen tubes and sired more seeds when in competition with pollen developed under warm conditions.

With more evidence emerging in support of the existence of gametophytic selection, the integration of early selection into plant breeding programs is promising. Although gametophytic selection does provide a time and cost efficient selection process, it should be considered a supplement to sporophytic selection and be used in tandem with a variety of techniques used for crop improvement.

References

Charlesworth D, and B Charlesworth. 1992a. The effects on genetic load of selection in the gametophytic stage. *In:* E Ottaviano, Mulcahy, DL, Sari Gorla, M, and Mulcahy, GB (eds), Angiosperm Pollen and Ovules, Springer-Verlag, New York, pp 401-407

Charlesworth, D. & B. Charlesworth. 1992b. The effects of selection in the gametophyte stage on mutational load. *Evolution* **46**, 703-720.

Chasan, R. 1992. Racing pollen tubes. *Plant Cell* **4**, 747-749.

Chi HS, TP Straathof, HJM Löffler, and JM Van Tuyl. 1999. *In vitro* selection for heat-tolerance in lilies. *In:* C Clement, Pacini, E, and J.C., A (eds), Anther and pollen, from biology to biotechnology, Springer, Berlin, pp 175-182

Chikkodi, S.B. & R.L. Ravikumar. 2000. Influence of pollen selection for *Alternaria helianthi* resistance on the progeny performance against leaf blight in sunflower (*Helianthus annuus* L.). *Sexual Plant Reproduction* **12**, 222-226.

Clarke, H.J., T.N. Khan & K.H.M. Siddique. 2004. Pollen selection for chilling tolerance at hybridisation leads to improved chickpea cultivars. *Euphytica* **139**, 65-74.

Clarke, H.J. & K.H.M. Siddique. 2004. Response of chickpea genotypes to low temperature stress during reproductive development. *Field Crops Research* **90**, 323-334.

Crispi ML, and LC Peirce. 1992. Gametophytic selection for early maturity in tomato (*Lycopersicon esculentum* Mill.). *In:* E Ottaviano, Mulcahy, DL, Sari Gorla, M, and Mulcahy, GB (eds), Angiosperm Pollen and Ovules, Springer-Verlag, New York, pp 370-376

Crone, D., J. Rueda, K.L. Marint, D.A. Hamilton & J.P. Mascarenhas. 2001. The differential expression of a heat shock promoter in floral and reproductive tissues. *Plant, Cell and Environment* **24**, 869-874.

Dahl, A.E. & M. Fredrikson. 1996. The timeTablele for development of maternal tissues sets the stage for male genomic selection in *Betula pendula* (*Betulaceae*). *American Journal of Botany* **83**, 895-902.

Delph, L.F., M.G. Johannsson & A.G. Stephenson. 1997. How environmental factors affect pollen performance: Ecological and evolutionary perspectives. *Ecology* **78**, 1632-1639.

Fehr, W.R. 1991. *Principles of cultivar development*. Iowa State University: Macmillian Publishing Company, New York. pp. 593

Frascaroli, E. & D.D. Songstad. 2001. Pollen genotype selection for a simply inherited qualitative factor determining resistance to chlorsulfuron in maize. *Theoretical and Applied Genetics* **102**, 342-346.

Frova, C., P. Portaluppi, M. Villa & M.S. Gorla. 1995. Sporophytic and gametophytic components of thermotolerance affected by pollen selection. *Journal of Heredity* **86**, 50-54.

Frova C, and M Sari Gorla. 1992. Pollen competition: genetics and implications for plant breeding. *In:* Y Dattée, Dumas, C, and Gallais, A (eds), Advances in Genetics, Academic Press, New York, pp 335-344

Frova C, G Taramino, MS Gorla, and E Ottaviano. 1992. Developmental synthesis of HSPs in the male gametophytic phase of heat tolerant and heat sensitive species. *In:* E Ottaviano, Mulcahy, DL, Gorla, MS, and Bergamini-Mulcahy, G (eds), Angiosperm Pollen and Ovules, Springer-Verlag, New York, pp 191-195

Frova, C., G. Taramino & E. Ottaviano. 1991. Sporophytic and gametophytic heat-shock protein-synthesis in *Sorghum-bicolor*. *Plant Science* **73**, 35-44.

Honys, D. & D. Twell. 2003. Comparative analysis of the *Arabidopsis* pollen transcriptome. *Plant Physiology* **132**, 640-652.

Honys, D. & D. Twell. 2004. Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*. *Genome Biology* **5**.

Hormaza, J.I. & M. Herrero. 1996. Male gametophytic selection as a plant breeding tool. *Scientia Horticulturae* **65**, 321-333.

Johannsson, M.H. & A.G. Stephenson. 1998a. Effects of temperature during microsporogenesis on pollen performance in *Cucurbita pepo* L. (*cucurbitaceae*). *International Journal of Plant Sciences* **159**, 616-626.

Johannsson, M.H. & A.G. Stephenson. 1998b. Variation in sporophytic and gametophytic vigor in wild and cultivated varieties of *Cucurbita pepo* and their F1 and F2 generations. *Sexual Plant Reproduction* **11**, 265-271.

Johnsen, O., T. Skroppa, G. Haug, I. Apeland & G. Ostreng. 1995. Sexual reproduction in a greenhouse and reduced autumn frost hardiness of *Picea abies* progenies. *Tree Physiology* **15**, 551-555.

Jourdren, C., D. Simonneaux & M. Renard. 1996. Selection on pollen for linolenic acid content in rapeseed, *Brassica napus* L. *Plant Breeding* **115**, 11-15.

Katiyar-Agarwal, S., M. Agarwal, D.R. Gallie & A. Grover. 2001. Search for the cellular functions of plant Hsp100/Clp family proteins. *Critical Reviews in Plant Sciences* **20**, 277-295.

Kovács G, and B Barnabás. 1992. Production of highly cold tolerant maize inbred lines by repeated gametophytic selection. *In:* E Ottaviano, Mulcahy, DL, Sari Gorla, M, and Mulcahy, GB (eds), Angiosperm Pollen and Ovules, Springer-Verlag, New York, pp 349-354

Lassere, T.B., S.B. Carroll & D.L. Mulcahy. 1996. Effect of pollen competition on offspring quality at varying stages of the life cycle in *Silene latifolia* 'Poiret' (*Caryophyllaceae*). *Bulletin of the Torrey Botanical Club* **123**, 175-179.

Mandhu B, M Cresti, and KR Shivanna. 1992. Effects of high temperature and humidity stresses on tobacco pollen and their progeny. *In:* E Ottaviano, Mulcahy, DL, Sari Gorla, M, and Mulcahy, GB (eds), Angiosperm Pollen and Ovules, Springer-Verlag, New York, pp 349-354

Mascarenhas, J.P. 1990. Gene activity during pollen development. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 317-338.

Mascarenhas, J.P. & D.F. Crone. 1996. Pollen and the heat shock response. *Sexual Plant Reproduction* **9**, 370-374.

McCormick, S. 2004. Control of male gametophyte development. *Plant Cell* **16**, S142-S153.

Mulcahy, D.L. 1971. A correlation between gametophytic and sporophytic characteristics in *Zea mays* L. *Science* **171**, 1155-1156.

Mulcahy, D.L. 1979. Rise of the angiosperms - A genecological factor. *Science* **206**, 20-23.

Mulcahy, D.L., M. SariGorla & G.B. Mulcahy. 1996. Pollen selection - past, present and future. *Sexual Plant Reproduction* **9**, 353-356.

Ottaviano, E., M.S. Gorla & E. Pe. 1982. Male gametophytic selection in maize. *Theoretical and Applied Genetics* **63**, 249-254.

Ottaviano E, and DL Mulcahy. 1986. Gametophytic selection as a factor of crop plant evolution. *In:* C Barigozzi (ed), The Origin and Domestication of Cultivated Plants, Elsevier, New York, pp 101-120

Ottaviano, E. & D.L. Mulcahy. 1989. Genetics of angiosperm pollen. *Advances in Genetics* **26**, 1-64.

Ottaviano E, ME Pè, and G Binelli. 1991. Genetic manipulation of male gametophytic generation in higher plants. *In:* BB Biswas and Harris, JR (eds), Subcellular Biochemistry: Plant Genetic Engineering, Plenum Press, New York, pp 107-142

Ottaviano E, and M Sari Gorla. 1993. Gametophytic and sporophytic selection. *In:* MD Hayward, Bosemark, NO, and Romagosa, I (eds), Plant breeding: Principles and prospects, Chapman & Hall, New York, pp 332-352

Ottaviano, E., M. Sarigorla & M. Villa. 1988. Pollen competitive ability in maize - within population variability and response to selection. *Theoretical and Applied Genetics* **76**, 601-608.

Pasonen, H.L., M. Kapyla & P. Pulkkinen. 2000. Effects of temperature and pollination site on pollen performance in *Betula pendula* 'Roth' - evidence for genotype-environment interactions. *Theoretical and Applied Genetics* **100**, 1108-1112.

Pasonen, H.L., P. Pulkkinen & M. Kapyla. 2001. Do pollen donors with fastest-growing pollen tubes sire the best offspring in an anemophilous tree, *Betula pendula* (*Betulaceae*)? *American Journal of Botany* **88**, 854-860.

Pedersen, S., V. Simonsen & V. Loeschcke. 1987. Overlap of gametophytic and sporophytic gene expression in Barley. *Theoretical and Applied Genetics* **75**, 200-206.

Petolino, J.F., N.M. Cowen, S.A. Thompson & J.C. Mitchell. 1990. Gamete selection for heat-stress tolerance in maize. *Journal of Plant Physiology* **136**, 219-224.

Ravikumar, R.L. & S.B. Chikkodi. 1998. Association between sporophytic reaction to *Alternaria helianthi* and gametophytic tolerance to pathogen culture filtrate in sunflower (*Helianthus annuus* L.). *Euphytica* **103**, 173-180.

Ravikumar, R.L. & B.S. Patil. 2004. Effect of gamete selection on segregation of wilt susceptibility-linked DNA marker in chickpea. *Current Science* **86**, 642-643.

Ravikumar, R.L., B.S. Patil & P.M. Salimath. 2003. Drought tolerance in sorghum by pollen selection using osmotic stress. *Euphytica* **133**, 371-376.

Sari-Gorla, M., S. Ferrario, E. Frascaroli, C. Frova, P. Landi & M. Villa. 1994. Soprophytic response to pollen selection for alachlor tolerance in maize. *Theoretical and Applied Genetics* **88**, 812-817.

Sari-Gorla, M., C. Frova, G. Binelli & E. Ottaviano. 1986. The extent of gametophytic-sporophytic gene-expression in maize. *Theoretical and Applied Genetics* **72**, 42-47.

Sari-Gorla, M., M.E. Pe, D.L. Mulcahy & E. Ottaviano. 1992. Genetic dissection of pollen competitive ability in maize. *Heredity* **69**, 423-430.

Sari-Gorla, M. & E. Rovida. 1980. Competitive ability of maize pollen. Intergametophytic effects. *Theoretical and Applied Genetics* **57**, 37-41.

Snow, A.A. & T.P. Spira. 1991. Pollen vigor and the potential for sexual selection in plants. *Nature* **352**, 796-797.

Snow, A.A. & T.P. Spira. 1996. Pollen-tube competition and male fitness in *Hibiscus moscheutos*. *Evolution* **50**, 1866-1870.

Snow, A.A., T.P. Spira & H. Liu. 2000. Effects of sequential pollination on the success of "fast" and "slow" pollen donors in *Hibiscus moscheutos* (*Malvaceae*). *American Journal of Botany* **87**, 1656-1659.

Sultan, S.E. 2005. An emerging focus on plant ecological development. *New Phytologist* **166**, 1-5.

Tanksley, S.D., D. Zamir & C.M. Rick. 1981. Evidence for extensive overlap of sporophytic and gametophytic gene-expression in *Lycopersicon esculentum*. *Science* **213**, 453-455.

Touraev, A., C.S. Fink, E. Stoger & E. HeberleBors. 1995. Pollen selection: A transgenic reconstruction approach. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 12165-12169.

Williams, J.H., W.E. Friedman & M.L. Arnold. 1999. Developmental selection within the angiosperm style: Using gamete DNA to visualize interspecific pollen competition. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 9201-9206.

Willing, R.P. & J.P. Mascarenhas. 1984. Analysis of the complexity and diversity of messenger-RNAs from pollen and shoots of *Tradescantia*. *Plant Physiology* **75**, 865-868.

Zamir, D. & I. Gadish. 1987. Pollen selection for low-temperature adaptation in tomato. *Theoretical and Applied Genetics* **74**, 545-548.

Chapter 2: Gametophytic Selection in *Phalaenopsis*

Introduction

Cultivar development and plant breeding programs focus on the improvement of a vast array of characteristics such as resistance to pathogens, pests and abiotic stresses as well as increased yields or enhanced aesthetic appeal. Due to the requirement of large population sizes in order to obtain the essential combination of characteristics for crop improvement, plant breeding can be a time, space and money consuming program (Hormaza and Herrero 1996). The unique alternation of generations that occurs in seed plants (angiosperms and gymnosperms) offers plant breeders a rare opportunity to streamline cultivar development. The frequency of fertilization by gametes carrying genes that confer better adaptability can be increased by applying selective pressures to the gametophytic phase (Ottaviano et al. 1991).

Traditional plant breeding programs have mainly focused on sporophytic selection. In angiosperms, sporophytes are the most prominent generation whereas the gametophyte is reduced in both size and lifespan (Hormaza and Herrero 1996). Early selection can be introduced into breeding programs via selective pressures applied to the gametophytes. Both male and female gametes are produced by the haploid gametophyte in the alternation of generations life cycle of angiosperms', however, male gametophytic selection is typically easier to manipulate. The largest screening potential exists within male populations of gametes due to several traits of pollen. Competition between the many male gametophytes during development in the anther, pollen germination and tube growth as well as the transition of the gametophyte auxotrophic development to an independent state following the release of the pollen from the anther creates the opportunity for more efficient and effective selection (Ottaviano and Sari Gorla 1993; Ottaviano *et al.* 1988; Sari-Gorla *et al.* 1992). Plant styles can screen large numbers of pollen grains in a short amount of time allowing MGS (male gametophytic selection) to occur with relatively little reduction in sporophytic fitness (Frova and Sari Gorla 1992; Mascarenhas 1990). This may allow angiosperms the unique opportunity to benefit from the possible adaptive value of viable recombinants while avoiding the negative effects of poorly functioning recombinants (Mulcahy 1979). Due to the large population size of pollen in typical pollinations and the haploid state of the male gametophyte, selection during this phase could be more efficient than selection during the sporophytic phase (Sari-Gorla et al. 1992).

Selection for pollen competitive ability and selection for tolerance to environmental stress are two aspects of MGS. The concept of selecting for pollen competitive ability relies on the assumption that the most vigorous pollen grains will confer vigor to the sporophyte (Ottaviano and Mulcahy 1989; Ottaviano *et al.* 1991). Tolerance of various environmental stresses by the sporophyte can be promoted by applying those same stresses to pollen during various stages of development, germination and tube growth in order to alter allele frequency to select for the desired resistance (Ottaviano et al. 1991). Selecting pollen that confers vigor or environmental tolerance to the resulting progeny is feasible when considering the transcriptome overlap between the sporophyte and gametophyte (Mascarenhas 1990; Ottaviano and Mulcahy 1989). Comparison of the sporophyte and gametophyte transcriptomes of *Arabidopsis thaliana* using microarray analysis revealed an extensive overlap of 61%; because their gene chip was estimated to cover 28% of the *Arabidopsis* transcriptome, some 1400 genes or 1 in 20 would be expected to be specifically or preferentially expressed in pollen (Honys and Twell 2003; Honys and Twell 2004). Many earlier studies demonstrated similar overlaps in various crops implying that transcriptome overlap is a common occurrence in angiosperms (Sari-Gorla *et al.* 1986; Tanksley *et al.* 1981; Willing and Mascarenhas 1984). Using transgenic tobacco pollen (Touraev et al. 1995) determined that selection applied to male gametes has a direct effect on gene frequency in the resulting sprorophyte. Strong evidence exists supporting the occurrence of male gametophytic selection.

Determining what characteristics the alteration of allele frequency confers to the sporophyte is the complicated aspect of studying MGS. Complex traits such as thermo-tolerance and disease resistance are generally controlled by multiple interactions acting on all levels of plant organization and are not often constitutively expressed (Ottaviano and Sari Gorla 1993). Selective pressure for non-constitutively expressed traits can be most effectively applied at two times: during pollen development or during pollen germination and tube growth. After release from the anther, but before germination, pollen is in a quiescent stage where some responses would not be inducible (Hormaza and Herrero 1996).

Gametophytic selection has been successfully used in producing more extreme temperature tolerant progeny in several different plants, especially maize. Increased tolerance to heat stress in the sporophytes has been observed in multiple studies where extreme heat treatments were applied during either pollen development or germination and subsequent tube growth (Frova et al. 1995; Frova et al. 1992; Mandhu et al. 1992; Petolino et al. 1990). Heat

tolerant seedlings were selected in *Lilium longiflorum* using heat treatments on pollen tube growth (Chi et al. 1999). In tobacco, the application of high temperatures to pollen effectively selected progeny that were heat resistant (Mandhu et al. 1992). Cold tolerance developed through gametophytic selection has been studied in maize as well. Researchers were able to obtain S6 lines with stronger cold tolerance than the original parents (Kovács and Barnabás 1992). Chickpea (*Cicer arietinum*) breeding lines developed through MGS by selecting pollen with cold temperature tolerance were found to have a "significant" advantage in cooler environments (Clarke et al. 2004). Low temperature adaptation via pollen selection was studied in tomato as well. A correlation between pollen selection at low temperatures and the response of the sporophyte under the same conditions was observed (Zamir and Gadish 1987).

In addition to the production of progeny with better thermo-tolerance, pollen produced under various temperature regimes or progeny derived from pollen resistant to extreme temperatures in some instances have both shown increased vigor. In *Curcurbita pepo*, pollen developed under cold conditions consistently sired more seeds than pollen developed under warm conditions. Seeds sired by cold-developed pollen also had significantly faster root growth rate (Johannsson and Stephenson 1998a). Tomato progeny derived from pollen treated with cold temperatures during germination and tube growth also exhibited higher rates of root elongation (Zamir and Gadish 1987). The superior performance of maize progeny produced through MGS for heat stress resistance was indicated by higher grain yields, and greater subsequent seedling vigor (Petolino et al. 1990). (Mandhu et al. 1992) observed increased vigor of seedlings that were products of MGS for heat stress resistance in tobacco under normal conditions as well.

MGS may be most useful for increasing thermo-tolerance in particularly temperature sensitive crops such as plants native to tropical and subtropical climates. Perhaps the greatest benefit of early selection through MGS is the greater efficiency brought to breeding programs seeking to introduce advantageous characteristics to plants that grow slowly and have extremely long developmental phases, i.e. potted flowering orchids.

Orchids are increasingly popular exotic flowering house plants. Second only to poinsettias in popularity in the United States, orchids are becoming more available in the flowering plant market (Griesbach 2002). According the USDA floriculture crops 2004 survey, the potted orchid industry in the United States is valued at $128 million and increased 5% from 2003 to 2004. The market for orchids has steadily increased for a number of years despite fluctuations in the floriculture industry overall (Service 2005). *Phalaenopsis,* or moth orchids,

are by far the most popular and constitute 50-99% of potted orchids marketed as cut flowers or potted plants in the world (Griesbach 2002; Laws 2005). Because this genus of orchid is native to tropical and subtropical climates, greenhouses are required for commercial production of *Phalaenopsis* in temperate climates such as Virginia. Costs of heating in the winter or cooling in the summer in greenhouses are major inhibitory factors to orchid production. If the environmental tolerance of *Phalaenopsis* can be increased, then greenhouse costs involved in temperature control during orchid production can be reduced.

The main allure of the moth orchid is the spectacular flowers produced on long arching spikes. During flowering, *Phalaenopsis* are the most marketable and at the same time the most sensitive to temperature extremes. Bud drop due to damage during airfreight and trucking can be as much as 15% (Laws 2005). As the availability of orchids broadens to non-traditional venues such as large nationwide retail, home improvement and grocery chains, the ability of a plant to withstand less than ideal conditions and still remain in bloom and marketable becomes increasingly important. According to the American Orchid Society (http://orchidweb.org/aos/index.aspx), *Phalaenopsis* are classified as warm growing orchids. Too cool temperatures can cause buds to drop (bud blast) or already open flowers to wilt. Cold temperatures also adversely affect the plants' general state of health, with obvious results such as yellowing and wilting of leaves. In addition rapid changes in temperature as well as too cold temperatures can cause bud and flower loss as well. Extreme cold temperatures and temperatures that cause heat stress can both adversely affect blooming and overall plant health. Extreme high temperatures during bud formation can cause flowers to be malformed. More importantly, plants will completely fail to bloom or even produce floral stalks under high temperature stress (Chen et al. 1997; Chou et al. 2000; Su et al. 2001). Due to the long life cycle of *Phalaenopsis* (3-5 years from seed germination to first flowering), gains from gamete selection could result in improved stress tolerance more quickly than those from sporophytic selection alone. Presumably deliberate selection of plants under stressful environments will result in progeny that are better able to adapt to such stress, e.g., high or low temperatures, outside the range that has been optimal for orchid production (Hormaza and Herrero 1996).

The main objective of this study was to determine if exposure of the male gametophyte of *Phalaenopsis* to different temperature regimes during pollination and fertilization results in the selection of male gametes with cool temperature or heat stress tolerance. The effect of gametophytic selection for cool temperature and heat stress tolerance on seed set, seed

germination, the rate of seedling root and shoot elongation, as well as overall seedling vigor has been evaluated.

Materials and Methods

Plant material

Mature hybrid *Phalaenopsis* plants were used for this experiment. *P.* (Taisoco Windian × Sogo Yukidian), *Phalaenopsis* hybrid unknown (Bedford Orchids, Montreal, Canada) *P.* Neon Stripe 'Red Avenger', *P.* Tropical Stripes 'Carmela', *P.* Brother Heather 'Nuclear' (Carmela Orchids, Hakalau, HI), *P.* Baldan's Kaleidoscope 'Golden Treasure', *P.* Sogo 'Little Angel', *P.* Pinlong Moristopher 'Dream', *P.* (Meller × Taisuco Kochdian), *P.* Taipei Gold 'Golden Star' (Floradise Orchids, Barboursville VA) and two diploid species *P. lindenii* and *P. equestris* 'Rosea' were used. All plants were maintained in a greenhouse under optimal conditions as specified for *Phalaenopsis* by the American Orchid Society (15-20^{o}C, 70% humidity) until inflorescences matured.

Pollinations and temperature treatments

Reciprocal crosses were carried out by hand as inflorescences matured and flowers opened. In most orchids, pollen is aggregated into masses known as pollinia. *Phalaenopsis* have two pollinia. The pollinia were removed from the pollen donor. The anther cap was gently detached and the caudicle was left intact with the two pollinia still attached. The pollina were immediately placed on the depression on the underside of the rostellum (modified stigma) of the recipient flower, which was then emasculated in order to emulate natural pollination as described best by (Arditti 1992). All crosses conducted are listed in Table 1.

After pollination, plants were placed in growth chambers set at warm or cold conditions. Two separate temperature treatments were chosen for thermotolerance selection. Three studies on high temperature effects on flower morphology, flower development and cytokinin levels in leaves of *Phalaenopsis* were used as reference papers for high temperature values (Chen et al. 1997; Chou et al. 2000; Su et al. 2001). Optimal conditions for *Phalaenopsis* were also considered. The most favorable conditions for *Phalaenopsis* vary from 21^{o}C during the day to 18^{o}C at night while 15^{o}C nights should induce floral stalk production. In this study warm temperature treatment consisted of exposure to 30ºC for daylight periods and 25^{o}C for night periods. The cool temperature treatment consisted of 14^{o}C for daylight periods and 9^{o}C for night.

After multiple failed cold pollinations the cold temperature treatments were increased to 15°C for daylight periods and 10°C night. Each plant was hand pollinated, labeled and then immediately placed into one of two growth chambers (25/30°C or 10/15°C) and exposed to photoperiods that were adjusted to those the plant was experiencing in the greenhouse and a relative humidity of 70%.

Plants undergoing cold pollination remained in the growth chamber for 7 days whereas warm pollinated plants underwent treatment for 3 days. The treatment lengths were determined by the observation of post pollination phenomena specific to *Phalaenopsis*. Once hyponasty, the inward folding of the petals, and stigmatic swelling were observed the pollination treatments were terminated. *Phalaenopsis* (Taisoco Windian × Sogo Yukidian) by *P*. hybrid unknown (tag was lost during shipping) and *P*. hybrid unknown by *P*. (Taisoco Windian × Sogo Yukidian) were the first crosses made. Each plant had one flower pollinated and was placed in a growth chamber to undergo the cold treatment. After 1 week plants were removed and another flower pollinated (same cross). The same two plants were then placed in a growth chamber to undergo the warm treatment. The same process was used in crosses between *P*. Brother Heather 'Nuclear', *P*. Tropical Stripes 'Carmela' and *P*. Neon Stripe 'Red Avenger'(Fig. 1). This was due to the availability of only a single plant of each hybrid. For *P*. Baldan's Kaleidoscope 'Golden Treasure', *P*. Sogo 'Little Angel', *P*. Pinlong Moristopher 'Dream', *P*. (Meller × Taisuco Kochdian), *P*. Taipei Gold 'Golden Star' we obtained two clonally propagated and presumed genetically identical plants of each hybrid. Each pollination was done on two flowers of each inflorescence and one clone was placed in the warm temperature treatment and the other clone placed in the cold temperature treatment (Fig. 2).

Pollination treatment synopsis

Seedlings produced from the cross between *P*. (Taisoco Windian × Sogo Yukidian) by *P*. hybrid unknown were used for this study. Plants were exposed to the cold pollination treatment directly after pollination of one flower. After 7 days, plants were removed from the cold incubator, a different flower on the same inflorescence was pollinated and then the plant was exposed to the warm pollination treatment for 3 days (Fig. 1).

Seed germination

Once temperature treatments were complete, plants were returned to the greenhouse under ambient conditions. Fruit was harvested after 150-190 days. Seeds were extracted from the first fruit (undehisced green capsule) and surfaced-sterilized first in a 70% ethanol solution then in a 30% sodium hypochlorite solution. Seeds plated from this method were contaminated so a different technique was employed on the second capsule. The following protocol is a revised version of the recommended dry pod sterilization technique described in Asymbiotic Technique of Orchid Seed Germination in order to accommodate specific needs of this experiment (Hicks 1999). Due to the extremely small size of orchid seed, seed number was not counted instead equal volumes of seeds were placed in microcentrifuge tubes. A saturated solution of calcium hypochlorite (17 g/L) containing Tween 20® was added in 1 ml volume to each tube. Each tube was vortexed in consecutive order until 12 min had elapsed. Individual tubes were then decanted onto a plate (100 × 20 mm) containing 35 ml Phytamax® media (Sigma Aldrich, St. Louis, MO) with 5% (total volume) coconut water. Plates were sealed with Parafilm® and placed on a temperature gradient table with temperatures ranging from 10ºC to 30ºC, for seed germination. Multiple plates (4-5 per table position) were arranged from the 30ºC temperature at position 1 to the 10ºC temperature at position 12. Each of the 12 positions differed by approximately 2ºC increments.

Protocorm evaluation and transfer

As in most orchids, *Phalaenopsis* seeds do not contain endosperm and do not produce an obvious cotyledon. Instead they germinate into a globular green mass called a protocorm. Protocorms were counted 72 days after initial plating and plates were rated according to number and size of protocorms. Protocorms were then divided and transferred to fresh germination media. One of each of the new plates was placed in a warm incubator set at 30^{o}C or a moderately cooler incubator set at 25^{o}C (Fig. 4). Temperatures much cooler would have prevented any growth of the seedlings (previous observations). Once the leaves and first roots developed, plates were evaluated on number of protocorms, leaf number, root number, and spontaneous clump formation. The second evaluation was conducted 125 days after initial plating.

Transfer to growth chambers and final seedling evaluation

Seedlings were allowed to grow on Phytamax® media without coconut water (35 ml per 100 × 20 mm) in incubators until plants were too large to remain on plates in culture. Seedlings were

transferred to dried sphagnum moss medium in Sigma® cell culture containers (P-4928) as compots with up to ten seedlings per pot and placed in growth chambers. All seedlings from the warm incubator were placed in the corresponding warm growth chamber set at 30°C during the day and 25°C at night. Seedlings from the cooler incubator were placed in the cold growth chamber set at 15°C day and 10°C night (Fig. 4). These temperatures were the original selected pollination temperatures. However, the cold growth chamber temperature range was increased to 18°C and 12°C because the original range was too cold to allow for any growth of the seedlings. One year after initial plating, seedlings were evaluated on the following criteria: fresh weight, number of leaves, leaf width, leaf length, leaf area, number of roots, and root length. Leaf area was estimated using a non-destructive method (Chen and Lin 2004).

Data analysis

All data were analyzed using SAS GLM. Mean comparisons were done using Ryan-Einot-Gabriel-Welsch Multiple Range Test. Pollination treatment, germination temperature and incubator/growth chamber effects were tested for significance at the $P<0.05$ level.

Results

Pollinations

In the course of this experiment 198 pollinations were completed (Table 1). Most of the pollinations were unsuccessful and did not yield capsules. A total of seven fruit was obtained, two from cold pollinations, and five from warm pollinations. One cross and its reciprocal yielded two cold pollinated fruit and two warm pollinated fruit. Although capsules matured, no seeds were produced. The cross between *P.* (Taisoco Windian × Sogo Yukidian) × *Phalaenopsis* hybrid unknown and the reciprocal cross *Phalaenopsis* hybrid unknown × *P.* (Taisoco Windian × Sogo Yukidian), yielded four capsules total. Each cross produced one warm pollinated capsule and one cold pollinated capsule (Fig. 1). Fruit differed in size according to pollination treatment. The cross between *P.* (Taisoco Windian × Sogo Yukidian) × *Phalaenopsis* hybrid unknown yielded a warm pollinated capsule 6.3 cm in length. The cold pollinated capsule derived from this cross was 5.2 cm in length. *Phalaenopsis* hybrid unknown × *P.* (Taisoco Windian × Sogo Yukidian) produced capsules from the warm and cold pollinations, 8.5 cm and 6.9 cm in length, respectively. During the germination process, the seeds collected from the cross between

Phalaenopsis hybrid unknown × *P.* (Taisoco Windian × Sogo Yukidian) were 90% contaminated. Losses due to fungal and bacterial contamination made data comparison impossible. Therefore, all of the data collected and analyzed focuses on the two capsules and seeds derived from the cross *P.* (Taisoco Windian × Sogo Yukidian) × *Phalaenopsis* hybrid unknown.

Germination and protocorms evaluation

Protocorm development was evaluated 72 days after initial plating. The coldest temperature at which seeds germinated was 14^{o}C. Below this temperature seeds did not germinate, therefore plates containing seeds located at lower germination temperatures were not considered. Each plate was evaluated on the number of protocorms that had developed. Because equal volumes of seeds were used for each plate, a comparison of the germination of seeds could be made. The results of the two-way ANOVA showed that the effect of the pollination treatment was significant ($P = 0.028$), whereas the effects of germination temperature and the interaction between pollination treatment and table position were highly significant ($P<0.0001$, $P<0.0001$) (Table 2). Cold pollinated seeds germinated better than warm pollinated seeds at almost all germination temperatures (Fig. 5). Plates at 28^{o}C and 22^{o}C germinated the best for cold pollinated seeds. Warm-pollinated seeds germinated best at 20^{o}C. More seeds derived from cold pollination temperature germinated overall than warm-pollination-derived seeds.

Seedling evaluation 125 days after initial plating

The second seedling evaluation was conducted 125 days after initial plating. At this point there was a varying degree of seedling development. Some protocorms had leaves and roots whereas others had yet to develop either organ. Protocorm leaf development, root development and mortality as well as the phenomenon of spontaneous proliferation of protocorms into large clumps of multiple plantlets were the characteristics evaluated and analyzed (Fig. 6). The number of protocorms per plate that had not developed roots or leaves, seedlings with only leaves, seedlings with leaves and roots, and those protocorms that formed clumps were divided by the total number of protocorms and seedlings still alive per plate in order to calculate the ratio of each.

The main effects of germination temperature (table position) and incubator were highly significant factors in protocorm mortality ($p = 0.0007$, $p= 0.0008$) (Table 3). The interactions

between pollination treatment and incubator, as well as germination temperature and incubator, were also significant ($p = 0.029$, $p = 0.012$). The highest percentage of protocorm death occurred on those located in the cold incubator (25°C) (Fig. 7). Warm pollinated protocorms germinated at 28°C and then transferred to the cold incubator (25°C) had almost 100% mortality. In the warm incubator, cold pollinated protocorms germinated at 26°C had the highest rate of mortality at a little over 80%. The warmer germination temperatures tended to have higher rates of mortality than cooler germination temperatures.

The percent of protocorms that had not formed leaves or roots was significantly affected by germination temperature, incubator, and the interaction between germination temperature and incubator ($p = 0.003$, $p < 0.0001$, $p = 0.005$) (Table 3). The effects of the cold incubator on leaf development are pronounced (Fig. 8). Over 50% of the seedlings in many treatments had yet to form leaves in comparison to those seedlings in the warm incubator (30°C). Approximately 80% of the protocorms derived from warm pollinations and germinated at the lowest temperatures (16-20°C) had not yet formed leaves or roots. A little over 40% of cold pollinated derived protocorms, germinated at 30°C and placed in the warm incubator also lacked leaves and roots.

The percentage of protocorms that had developed leaves, but not roots, was significantly affected by germination temperature ($p = 0.036$), although the effect of the interaction between germination temperature and incubator was approaching significance ($p = 0.088$) (Table 3). Both warm and cold-pollination-derived seedlings germinated at 24°C and transferred to the cold incubator had the highest percentage of seedlings that had formed leaves but not roots (Fig. 9). More cold pollinated seedlings developed leaves while growing in the cold incubator. More warm-pollination-derived seedlings developed leaves and roots than cold-pollination-derived seedlings in the warm incubator. Warm germination temperatures also seemed to produce a higher percentage of seedlings with leaves but not roots in the warm incubator.

The percentage of protocorms that had both leaves and roots was significantly affected by the incubator alone ($p = 0.008$) (Table 3). Although the effect of the incubator was the only statistically significant effect, those seedlings derived from cold pollinations and germinated in warmer temperatures tended to have the highest percentage of seedlings that had produced both leaves and roots (Fig. 10). Overall the warm incubator (30°C) had the highest percentages of seedlings that had developed both roots and leaves.

The effects of germination temperature and incubator temperature on the percentage of protocorms that spontaneously formed clumps of multiple seedlings were highly significant

(Table 3) ($p = 0.0037$, $p = 0.0002$). Higher percentages of protocorms forming "clumps" were produced in the warm incubator (30°C) (Fig. 11). In addition, germination temperatures of both 28 and 26°C produced closed to 100% clump formation for warm-pollination and cold-pollination-derived seedlings. In the cold incubator the greatest percentages of seedlings forming "clumps" was highest for both warm and cold-pollination-derived seedlings at 26°C germination temperature.

The comparison of means between the warm and cold incubator showed significant differences between all characteristics observed at 125 days after initial plating (Table 4). The percentage of protocorms that died in the cold incubator was twice that of the warm incubator. In addition, the percentage of protocorms in the cold incubator that had not yet formed roots and leaves was almost 9 times that of the percentage of protocorms at the same developmental stage located in the warm incubator. The percentage of seedlings that had developed leaves and roots in the warm incubator was 8 times the percentage of seedlings that had also produced leaves and roots in the cold incubator. The warm incubator produced 3 times the percentage of spontaneous "clumps" than the cold incubator.

The mean comparison between germination temperatures indicates that the warmest temperatures during germination had the greatest effect on mortality with close to 50% of protocorms produced at temperatures between 30-26°C dying after germination (Table 5). The coldest temperatures during germination tended to produce the highest rates of protocorms that had not developed leaves or roots after 125 days. Germination temperature did not significantly affect the percentage of seedlings with leaves or those that had produced both leaves and roots. The intermediate germination temperatures produced the highest percentages of clumps although by far the greatest clump production was observed at 26°C with 67% of protocorms forming clumps. The highest and lowest germination temperatures produced the smallest percentages of clumps. Mean comparison between pollination treatments for clump formation yielded no significant differences.

Seedling evaluation 1 year after initial plating

The final evaluation of seedlings was completed one year after initial plating. Seedling fresh weight, leaf number, leaf length, leaf width, root number and root length were all evaluated. Leaf area was calculated using the equation [(maximum leaf length × maximum leaf width)0.72] derived from a nondestructive estimation of leaf area for Grandiflora cultivars of *Phalaenopsis*

(Chen and Lin 2004). Each individual leaf area was calculated first and then the mean leaf area calculated from those data. The main effect of the warm and cold incubator and growth chamber was significant for all seedling characteristics evaluated; weight, leaf number, leaf length, leaf width, leaf area, root number and root length. As expected, *Phalaenopsis* favored warmer growing conditions.

Comparison of the mean weight of seedlings showed that the main effect of germination temperature (table position) and the interaction of pollination treatment × germination temperature were both highly significant ($p = 0.001$, $p = 0.0001$). Seedlings derived from cold pollinations were typically heavier than warm-pollination-derived seedlings, except for those seedlings germinated at the coldest table positions (Table 6). The warm pollination treatment along with the two coldest seed germination temperatures, 18°C and 16°C, produced the largest mean weight of seedlings grown in the cold incubator/growth chamber. The mean weight of warm-pollination-derived seedlings in the warm incubator was typically higher than cold-pollination-derived seedlings (Fig. 12).

The main effect of the incubator/ growth chamber and the interaction between pollination treatment and incubator/growth chamber were both highly significant ($p<0.0001$ and $p = 0.0004$ respectively) (Table 6). Plants grown in the warm incubator and transferred to the warm growth chamber had a mean of 2.5 and 3 leaves per plant, whereas plants located in the cold incubator had between 1 and 2.5 mean leaves (Fig. 13). In addition warm-pollination-derived seedlings located in the warm incubator, regardless of germination temperature, had a greater number of leaves per plant than cold-pollination-derived seedlings. The opposite is observed in the cold incubator. Seedlings derived from cold pollinations had a greater number of leaves per plant.

No statistically significant effects were found for leaf width. The main effects of incubator/growth chamber and germination temperature as well as the interaction between pollination treatment and incubator/growth chamber on leaf length were highly significant ($p<0.0001$, $p= 0.0085$, and $p<0.0001$, respectively) (Table 6). Warm-pollination-derived seedlings exhibited a longer mean leaf length in the warm growth chamber than cold-pollination-derived seedlings. Conversely, cold-pollination-derived seedlings exhibited a longer mean leaf length than warm-pollination-derived seedlings when grown in the cold incubator. However, warm-pollination-derived seedlings germinated under the coolest temperatures grew longer leaves than when grown in the cold growth chamber (Fig. 14). The main effect of the incubator/growth chamber on leaf area was highly significant ($p = 0.0016$) (Table 6). Those

seedlings derived from cold pollination, located in the warm growth chamber, and germinated at 24^{o}C had by the far the largest leaf area of any of the treatment combinations (Fig. 15).

Root number was significantly affected by the incubator/growth chamber, pollination treatment, and the interaction between the pollination treatment and the incubator/growth chamber ($p < 0.0001$, $p = 0.02$, $p = 0.04$, respectively) (Table 6). The interaction between germination temperature and pollination treatment was approaching significance ($p = 0.057$). Cold-pollination-derived seedlings grown in the cold incubator/growth chamber had a larger mean number of roots in comparison to warm-pollination-derived seedlings except for those warm-pollination-derived seedlings that were germinated under the coldest temperatures, 18 and 16^{o}C (Fig. 16). The interaction between pollination treatment and incubator/growth chamber on mean root number was more pronounced under the cooler conditions. There does not appear to be a discernible trend towards the warm-pollination-derived seedlings having an advantage in the warm incubator so far as root number is concerned.

The mean root length of seedlings measured 1 year after initial plating was significantly affected by the incubator/growth chamber as well as the interactions between pollination treatment and incubator/growth chamber and pollination treatment and germination temperature ($p < .0001$, $p = 0.0016$, $p = 0.0053$, respectively) (Table 6). The main effect of pollination treatment was approaching significance ($p = 0.088$). The mean root length of most seedlings located in the warm incubator was approximately 2-3 cm whereas the mean root length of seedlings located in the cold incubator was less than 2 cm (Fig. 17). Warm-pollination-derived seedlings located in the warm incubator/growth chamber had a greater mean root length than cold-pollination-derived seedlings whereas cold-pollination-derived seedlings located in the cold incubator/growth chamber generally had a greater mean root length. However, warm-pollination-derived seedlings that were germinated under the coldest two germination temperatures (18 and 16^{o}C) had the highest mean root length of those seedlings located in the cold incubator/growth chamber. Warm-pollination-derived seedlings germinated at the second warmest temperature (28^{o}C) had the highest mean root length in the warm incubator/growth chamber.

Mean comparison between warm and cold incubator/growth chambers, as expected, showed significant differences between seedlings (Table 7). Seedlings grown in the warm incubator/growth chamber had larger means for all characteristics measured. Mean comparison between pollination treatments showed one significant difference between the mean number of roots produced (Table 8). Cold-pollination-derived seedlings produced 4.2 roots per seedling

whereas warm-pollination-derived seedlings produced only 3.6 roots per seedling. Mean comparison between germination temperatures yielded no significant differences in number of leaves, leaf width, leaf area, or number of roots (Table 9). Seedlings germinated at the lowest temperature, 16^{o}C, had a significantly higher mean weight and root length than any of the other seedlings germinated at different temperatures. In addition seedlings germinated at 16^{o}C also had the greatest mean leaf length.

Discussion

The main objective of this study was to determine if exposure of the male gametophyte to different temperature regimes during pollination and fertilization resulted in the selection of male gametophytes with cool temperature or heat stress tolerance. Our results demonstrate that exposure to selective temperatures during the initial stages of pollen germination in *Phalaenopsis* can influence seedling thermotolerance. Seedlings measured 1 year after initial plating revealed different reactions to their environments as evidenced in significant pollination treatment by incubation condition interactions. Seedlings that were derived from cold pollinations and then grown in the cool incubator and growth chamber, exhibited greater seedling weight, mean leaf number, mean leaf length, mean root number and mean root length than warm-pollination-derived seedlings grown under cool conditions. Conversely, seedlings derived from warm temperature pollinations, and then grown in the warm incubator and growth chamber, exhibited greater weight, leaf number, root number, leaf length and root length than cold-pollination-derived seedlings grown under the same warm conditions. This indicates that progeny derived from the two pollination treatments are indeed better adapted to more extreme temperatures that correlate with the pollination treatments.

Mean leaf width and leaf area did not exhibit important significant differences. This is most likely due to the growth habit and structure of orchid leaves. Leaves will remain the same width while elongating during later stages of seedling growth. Leaf area was calculated based on the mean leaf width and length measurements. Although the mean leaf length showed significant results, including influence of the pollination treatment and growth chamber interaction, the addition of the mean leaf width into the equation did not adequately reflect the difference in mean leaf size. One consideration that may have greatly affected the mean leaf area calculations is that the equation used for non-destructive leaf area measurements was developed using mature *Phalaenopsis* hybrids (Chen and Lin 2004). Developing seedlings may not have a similar enough

leaf to width ratio to be able to apply the same equation used on larger, mature plants. However, the differences in mean leaf length are sufficient to demonstrate that leaf development was significantly affected by the pollination treatment and growth chamber interaction.

Measurements taken on seedling characteristics 125 days after initial plating were not necessarily indicative of the capability of the progeny. Germination temperature and incubator temperatures were the significant factors in leaf development, root development and mortality. *Phalaenopsis* species and hybrids are categorized by the American Orchid Society as warm growing orchids, so it is not surprising that colder germination temperatures produced fewer seedlings. Growth was also slower in the cool incubator for both warm- and cold-pollination-derived seedlings. However, the two incubators that protocorms were transferred to after initial germination only differed by 5^{o}C. The cooler incubator was set at 25^{o}C while the warm incubator was set at 30^{o}C. This temperature difference was enough to significantly affect leaf and root production of protocorms. Initially the original pollination temperatures were selected and the incubators were to set at 30^{o}C and 15^{o}C. Due to the slow growing nature of orchid seedlings and the intermediate protocorm phase, the cooler incubator was reset to a warmer temperature to allow for some growth. If a greater temperature difference had been used, seedling development differences may have been more pronounced. It is likely that a more extreme incubator temperature used for cooler growing conditions would have inhibited development of the protocorms. Incubators set at a range of temperatures differing by 5^{o}C increments may be useful means in a future study to identify more precise temperatures that inhibit leaf and root development of protocorms. In this research, once seedlings developed sufficiently to be removed from nutrient media and placed on sphagnum moss at a developmental stage when they would ordinarily be transferred to a greenhouse, we returned them to cool growing conditions that reflected the more extreme temperature regime used in the pollination treatment (10/15^{o}C).

One unexpected characteristic observed in seedlings 125 days after initial plating was the spontaneous proliferation of plantlets from a single protocorm or clump formation. Anywhere from two to over 15 plantlets arose from a single seed. Polyembryony is a naturally occurring phenomenon in most orchids. *Phalaenopsis* has been observed to contain two embryos per seed (Arditti 1992; Singh and Thimmappaiah 1982). This would explain only a small portion of the "clumping" observed in the first seedling evaluation. In studies conducted on other species of orchids, especially terrestrial species, seedling development in asymbiotic culture has been described as abnormal. Development and shape of protocorms, leaves and roots may differ from

naturally germinated seeds (Vinogradova and Andronova 2002). Seeds in this experiment were germinated asymbiotically on a nutrient medium containing coconut water. Coconut water is liquid endosperm and contains plant hormones that may affect germination and protocorm growth. Observations of some orchid seeds proliferating into masses of undifferentiated cells giving rise to a "considerable" number of plants when germinated on a nutrient medium have been documented. This phenomenon has been attributed to cleavage embryony occurring during germination and is considered genotype dependent (Singh and Thimmappaiah 1982). In this experiment, "clumping" was significantly affected by germination temperature and incubator temperature. Optimal germination temperatures were 28 and 26^{o}C, whereas the warm incubator set at 30^{o}C produced the highest percentage of protocorms spontaneously forming clumps. Optimal temperature conditions during germination and initial protocorm development may provide a more conducive environment for undifferentiated cells. Clumping should produce genetically identical plantlets all sharing the same characteristics. This may be particularly useful in future research because different conditions can be applied to plants with the same genetic make-up to test responses to different environmental conditions. In addition, if a particularly beneficial characteristic is demonstrated by one of these plantlets, all of the sister plantlets produced from the spontaneous clump formation should exhibit the same characteristic.

Warm-pollination-derived seedlings germinated at the two coldest germination temperatures, 16 and 18^{o}C, germinated poorly and developed slowly. However, they exhibited a low rate of mortality. Measurements recorded from these two groups of seedlings after 1 year showed that they performed equally well or even better than cold-pollination-derived seedlings in the cold incubator. The general trend of cold-pollination-derived seedlings performing better in the cooler environment held true except for these warm-pollination-derived seedlings germinated under the coldest temperatures. These seedlings had the greatest mean weight of seedlings of any in the cold growth chamber. In addition mean root length and mean root number exceeded the means shown by the cold-pollination-derived seedlings. Mean leaf length was equal to the greatest mean leaf lengths of the cold-pollination-derived seedlings. These seedlings also exhibited the greatest means of seedling characteristics measured 1 year after initial plating, when grown in the warm growth chamber. The germination temperature of 16^{o}C resulted in significantly greater mean values for weight, leaf length, and root length (Table 9). This can be attributed to the performance of the warm-pollination-derived seedlings. Cold-pollination-derived seedlings germinated at warmer temperatures, 28 and 26^{o}C, tended to have the greater

mean values of all of their cohort cold-pollination-derived seedlings. Although any trend affecting the cold-pollination-derived seedlings was not as obvious as the trends of the warm-pollination-derived seedlings just discussed. One possible reason for this could be the density at which the seedlings were placed in the dried sphagnum media. Fewer warm pollination plants were produced due to poorer germination. Fewer warm-pollination-derived seedlings were placed in the same sized containers used for the growth chambers. Warm-pollination-derived seedlings may have had a slight benefit from the lower plant density therefore showing the trend of outgrowing cold-pollination derived seedlings. However, this does not explain the discrepancy between other warm-pollination-derived seedlings germinated at different temperatures not growing equally as well. In addition seedlings transferred from culture to the greenhouse in other experiments have been observed to flourish in pots with a high density of plants in comparison to plants contained individually. Seedling density is a possible explanation but should be researched more so that the exact implications are understood.

Results from this research indicate that gametophytic selection affected thermotolerance in progeny produced through selective temperature applications. The exact process by which male gametophytic selection was effective will not be completely understood until more research is continued on these or similar plants. Part of the difficulty of understanding the mechanics behind male gametophytic selection in this particular plant system is due to the complexities of orchids themselves. Due to the time required for seed production and plants to mature few studies on orchid heredity have been completed. In addition to growing extremely slowly, *Phalaenopsis* have a large and complex genome containing 40 chromosomes (Arditti 1992). *Grandiflora* hybrids such as those used in this experiment are most likely tetraploid; however octaploids are also common. A protocol for the flow cytometric analysis of *Phalaenopsis* to aid in karyotyping is currently being developed. Orchid biology and genetics are complex topics. Applying male gametophytic selection to such a plant system has provided a number of interesting challenges.

Selecting the most desirable length of time to apply the pollination treatments for the best possible response of the progeny is a complex and challenging issue due to some unique aspects of orchid biology. In this research, the length of pollination treatments was determined by post pollination phenomena of orchids including hyponasty, the inward folding of the petals to enclose the column and lip, as well as stigmatic swelling (Arditti 1976). However, ovary development and ovule differentiation in orchids are induced only by pollination. This means

that ovules have yet to form when pollination occurs. The initial "wilting" response of the flower is to simply signal pollinators to visit other flowers that have yet to be pollinated (Arditti 1992). Fertilization in orchids does not occur immediately. In *Phalaenopsis*, pollen germination occurs 5-7 days after pollen deposition. Pollen tubes enter and grow along the ovary wall for 10 to 35 days. Once in this position, pollen will remain quiescent until a single archesporial cell is present in an ovule approximately 40 days after pollination. At this point pollen tubes will begin to grow towards the ovules. Fertilization occurs approximately 80-85 days after initial pollination soon after the maturation of the ovules (Nadeau *et al.* 1996; Oneill *et al.* 1993; Zhang and O'Neill 1993).

Hypothetically, selective pressures applied during pollen germination and tube growth should extend through the entire 85 days between pollination and fertilization. Realistically, treatment for this period is not feasible. The temperature treatments selected for this experiment are too extreme and would cause the reproductive tissues to fail. This occurred in the 3 to 7 day period in many of the crosses that were attempted. Only two pollinations were attempted at a time on a single plant and extreme care was taken emulating natural pollination progression so that spontaneous abortion would be less likely to occur. Less extreme temperatures could be used, but selective pressures may not be strong enough to ensure the progeny would demonstrate any alteration in allele frequency favoring thermotolerance (Hormaza and Herrero 1996). Due to the lag between pollination and the maturation of the ovules, pollen tube growth is arrested and the sperm and vegetative cell are not active. This might also be a time when temperature treatments would be unsuccessful. Gametophytic selection for thermotolerance is thought to be most effective while pollen is active: during microspore development or pollen germination and tube growth (Frova *et al.* 1995; Hormaza and Herrero 1996). Male gametophytic selection in this research most likely occurred through the death of pollen that was not resistant to the temperatures applied during the pollination treatments. Although the temperature treatments were applied only during the very initial stages of pollen germination, because of the extreme nature of the selective pressure, male gametophytic selection may have been successful in producing progeny with the ability to outperform in a selected environment.

There may be some question as to the effect of the treatments on the tissues that give rise to the ovules, because female gametophytes had yet to form when the temperature treatments were applied. Ovule differentiation is triggered by pollination. At the time of the temperature treatments the placental ridge, the site of ovule differentiation, is the only visible presence of the

future female gametophytes (Zhang and O'Neill 1993). These cells may have been affected by the temperature treatments; however, one of the difficulties of applying selective pressures to the female gametophytes of any species of seed plant is the insulation of the megaspores by the female reproductive tissues (Hormaza and Herrero 1996). In addition, at no point during development or after maturation, do female gametophytes experience an independent phase such as male gametophytes experience. Environmental responses are regulated by the sporophyte, which would most likely negate any selection on the gametophyte (Ottaviano and Sari Gorla 1993). The possibility that the temperature treatments affected the maternal sporophyte to produce ovules with increased thermotolerance exists. However, the warm temperature treatment was applied for 3 days and the cold treatment for 7 days. The placental protuberances that eventually give rise to the ovules have only begun to differentiate from a single epidermal layer of the placenta at 14 days after pollination. If the temperature treatments had extended to include more of the development of the ovules, then female gamete selection could be considered a greater possibility. Due to the brevity of the temperature treatments, the insulation of the reproductive cells by the floral tissues and the increased sensitivity to temperature extremes of the pollen, it is highly probable that the male gametophytes and not the developing ovules were affected by the temperature treatments (Hormaza and Herrero 1996).

According to the data collected, cold-pollination-derived seeds germinated more frequently (Fig. 5). Although seed capsules produced from cold pollinations were smaller in size and the volume of seeds plated less, more cold-pollination-derived seeds germinated. Cold-pollination-derived seeds germinated best at 28 and 22^{o}C, specifically. Warm-pollination-derived seeds germinated best at a lower temperature, 20^{o}C. Even at this optimal temperature, germination of warm-pollination-derived seeds was less than half that of the cold-pollination-derived seeds that were germinated at 28 and 22^{o}C. Optimal temperatures for seed germination are most likely genotype dependent so variability is expected. However, the disparity between the warm and cold-pollination-derived seedlings in the number of seedlings produced regardless of germination temperature, suggests a correlation between seed germination and the selection processes applied during pollination.

Aside from the initial germination comparison, there was little indication of a consistent trend demonstrating that cold or warm pollinated seeds outperforming each other, regardless of growing conditions in more mature seedlings. Pollination treatment was found to significantly affect the number of roots of seedlings measured 1 year after initial plating. Comparison of

pollination treatment means showed that cold pollinated seedlings produced significantly more roots than warm pollinated seedlings. Root length was not significantly different. The root growth rate in *Cucurbita pepo* and an F_1 hybrid of *Lycopersicon esculentum* × *hirsutum* was also observed to improve in progeny derived from cool temperature pollinations (Johannsson and Stephenson 1998a; Zamir and Gadish 1987). Any other positive effects on overall growth, regardless of growing conditions, gained from the pollination treatments did not seem to transfer to maturing seedlings. Sporophytic provisioning of pollen and seeds has been a favored explanation in many of the studies, which demonstrated that progeny derived from one pollination treatment consistently outgrew another, in all of the post pollination environmental conditions (Johannsson and Stephenson 1998b). Sporophytes grown under cooler conditions may provide more resources to developing pollen thus allowing for paternal provisioning to affect the future progeny (Delph et al. 1997). However, pollen used in this experiment for both pollination treatments was produced under the same conditions. Any effect of paternal provisioning during pollen development would be irrelevant, because pollen used in both the warm and cold temperature treatments would have had the same resources allocated from the sporophyte. However, if paternal provisioning extends beyond pollen development, then some effects could be due to paternal provisioning. Plants crossed in this research were first exposed to warm temperatures to produced warm-pollination-capsules. Once a successful pollination occurred the same plants were then exposed to cold conditions in order to produce the cold-pollination-capsules. If the plants responded to the initial exposure to the warm temperatures by reallocating resources to already existing pollinia some of the responses of the cold-pollination-derived seedlings could have derived from paternal provisioning. This still seems an unlikely scenario. Pollinia were already developed at the time of the pollination treatments. In addition the only seedling characteristic affected by pollination treatment was the mean number of roots per seedlings. Paternal allocation of resources would most likely affect more than a single seedling characteristic.

Sporophytic provisioning that affects seed germination can also be attributed to maternal effects. Those pollen grains that are the most vigorous and grow the fastest fertilize ovules in the portion of the ovary that will best provision the seed. As mentioned before, orchid pollen grows into the ovary wall and then enters a quiescent stage until ovule development is complete (Zhang and O'Neill 1993). This would seem to exclude orchid pollen from intergametophytic competition by restricting growth once pollen tubes have reached the same position in the ovary.

However, once the pollen is signaled that ovules are beginning to mature, it still takes the pollen up to 40 days to reach the ovules and for fertilization to transpire. During this period pollen competition may occur leading to the fastest growing pollen tubes producing seeds with the highest germinability (Hormaza and Herrero 1996). Intergametophytic competition would have occurred only between pollen that was better adapted to germinate under warmer or cooler conditions with pollen that was less able to endure the selected temperatures. In essence, the male gametes exhibiting the greatest fitness in either of the warm or cold conditions would outcompete those male gametes less able to tolerate the more extreme conditions. During the final period of tube growth, pollination treatments were not applied to the plants. This would factor out intergametophytic competition between pollen better adapted to cold or warm temperatures growing faster than poorly adapted pollen because no temperature treatment was applied during this period. Irrespective of the level of pollen competition in the fertilization process, orchids do not tend to produce endosperm. In *Phalaenopsis*, a triploid endosperm nucleus is formed but degenerates. Seeds are not provided with any endosperm, therefore limiting the effect of maternal provisioning on the progeny (Vinogradova and Andronova 2002; Wing Yam *et al.* 2002). Both paternal and maternal provisioning of pollen and seeds in this research seem a remote explanation for the difference in germination between cold and warm-pollination-derived seeds.

Another explanation for the improved germinability of cold-pollination-derived seeds is a possible link between cold tolerance and germinability. Selection for pollen with increased cold tolerance could also select for germinability indicating a link between these two traits. Thermotolerance is a complex trait that is regulated at all levels of plant organization including components on the cellular and subcellular level that are difficult to detect (Ottaviano and Sari Gorla 1993). Many genes that are active in pollen mediate basic metabolic activities such as those involved with energy production and starch synthesis (Ottaviano and Mulcahy 1989). Due to the overlap between sporophytic and gametophytic transcriptomes, it is likely that genes conferring adaptability to cold temperatures could also improve germinability through enhanced cellular or sub-cellular activity.

According to the results of this study, progeny derived from warm and cold pollination treatments were better able to survive and grow at higher and lower temperatures, respectively. However, better germinability was conferred to seeds selected through cooler conditions applied during pollen germination. Warm-pollination-derived seedlings did not germinate as

successfully, but still performed better than cold-pollination-derived seedlings under warmer conditions. Observations from an analysis of a multi-locus selection model questioned that, if gametophytic selection was more than a marginal effect, then pollen with the greatest fitness would always produce progeny with greater fitness, which would lead to fixation of alleles for the best competing phenotype, and result in a loss of genetic variation (Charlesworth and Charlesworth 1992a; Charlesworth and Charlesworth 1992b). The correlation between cold temperature tolerance and better seed germination in the population of *Phalaenopsis* seedlings generated in this research emphasizes the maintenance of genetic variability. Pollen that is able to successfully fertilize ovules because of a better level of fitness under certain conditions does not lead to a fixation of alleles for the best competing phenotype, because this pollen does not necessarily produce the fittest phenotype in every aspect of the future progeny's existence. The ability of orchid seeds to germinate more easily in nature is imperative for species survival due to a lack of endosperm and the need for a symbiosis with mycorrhizae. Genotypes that confer fitness in one environment may not confer the same level of fitness in another environment. This differential gametophytic selection under varying environments would maintain the genetic variability of the species as a whole (Delph et al. 1997; Mulcahy et al. 1996). The microenvironment in which some species exist may be more important for determining mating success than its genotype (Pasonen et al. 2000). Variation in pollen performance may be maintained by genotype × environment interactions or negative genetic correlation (Charlesworth and Charlesworth 1992b; Pasonen *et al.* 2001; Snow and Spira 1996).

Although characteristics measured in this research demonstrated an increased thermotolerance of seedlings to more extreme environmental conditions, further research is needed. Due to many setbacks, only one cross between cross *P.* (Taisoco Windian × Sogo Yukidian) × *Phalaenopsis* hybrid unknown produced both warm and cold-pollination-derived seedlings that could be used for study. These are two genetically complex *Phalaenopsis* polyploids. The attempted pollination of two known diploid species, *P. equestris* 'Rosea' and *P. lindenii*, for comparison in this study would have greatly enhanced the findings. However, the pollinations of these species under the temperature treatments were unsuccessful. The implications of the pollination treatment effects are of greatest concern when viewed in light of the application of the temperature treatments to the plant that produced the two capsules used for analysis in this study. The cold pollination treatment was applied after the first pollination of a single flower. Once that flower appeared to have been successfully pollinated, a second flower

on the warm pollination treatment was then applied to the same plant (Figure 1). It is more than likely that the pollen initially exposed to the cold temperature pollination treatment during the very early stages of germination was selected for again under the warm conditions unintentionally. This issue was recognized early in the study and a second pollination method was developed to avoid this double selection issue (Figure 2). The pollinations using the second method involving the clonally propagated plants did not result in pairs of warm and cold pollinated pods with similar parentage. Another issue that would greatly enhance any findings in respect to the response of seedlings to germination temperature and growing conditions would be the inclusion of seedlings that had been pollinated under optimal conditions for *Phalaenopsis* without the imposition of temperature extremes during pollination. This would demonstrate the ability of unselected seedlings to germinate and grow under different temperature extremes, and offer a comparison to further uphold the effectiveness of early selection. The limiting factor in many aspects of this study was the availability of plant material. As plant material is generated for research purposes more in-depth studies can be designed that address the flaws in this initial evaluation of gametophytic selection in *Phalaenopsis*. Due to the lack of successful pollinations the scope of this study is limited. However, the results imply that gametophytic selection has a great wealth of possibilities for the future selection of *Phalaenopsis* hybrids for thermotolerance.

Due to the availability of more advanced analysis techniques, whether or not male gametophytic selection has indeed altered allele frequency in progeny can be definitively answered. Marker analysis on the seedlings produced in this research would support whether or not gametophytic selection is merely epigenetic or is a means to implement early selection into a plant breeding program. Another important future endeavor would be the evaluation of the thermotolerance capabilities of these plants during bloom. It is important to determine if the seedlings produced in this research are better able to maintain buds during more extreme temperatures or are better adapted to flowering during temperature fluctuations.

Conclusion

The implications of early selection for breeding programs in *Phalaenopsis* could be vast. Traditional breeding in *Phalaenopsis* has focused on flower color, time and number. Due to the increase in popularity of these orchids as potted house plants, breeding for other characteristics such as foliage appearance and environmental stress resistance has become more important (Griesbach 2002). Male gametophytic selection has long been considered an ideal addition to

plant breeding programs of species that have long juvenile phases (Hormaza and Herrero 1996). *Phalaenopsis* grown from seed require 3-6 years before bloom. The financial losses suffered from plant damage due to temperature extremes during shipping and marketing seem to indicate that male gametophytic selection for thermotolerance could benefit the potted orchid industry (Laws 2005). Future research on the seedlings generated from this research might determine if these plants are better able to produce and maintain buds during more extreme temperatures.

References

Arditti, J. 1976. Post-pollination phenomena in orchid flowers. A pictorial view. *Orchid Review* **84:** 261-268.

Arditti, J. 1992. *Fundamentals of Orchid Biology*. John Wiley and Sons, Inc., New York.

Charlesworth, D. and B. Charlesworth. 1992a. The effects on genetic load of selection in the gametophytic stage. In *Angiosperm Pollen and Ovules* (eds. E. Ottaviano D.L. Mulcahy M. Sari Gorla, and G.B. Mulcahy), pp. 401-407. Springer-Verlag, New York.

Charlesworth, D. and B. Charlesworth. 1992b. The effects of selection in the gametophyte stage on mutational load. *Evolution* **46:** 703-720.

Chasan, R. 1992. Racing pollen tubes. *Plant Cell* **4:** 747-749.

Chen, C. and R.S. Lin. 2004. Nondestructive estimation of dry weight and leaf area of *Phalaenopsis* leaves. *Applied Engineering in Agriculture* **20:** 467-472.

Chen, W.S., H.W. Chang, W.H. Chen, and Y.S. Lin. 1997. Gibberellic acid and cytokinin affect *Phalaenopsis* flower morphology at high temperature. *HortScience* **32:** 1069-1073.

Chi, H.S., T.P. Straathof, H.J.M. Löffler, and J.M. Van Tuyl. 1999. *In vitro* selection for heat-tolerance in lilies. In *Anther and pollen, from biology to biotechnology* (eds. C. Clement E. Pacini, and A. J.C.), pp. 175-182. Springer, Berlin.

Chikkodi, S.B. and R.L. Ravikumar. 2000. Influence of pollen selection for *Alternaria helianthi* resistance on the progeny performance against leaf blight in sunflower (*Helianthus annuus* L.). *Sexual Plant Reproduction* **12:** 222-226.

Chou, C.C., W.S. Chen, K.L. Huang, H.C. Yu, and L.J. Liao. 2000. Changes in cytokinin levels of *Phalaenopsis* leaves at high temperature. *Plant Physiology and Biochemistry* **38:** 309-314.

Clarke, H.J., T.N. Khan, and K.H.M. Siddique. 2004. Pollen selection for chilling tolerance at hybridisation leads to improved chickpea cultivars. *Euphytica* **139:** 65-74.

Clarke, H.J. and K.H.M. Siddique. 2004. Response of chickpea genotypes to low temperature stress during reproductive development. *Field Crops Research* **90:** 323-334.

Crispi, M.L. and L.C. Peirce. 1992. Gametophytic selection for early maturity in tomato (*Lycopersicon esculentum* Mill.). In *Angiosperm Pollen and Ovules* (eds. E. Ottaviano D.L. Mulcahy M. Sari Gorla, and G.B. Mulcahy), pp. 370-376. Springer-Verlag, New York.

Crone, D., J. Rueda, K.L. Marint, D.A. Hamilton, and J.P. Mascarenhas. 2001. The differential expression of a heat shock promoter in floral and reproductive tissues. *Plant, Cell and Environment* **24:** 869-874.

Dahl, A.E. and M. Fredrikson. 1996. The timetable for development of maternal tissues sets the stage for male genomic selection in *Betula pendula* (*Betulaceae*). *American Journal of Botany* **83:** 895-902.

Delph, L.F., M.G. Johannsson, and A.G. Stephenson. 1997. How environmental factors affect pollen performance: Ecological and evolutionary perspectives. *Ecology* **78:** 1632-1639.

Fehr, W.R. 1991. *Principles of cultivar development*. Iowa State University: Macmillian Publishing Company, New York. pp. 593.

Frascaroli, E. and D.D. Songstad. 2001. Pollen genotype selection for a simply inherited qualitative factor determining resistance to chlorsulfuron in maize. *Theoretical and Applied Genetics* **102:** 342-346.

Frova, C., P. Portaluppi, M. Villa, and M.S. Gorla. 1995. Sporophytic and gametophytic components of thermotolerance affected by pollen selection. *Journal of Heredity* **86:** 50-54.

Frova, C. and M. Sari Gorla. 1992. Pollen competition: genetics and implications for plant breeding. In *Advances in Genetics* (eds. Y. Dattée C. Dumas, and A. Gallais), pp. 335-344. Academic Press, New York.

Frova, C., G. Taramino, M.S. Gorla, and E. Ottaviano. 1992. Developmental synthesis of HSPs in the male gametophytic phase of heat tolerant and heat sensitive species. In *Angiosperm Pollen and Ovules* (eds. E. Ottaviano D.L. Mulcahy M.S. Gorla, and G. Bergamini-Mulcahy), pp. 191-195. Springer-Verlag, New York.

Frova, C., G. Taramino, and E. Ottaviano. 1991. Sporophytic and gametophytic heat-shock protein-synthesis in *Sorghum-bicolor*. *Plant Science* **73:** 35-44.

Griesbach, R.J. 2002. Development of *Phalaenopsis* orchids for the mass-market. In *Trends in new crops and new uses. Proceedings of the Fifth National Symposium,* eds J. Janick and A. Whipkey), pp. 458-465, Atlanta, GA, USA.

Hicks, A.J. 1999. *Asymbiotic Technique of Orchid Seed Germination*. Orchid Seedbank Project, Socorro, NM, USA.

Honys, D. and D. Twell. 2003. Comparative analysis of the *Arabidopsis* pollen transcriptome. *Plant Physiology* **132:** 640-652.

Honys, D. and D. Twell. 2004. Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*. *Genome Biology* **5**.

Hormaza, J.I. and M. Herrero. 1996. Male gametophytic selection as a plant breeding tool. *Scientia Horticulturae* **65:** 321-333.

Johannsson, M.H. and A.G. Stephenson. 1998a. Effects of temperature during microsporogenesis on pollen performance in *Cucurbita pepo* L. (*cucurbitaceae*). *International Journal of Plant Sciences* **159:** 616-626.

Johannsson, M.H. and A.G. Stephenson. 1998b. Variation in sporophytic and gametophytic vigor in wild and cultivated varieties of *Cucurbita pepo* and their F1 and F2 generations. *Sexual Plant Reproduction* **11:** 265-271.

Johnsen, O., T. Skroppa, G. Haug, I. Apeland, and G. Ostreng. 1995. Sexual reproduction in a greenhouse and reduced autumn frost hardiness of *Picea abies* progenies. *Tree Physiology* **15:** 551-555.

Jourdren, C., D. Simonneaux, and M. Renard. 1996. Selection on pollen for linolenic acid content in rapeseed, *Brassica napus* L. *Plant Breeding* **115:** 11-15.

Katiyar-Agarwal, S., M. Agarwal, D.R. Gallie, and A. Grover. 2001. Search for the cellular functions of plant Hsp100/Clp family proteins. *Critical Reviews in Plant Sciences* **20:** 277-295.

Kovács, G. and B. Barnabás. 1992. *Production of highly cold tolerant maize inbred lines by repeated gametophytic selection*.

Lassere, T.B., S.B. Carroll, and D.L. Mulcahy. 1996. Effect of pollen competition on offspring quality at varying stages of the life cycle in *Silene latifolia* 'Poiret' (*Caryophyllaceae*). *Bulletin of the Torrey Botanical Club* **123:** 175-179.

Laws, N. 2005. The world's fascination with potted orchids. In *Floraculture International*. Ball Publishing. http://www.floracultureintl.com/archive/articles/1186.asp .

Mandhu, B., M. Cresti, and K.R. Shivanna. 1992. Effects of high temperature and humidity stresses on tobacco pollen and their progeny. In *Angiosperm Pollen and Ovules* (eds. E. Ottaviano D.L. Mulcahy M. Sari Gorla, and G.B. Mulcahy), pp. 349-354. Springer-Verlag, New York.

Mascarenhas, J.P. 1990. Gene activity during pollen development. *Annual Review of Plant Physiology and Plant Molecular Biology* **41:** 317-338.

Mascarenhas, J.P. and D.F. Crone. 1996. Pollen and the heat shock response. *Sexual Plant Reproduction* **9:** 370-374.

McCormick, S. 2004. Control of male gametophyte development. *Plant Cell* **16:** S142-S153.

Mulcahy, D.L. 1971. A correlation between gametophytic and sporophytic characteristics in *Zea mays* L. *Science* **171:** 1155-1156.

Mulcahy, D.L. 1979. Rise of the angiosperms - A genecological factor. *Science* **206:** 20-23.

Mulcahy, D.L., M. SariGorla, and G.B. Mulcahy. 1996. Pollen selection - past, present and future. *Sexual Plant Reproduction* **9:** 353-356.

Nadeau, J.A., X.S. Zhang, J. Li, and S.D. O'neill. 1996. Ovule development: Identification of stage-specific and tissue-specific cDNAs. *Plant Cell* **8:** 213-239.

Oneill, S.D., J.A. Nadeau, X.S. Zhang, A.Q. Bui, and A.H. Halevy. 1993. Interorgan regulation of ethylene biosynthetic genes by pollination. *Plant Cell* **5:** 419-432.

Ottaviano, E., M.S. Gorla, and E. Pe. 1982. Male gametophytic selection in maize. *Theoretical and Applied Genetics* **63:** 249-254.

Ottaviano, E. and D.L. Mulcahy. 1986. Gametophytic selection as a factor of crop plant evolution. In *The Origin and Domestication of Cultivated Plants* (ed. C. Barigozzi), pp. 101-120. Elsevier, New York.

Ottaviano, E. and D.L. Mulcahy. 1989. Genetics of angiosperm pollen. *Advances in Genetics* **26:** 1-64.

Ottaviano, E., M.E. Pè, and G. Binelli. 1991. Genetic manipulation of male gametophytic generation in higher plants. In *Subcellular Biochemistry: Plant Genetic Engineering* (eds. B.B. Biswas and J.R. Harris), pp. 107-142. Plenum Press, New York.

Ottaviano, E. and M. Sari Gorla. 1993. Gametophytic and sporophytic selection. In *Plant breeding: Principles and prospects* (eds. M.D. Hayward N.O. Bosemark, and I. Romagosa), pp. 332-352. Chapman & Hall, New York.

Ottaviano, E., M. Sarigorla, and M. Villa. 1988. Pollen competitive ability in maize - within population variability and response to selection. *Theoretical and Applied Genetics* **76:** 601-608.

Pasonen, H.L., M. Kapyla, and P. Pulkkinen. 2000. Effects of temperature and pollination site on pollen performance in *Betula pendula* 'Roth' - evidence for genotype-environment interactions. *Theoretical and Applied Genetics* **100:** 1108-1112.

Pasonen, H.L., P. Pulkkinen, and M. Kapyla. 2001. Do pollen donors with fastest-growing pollen tubes sire the best offspring in an anemophilous tree, *Betula pendula* (*Betulaceae*)? *American Journal of Botany* **88:** 854-860.

Pedersen, S., V. Simonsen, and V. Loeschcke. 1987. Overlap of gametophytic and sporophytic gene expression in Barley. *Theoretical and Applied Genetics* **75:** 200-206.

Petolino, J.F., N.M. Cowen, S.A. Thompson, and J.C. Mitchell. 1990. Gamete selection for heat-stress tolerance in maize. *Journal of Plant Physiology* **136:** 219-224.

Ravikumar, R.L. and S.B. Chikkodi. 1998. Association between sporophytic reaction to *Alternaria helianthi* and gametophytic tolerance to pathogen culture filtrate in sunflower (*Helianthus annuus* L.). *Euphytica* **103:** 173-180.

Ravikumar, R.L. and B.S. Patil. 2004. Effect of gamete selection on segregation of wilt susceptibility-linked DNA marker in chickpea. *Current Science* **86:** 642-643.

Ravikumar, R.L., B.S. Patil, and P.M. Salimath. 2003. Drought tolerance in sorghum by pollen selection using osmotic stress. *Euphytica* **133:** 371-376.

Sari-Gorla, M., S. Ferrario, E. Frascaroli, C. Frova, P. Landi, and M. Villa. 1994. Soprophytic response to pollen selection for alachlor tolerance in maize. *Theoretical and Applied Genetics* **88:** 812-817.

Sari-Gorla, M., C. Frova, G. Binelli, and E. Ottaviano. 1986. The extent of gametophytic-sporophytic gene-expression in maize. *Theoretical and Applied Genetics* **72:** 42-47.

Sari-Gorla, M., M.E. Pe, D.L. Mulcahy, and E. Ottaviano. 1992. Genetic dissection of pollen competitive ability in maize. *Heredity* **69:** 423-430.

Sari-Gorla, M. and E. Rovida. 1980. Competitive ability of maize pollen. Intergametophytic effects. *Theoretical and Applied Genetics* **57:** 37-41.

Service, N.A.S. 2005. USDA Floriculture Crops 2004 Summary (ed.s USDA).

Singh, F. and M. Thimmappaiah. 1982. Polyembryony in orchid seeds. *Seed Science and Technology* **10:** 29-33.

Snow, A.A. and T.P. Spira. 1991. Pollen vigor and the potential for sexual selection in plants. *Nature* **352:** 796-797.

Snow, A.A. and T.P. Spira. 1996. Pollen-tube competition and male fitness in *Hibiscus moscheutos*. *Evolution* **50:** 1866-1870.

Snow, A.A., T.P. Spira, and H. Liu. 2000. Effects of sequential pollination on the success of "fast" and "slow" pollen donors in *Hibiscus moscheutos* (*Malvaceae*). *American Journal of Botany* **87:** 1656-1659.

Su, W.R., W.S. Chen, M. Koshioka, L.N. Mander, L.S. Hung, W.H. Chen, Y.M. Fu, and K.L. Huang. 2001. Changes in gibberellin levels in the flowering shoot of *Phalaenopsis hybrida* under high temperature conditions when flower development is blocked. *Plant Physiology and Biochemistry* **39:** 45-50.

Sultan, S.E. 2005. An emerging focus on plant ecological development. *New Phytologist* **166:** 1-5.

Tanksley, S.D., D. Zamir, and C.M. Rick. 1981. Evidence for extensive overlap of sporophytic and gametophytic gene-expression in *Lycopersicon esculentum*. *Science* **213:** 453-455.

Touraev, A., C.S. Fink, E. Stoger, and E. HeberleBors. 1995. Pollen selection: A transgenic reconstruction approach. *Proceedings of the National Academy of Sciences of the United States ff America* **92:** 12165-12169.

Vinogradova, T. and E.V. Andronova. 2002. Development of orchid seed and seedlings. In *Orchid Biology: Reviews and Perspectives, VIII* (eds. T. Kull and J. Arditti), pp. 167-234. Kluwer Academic Publishers, Boston.

Williams, J.H., W.E. Friedman, and M.L. Arnold. 1999. Developmental selection within the angiosperm style: Using gamete DNA to visualize interspecific pollen competition. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 9201-9206.

Willing, R.P. and J.P. Mascarenhas. 1984. Analysis of the complexity and diversity of messenger-RNAs from pollen and shoots of *Tradescantia*. *Plant Physiology* **75:** 865-868.

Wing Yam, T., E.C. Yeung, X.L. Ye, S.Y. Zee, and J. Arditti. 2002. Orchid embryos. In *Orchid Biology: Reviews and Perspectives, VIII* (eds. T. Kull and J. Arditti), pp. 287-386. Kluwer Academic Publishers, Boston.

Zamir, D. and I. Gadish. 1987. Pollen selection for low-temperature adaptation in tomato. *Theoretical and Applied Genetics* **74:** 545-548.

Zhang, X.S. and S.D. O'Neill. 1993. Ovary and gametophyte development are coordinately regulated by auxin and ethylene following pollination. *Plant Cell* **5:** 403-418.

Table 1: Reciprocal pollinations between hybrid *Phalaenopsis*. Red panels indicate warm pollination treatment (30/25°C) and blue panels indicate cold pollination treatment (15/10°C). Ratios of the crosses that were attempted indicate the number of fruit that were produced compared to the number of pollinations attempted (individual flowers). Pollinations were attempted over a two-year period during which plants bloomed twice. * indicates fruit production, without seed generation (capsules did not contain seeds when harvested).

Female ↓ Male →	*P.* (Taisuco Windian × Sogo Yukidian)	*P.* Hybrid Unknown	*P.* Tropical Stripes 'Carmela'	*P.* Brother Heather 'Nuclear'	*P.* Neon Stripe 'Red Avenger'	*P.* Sogo 'Little Angel'	*P.* Pinlong Moristopher 'Dream'	*P.* Baldan's Kaleidoscope 'Golden Treasure'	*P.* (Meller × Taisuco Kochdian)	*P.* Taipei Gold 'Golden Star'
P. (Taisuco Windian × Sogo Yukidian)		**1/3**	0/2							
		1/3	0/2							
P. Hybrid Unknown	**1/3**									
	1/3									
P. Tropical Stripes 'Carmela'	0/2	0/2		1/2	0/3	0/2				
	0/2	0/2		0/3	0/3	0/2				
P. Brother Heather 'Nuclear'			1/2		0/3					
			0/3		0/3					
P. Neon Stripe 'Red Avenger'			0/3	0/3						
			0/3	0/3						
P. Sogo 'Little Angel'			0/2				1/2	0/8		
			0/2				0/2	0/8		
P. Pinlong Moristopher 'Dream'						0/5		0/5	0/6	
						0/5		0/5	0/6	
P. Baldan's Kaleidoscope 'Golden Treasure'						0/10	0/5		0/4	0/3
						0/10	0/5		0/4	0/3
P. (Meller × Taisuco Kochdian)							0/3	0/4		1/3 *
							0/3	0/4		1/3 *
*P.*TaipeiGold 'Golden Star'								0/2	0/5	
								0/2	0/5	

Table 2: Two-way ANOVA for mean number of protocorms 72 days after initial plating before transfer of protocorms to the incubator. P= pollination treatment, T= germination temperature (including all 9 temperatures at which protocorms developed and were evaluated in Figure 5) and P × T = the interaction between pollination treatment and germination temperature. df = degrees of freedom, MS= mean squares.

Source of Variation	**df**	**MS**	**P value**
Pollination trt. (P)	1	11	0.028 *
Germination Temp. (T)	8	29.1	<0.0001 **
P × T	8	39.1	<0.0001 **
Error	17	1.9	
Total	34		

* indicates significance at P<0.05;**indicates significance at P<0.01

Table 3: Three-way ANOVA for mortality, leaf and root development and spontaneous clumping of protocorms and seedlings measured 125 days after initial plating. Treatments measured include: P= warm and cold pollination treatments, T= germination temperature (including all 8 temperature increments), I = warm and cold incubators, P × T = the interaction between pollination treatment and table position, T × I = interaction between the germination temperature and the incubators effects, P × I = the interaction between pollination treatments and incubator and P × T × I = the interaction between pollination treatment, incubator and germination temperature. df = degrees of freedom, MS= mean squares.

Source of Variation	df	Mortality % dead MS	% of Protocorms without leaves or roots MS	% of Seedlings with leaves but no roots MS	% of Seedlings with leaves and roots MS	% of protocorms/ seedlings in clumps MS
Pollination Trt (P)	1	0.00009	0.026	0.11	0.00084	0.004
Germination Temp (T)	7	0.12 **	0.13 **	0.000023*	0.019	0.18 **
Incubator (I)	1	0.29 **	1.98 **	0.0018	0.46 **	0.86 **
P × T	5	0.039	0.033	0.020	0.039	0.038
T × I	7	0.065 *	0.12 **	0.082	0.0046	0.015
P × I	1	0.097 *	0.0011	0.017	0.0074	0.025
P × T × I	4	0.027	0.026	0.041	0.038	0.05
Error	16	0.27	0.025	0.037	0.049	0.036
Total	42					

* indicates significance at P<0.05;**indicates significance at P<0.01.

Table 4 : Means comparison of the mortality of seedlings, and the percentages of leaf and root development as well as spontaneous clump formation measured 125 days after initial plating in response to incubator. Contaminated plates were discarded and not included in data analysis.

Incubator	N	Mortality % dead	% Protocorms without leaves or roots	% Seedlings with leaves but no roots	% Seedlings with leaves and roots	% of protocorms/ seedlings in clumps
Cold (25° C)	22	31 [a]	51 [a]	26 [a]	3 [b]	16 [b]
Warm (30° C)	21	15 [b]	6 [b]	25 [a]	24 [a]	45 [a]

Mean separation by Ryan-Einot-Gabriel-Welsch Multiple Range Test. Letters denote significant differences between the means. N = the number of plates where protocorms were compared.

Table 5: Means comparison of the percentages of seedling mortality, leaf and root development as well as spontaneous clump formation measured 125 days after initial plating in response to germination temperature. Contaminated plates were discarded and not included in data analysis.

Germination temperature	N	Mortality % dead	% Protocorms without leaves or roots	% Seedlings with leaves but no roots	% Seedlings with leaves and roots	% of protocorms/ seedlings in clumps
1 (30°C)	2	0.41 ^{abc}	0.30 ^{ab}	0.40 ^{a}	0.23 ^{a}	0.0 ^{b}
2 (28°C)	4	0.42 ^{ab}	0.18 ^{ab}	0.05 ^{a}	0.11 ^{a}	0.42 ^{ab}
3 (26°C)	6	0.47 ^{a}	0.13 ^{b}	0.06 ^{a}	0.15 ^{a}	0.67 ^{a}
4 (24°C)	7	0.16 ^{bcd}	0.097 ^{b}	0.44 ^{a}	0.23 ^{a}	0.26 ^{b}
5 (22°C)	4	0.12 ^{d}	0.31 ^{ab}	0.22 ^{a}	0.15 ^{a}	0.31 ^{ab}
6 (20°C)	7	0.22 ^{abcd}	0.45 ^{ab}	0.21 ^{a}	0.11 ^{a}	0.25 ^{b}
7 (18°C)	8	0.10 ^{d}	0.36 ^{ab}	0.33 ^{a}	0.09 ^{a}	0.21 ^{b}
8 (16°C)	5	0.13 ^{dc}	0.50 ^{a}	0.30 ^{a}	0.06 ^{a}	0.14 ^{b}

Mean separation by Ryan-Einot-Gabriel-Welsch Multiple Range Test. Letters denote significant differences between the means. N = the number of plates where protocorms were compared.

Table 6: Three-way ANOVA of mean weight, mean leaf number, mean leaf length, mean leaf area mean root number and mean root length of seedlings measured one year after initial plating. Mean leaf width was not significantly affected by any of the treatments measured. Treatments measured include: P= warm and cold pollination treatments, T= germination temperature (including all 7 temperature increments), I = warm (30^{o}C) and cold (25^{o}C) incubators, P × T = the interaction between pollination treatment and germination temperature, T × I = interaction between the germination temperature and the incubators effects, P × I = the interaction between pollination treatments and incubator and P × T × I = the interaction between pollination treatment, incubator and germination temperature. df = degrees of freedom, MS= mean squares.

Source of Variation	df	Mean weight of seedlings MS	Mean no. leaves MS	Mean leaf length MS	Mean leaf area MS	Mean no. of roots MS	Mean root length MS
Pollination Trt (P)	1	0.35	0.19	0.51	22.27	16.55 *	3.54
Germination temp (T)	6	1.67 **	0.48	1.66 **	19.38	2.21	2.16
Incubator/ Growth Chamber (I)	1	28.99 **	8.82 **	82.64 **	246.34**	79.88 **	31.41 **
P × T	4	2.39 **	0.26	0.58	16.85	6.68 *	4.57 **
T × I	6	0.21	0.33	0.37	11.56	0.44	1.49
P × I	1	5.69 *	5.69 **	10.64 **	0.43	3.01 *	12.33 **
P × T × I	2	1.25	0.27	0.56	9.68	4.49	2.19
Error	269	0.49	0.44	0.57	24.10	2.88	1.21
Total	290						

Indicates significance at P<0.05;**indicates significance at P<0.01

Table 7: Means comparison of seedling weight, mean leaf number, mean leaf length, mean leaf width, mean leaf area, mean root number and mean root length measured 1 year after initial plating in response to the temperature of the incubator/ growth chamber. Protocorms were transferred to incubators set at 25°C and 30°C. Seedlings mature enough to be removed from culture were transferred to growth chambers set at 10/15°C and 25/30°C (night and day temperatures).

Incubator/ Growth chamber	N	Mean Weight (g)	Mean number of leaves	Mean leaf length (cm)	Mean leaf width (cm)	Mean leaf area (cm^2)	Mean number of roots	Mean root length (cm)
Cold	172	0.5 [b]	2.1 [b]	1.1 [b]	0.9 [b]	0.8 [b]	3.4 [b]	1.7 [b]
Warm	119	1.1 [a]	2.5 [a]	2.2 [a]	1.6 [a]	2.6 [a]	4.4 [a]	2.3 [a]

Mean separation by Ryan-Einot-Gabriel-Welsch Multiple Range Test. Letters denote significant differences between the means. N = the number of protocorms compared.

Table 8: Means comparison of seedling weight, mean leaf number, mean leaf length, mean leaf width, mean leaf area, mean root number and mean root length measured 1 year after initial plating in response to the warm (30/25°C, 3 days) or cold (15/10°C, 7days) pollination treatment.

Pollination Treatment	N	Mean Weight (g)	Mean number of leaves	Mean leaf length (cm)	Mean leaf width (cm)	Mean leaf area (cm^2)	Mean number of roots	Mean root length (cm)
Cold (15/10°C)	212	1.0 [a]	2.3 [a]	1.8 [a]	1.4 [a]	1.9 [a]	4.2 [a]	2.0 [a]
Warm (30/25°C)	79	0.8 [a]	2.4 [a]	1.8 [a]	1.0 [a]	1.6 [a]	3.6 [b]	2.2 [a]

Mean separation by Ryan-Einot-Gabriel-Welsch Multiple Range Test. Letters denote significant differences between the means. N = the number of protocorms compared.

Table 9: Means comparison of seedlings weight, mean leaf number, mean leaf length, mean leaf width, mean leaf area, mean root number and mean root length measured 1 year after initial plating in response to germination temperature.

Germination Temperature	N	Mean Weight	Mean leaf number	Mean leaf length	Mean leaf width	Mean leaf area	Mean root number	Mean root length
2 (28°C)	5	0.8 b	2.4 a	1.7 bc	1.1 a	1.5 a	4.1 a	2.1 b
3 (26°C)	58	0.5 b	2.4 a	1.4 c	0.9 a	1.0 a	3.5 a	1.7 b
4 (24°C)	49	0.9 b	2.3 a	1.6 bc	2.3 a	3.0 a	3.9 a	2.0 b
5 (22°C)	47	0.9 b	2.3 a	2.0 ab	1.1 a	1.9 a	4.6 a	2.3 b
6 (20°C)	49	0.9 b	2.3 a	1.8 bc	1.0 a	1.5 a	3.9 a	2.1 b
7 (18°C)	39	1.0 b	2.3 a	2.0 ab	1.3 a	2.0 a	4.1 a	2.1 b
8 (16°C)	44	2.0 a	2.6 a	2.6 a	1.3 a	2.8 a	4.6 a	3.4 a

Mean separation by Ryan-Einot-Gabriel-Welsch Multiple Range Test. Letters denote significant differences between the means. N = the number of protocorms compared.

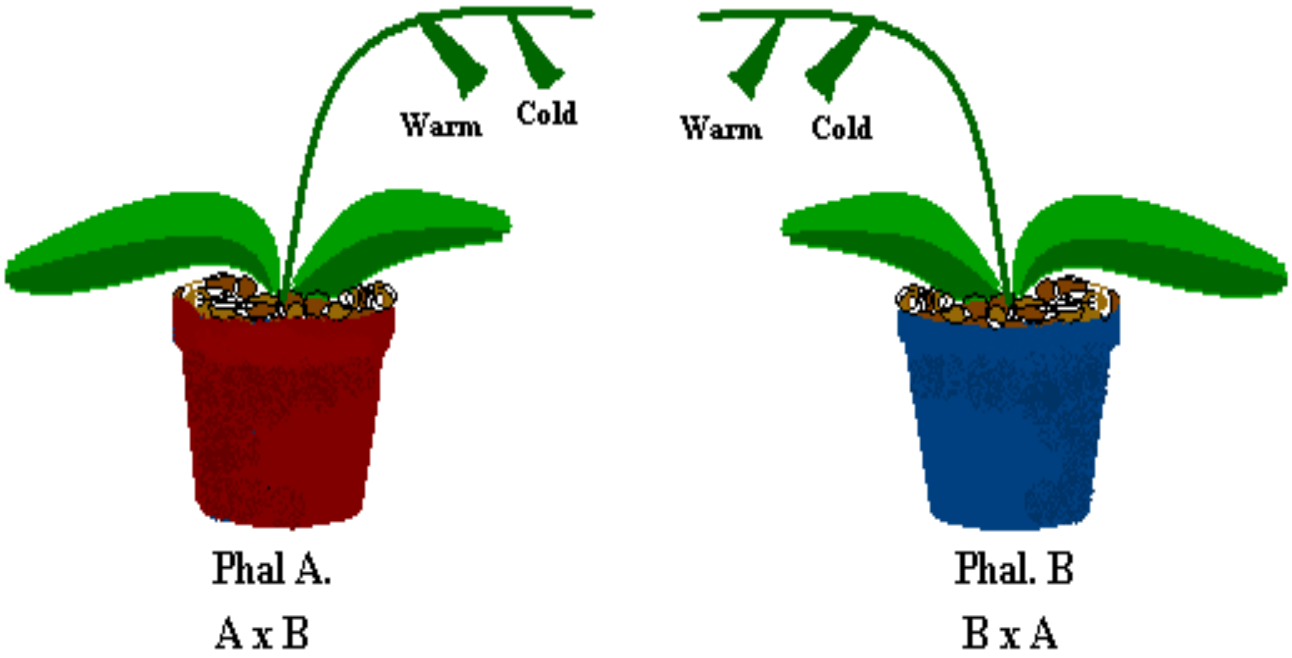

Figure 1: Initial pollinations and crosses under two temperature treatments (30/25°C and 15/10°C) made between hybrid *Phalaenopsis*. Each parent was exposed to the warm temperature treatment for 3 days followed by the cold temperature treatment for 7 days in order to produce both a warm and cold pollinated capsule. Fruit used in this experiment were derived from this pollination method**.**

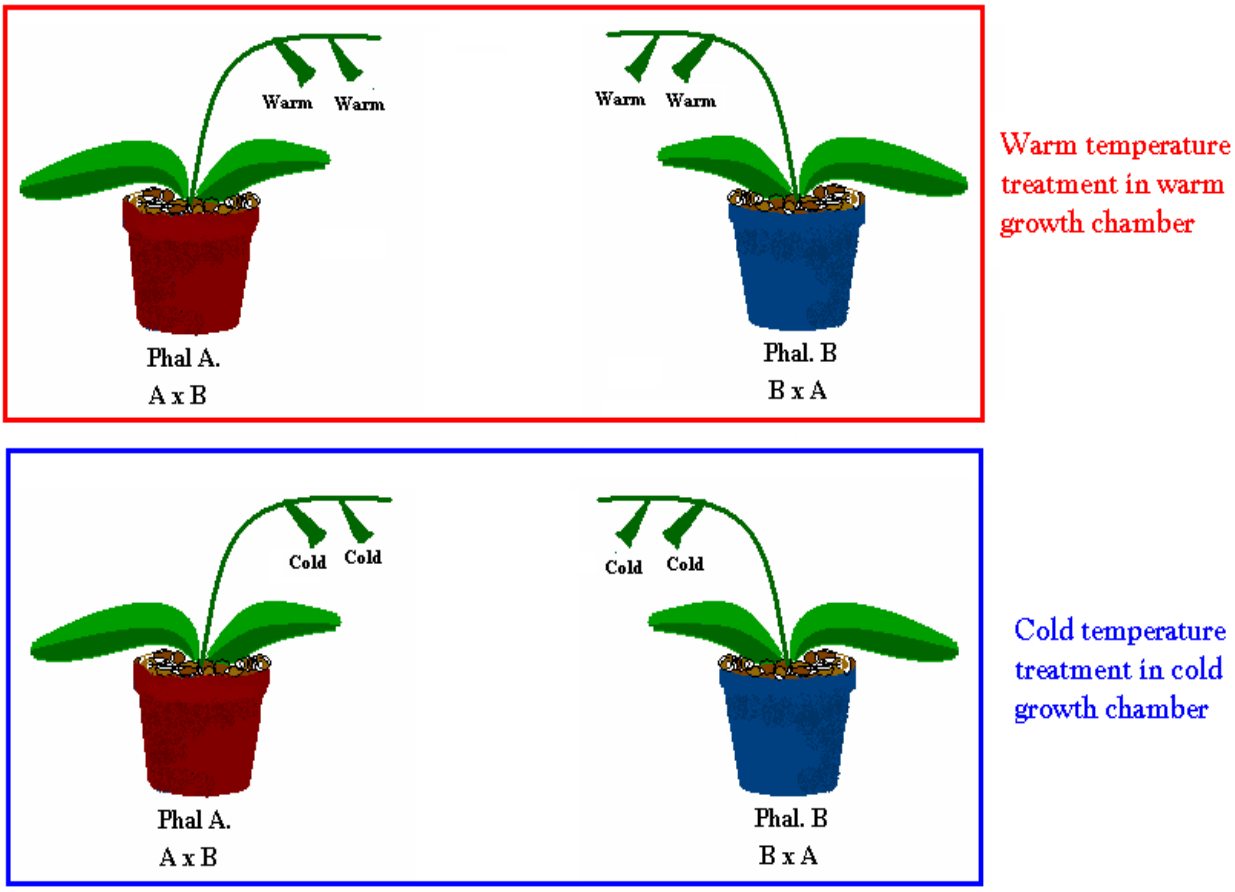

Figure 2: Protocol of pollination treatments designed for pairs of "cloned" *Phalaenopsis* hybrids. Individual plants of a pair would be exposed to only a single treatment, because of the availability of two presumed genetically identical plants derived from clonal propagation. No fruit were produced that were used in this experiment.

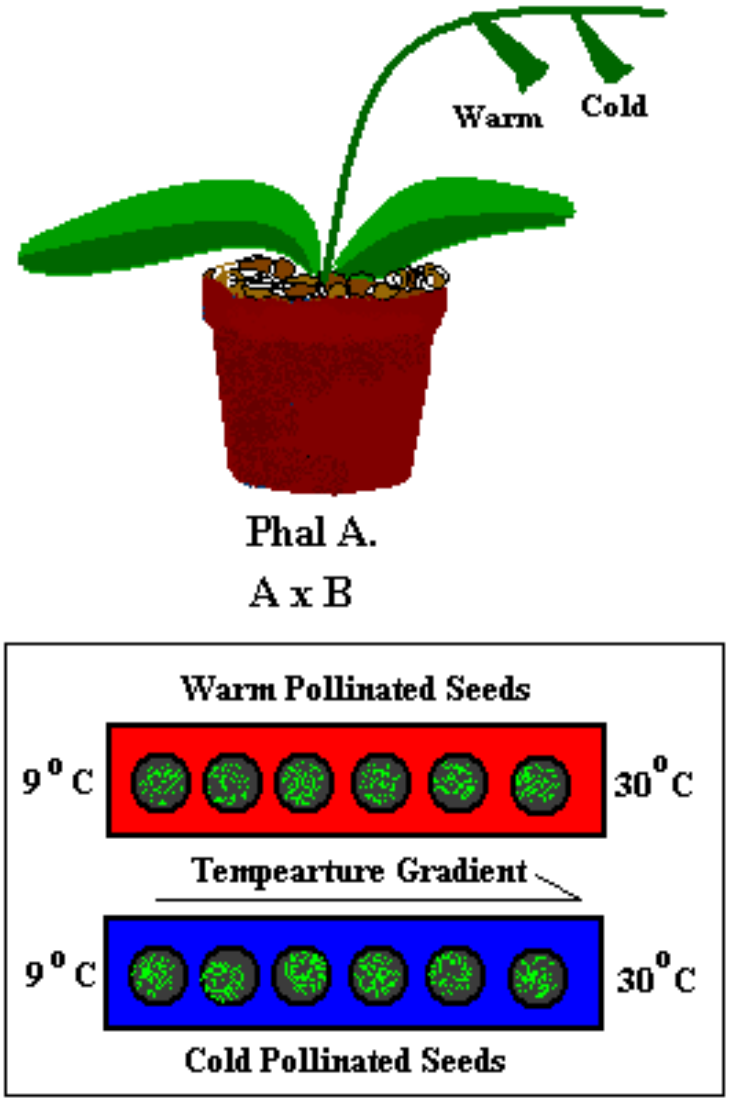

Figure 3: Seeds derived from the same *Phalaenopsis* hybridization, but pollinated under cold (C) or warm (W) conditions, were germinated in nutrient media on a temperature gradient table. The temperature gradient table had 12 positions ranging incrementally from 30°C to 9°C.

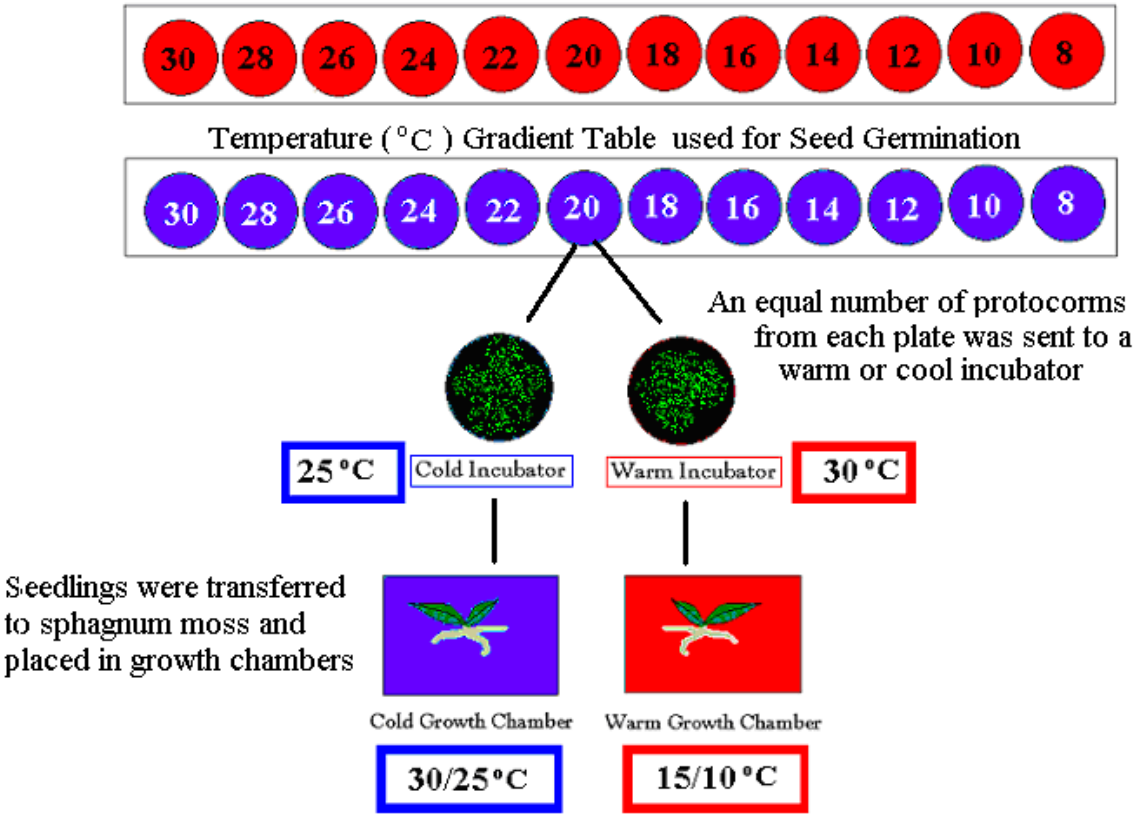

Figure 4: Protocorms germinated on the temperature gradient table (ranging from 30-9°C) were divided in half and transferred to fresh media and to either a warm incubator (30°C) or a cold incubator (25°C). Seedlings mature enough to normally be transferred to a greenhouse were removed from culture and placed on dried sphagnum moss. Seedlings were then transferred to a warm or cold growth chamber depending on whether grown in a warm or cold incubator. Growth chambers were set at 10/15°C and 25/30°C.

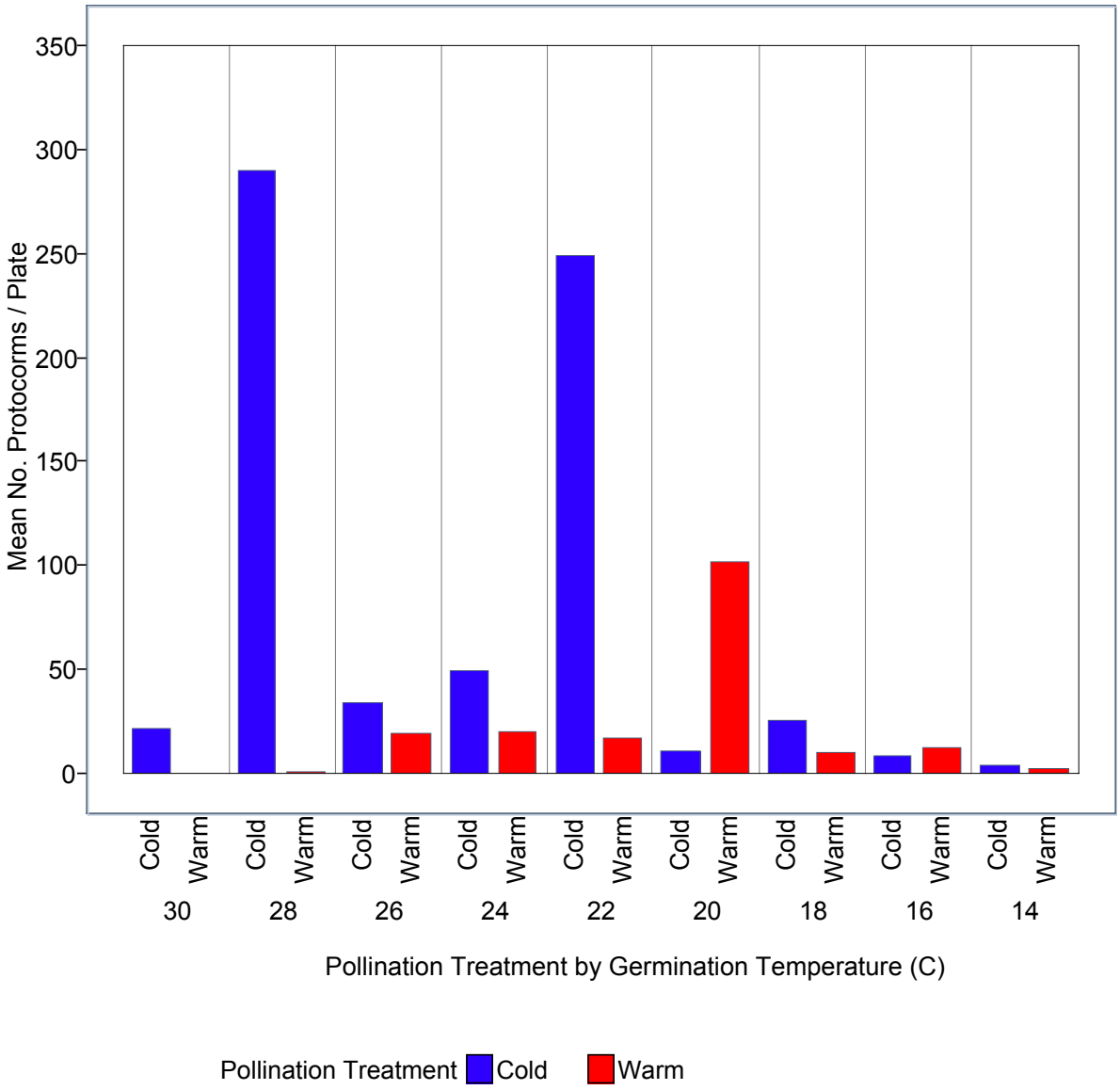

Figure 5: Evaluation of the mean number of protocorms produced per plate post germination by pollination treatment and germination temperature (^{o}C). Seeds were derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator/ growth chamber. All seeds collected from each fruit were placed on Phytamax® media with 5% coconut water in equal volumes per plate for comparison of germination.

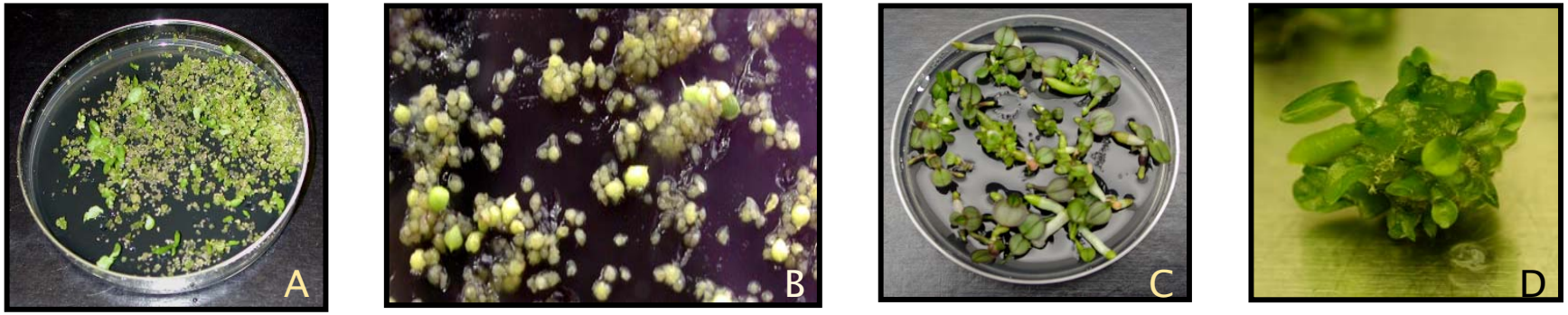

Figure 6: The progression of orchids seeds from protocorms to seedlings. A. A typical plate containing hundreds of orchid protocorms. B. Close-up of orchid protocorms. C. Leaf and root production after protocorm phase. D. Example of spontaneous protocorm proliferation. A single protocorm can give rise to a clump of multiple plantlets.

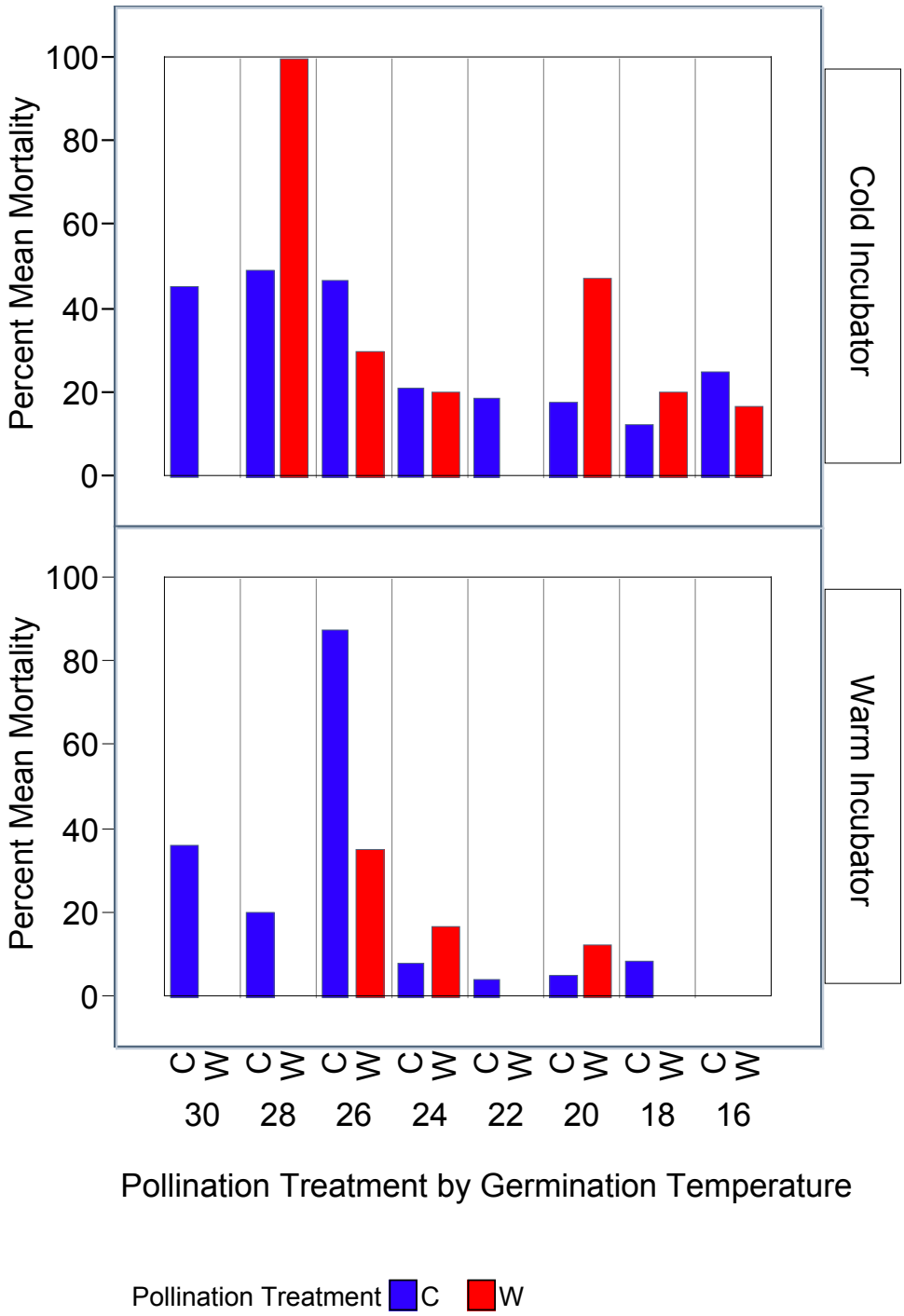

Figure 7: Percent mortality of protocorms of seeds derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator. The ratio of dead protocorms to the total number of protocorms transferred to each incubator was evaluated 125 days after initial plating. Germination temperature is in ^{o}C.

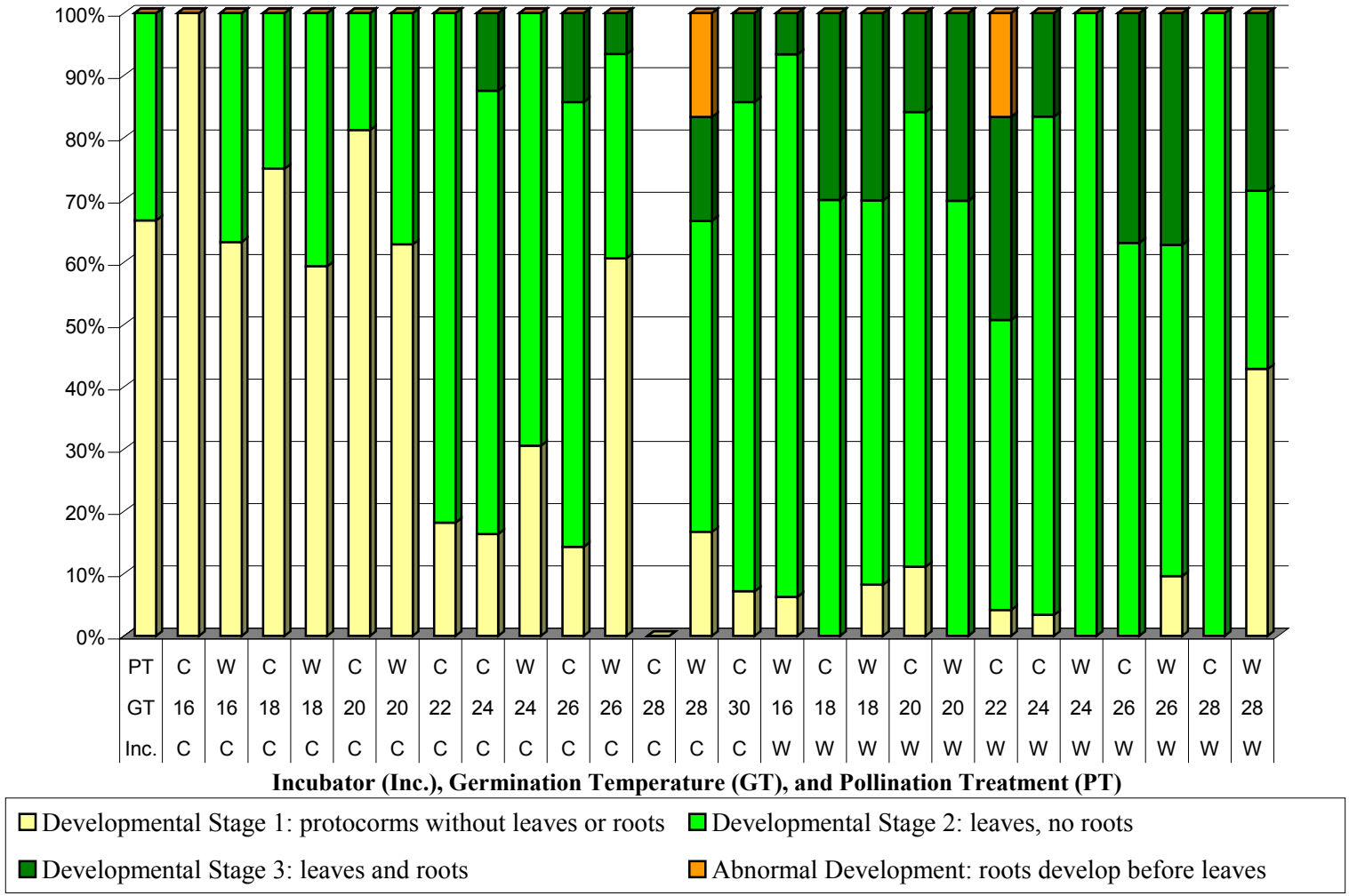

Figure 8: Comparison of seedling developmental stages 125 days after initial plating. Seeds were derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator. Developmental stage 1 is characterized as the protocorm stage lacking leaves and roots. Stage 2 is characterized by the development of leaves. Stage 3 is characterized by the formation of leaves and roots. Occasionally abnormal development occurred with roots forming before leaves. Percentages of each developmental stage were calculated using the total number of live protocorms. Dead and contaminated seedlings were not included.

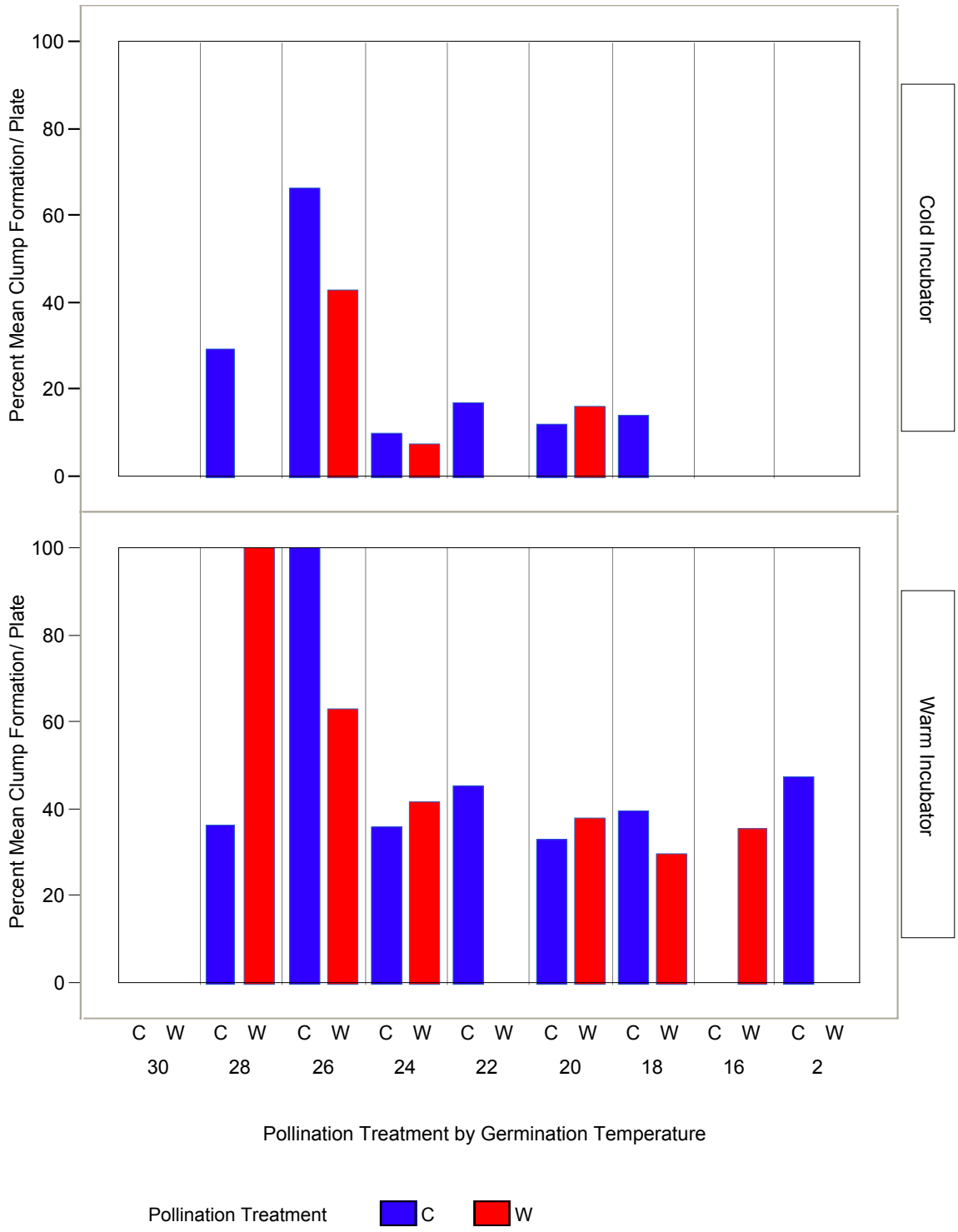

Figure 9: Percent of spontaneous clump formation from protocorms of seeds derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator. Clump formation was evaluated 125 days after initial plating. (Germination temperature = ^{o}C)

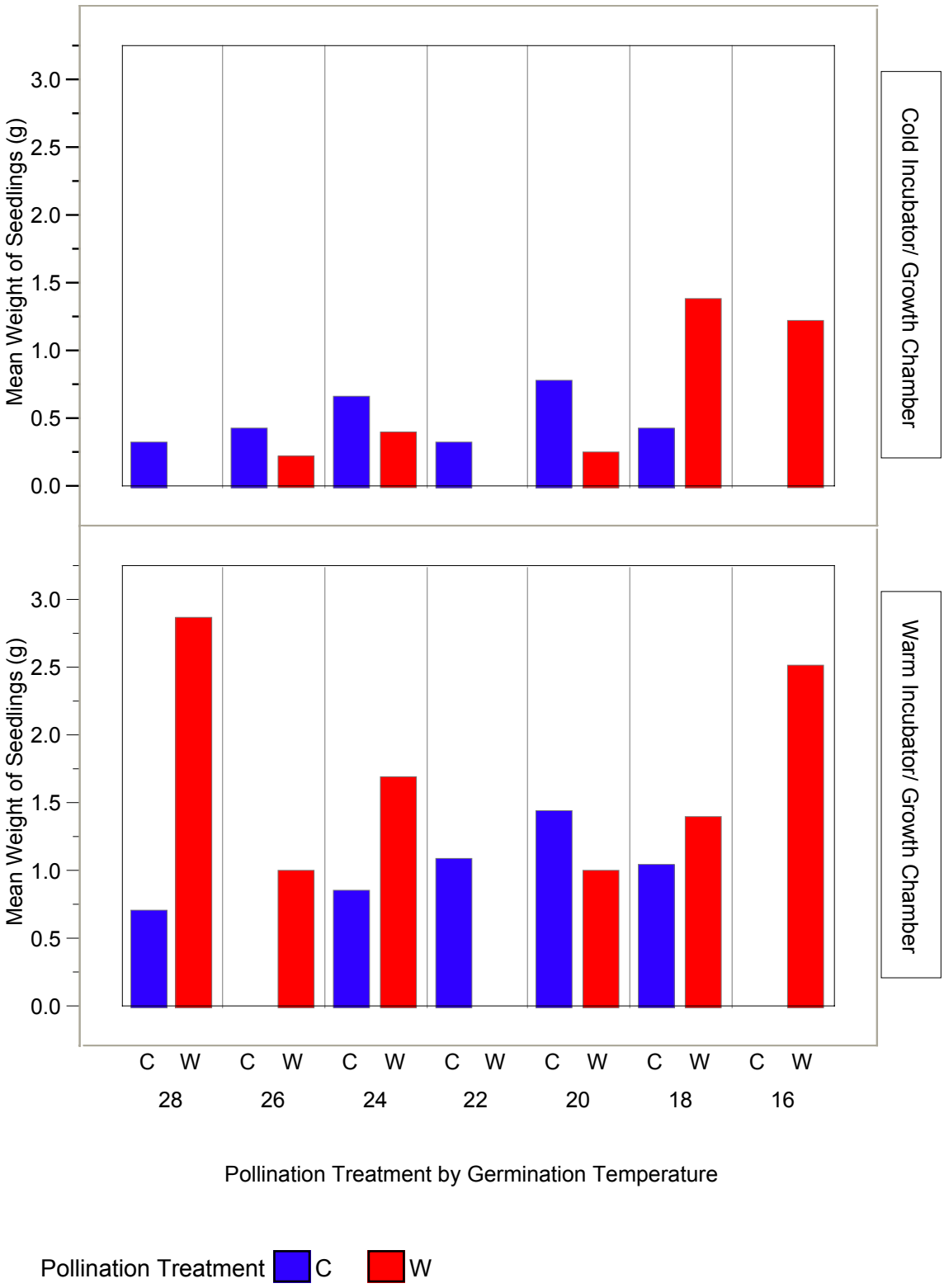

Figure 10: Comparison of the mean wet weight (g) of seedlings 1 year after initial plating. Seedlings were derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator/ growth chamber. Germination temperature is in ^{o}C.

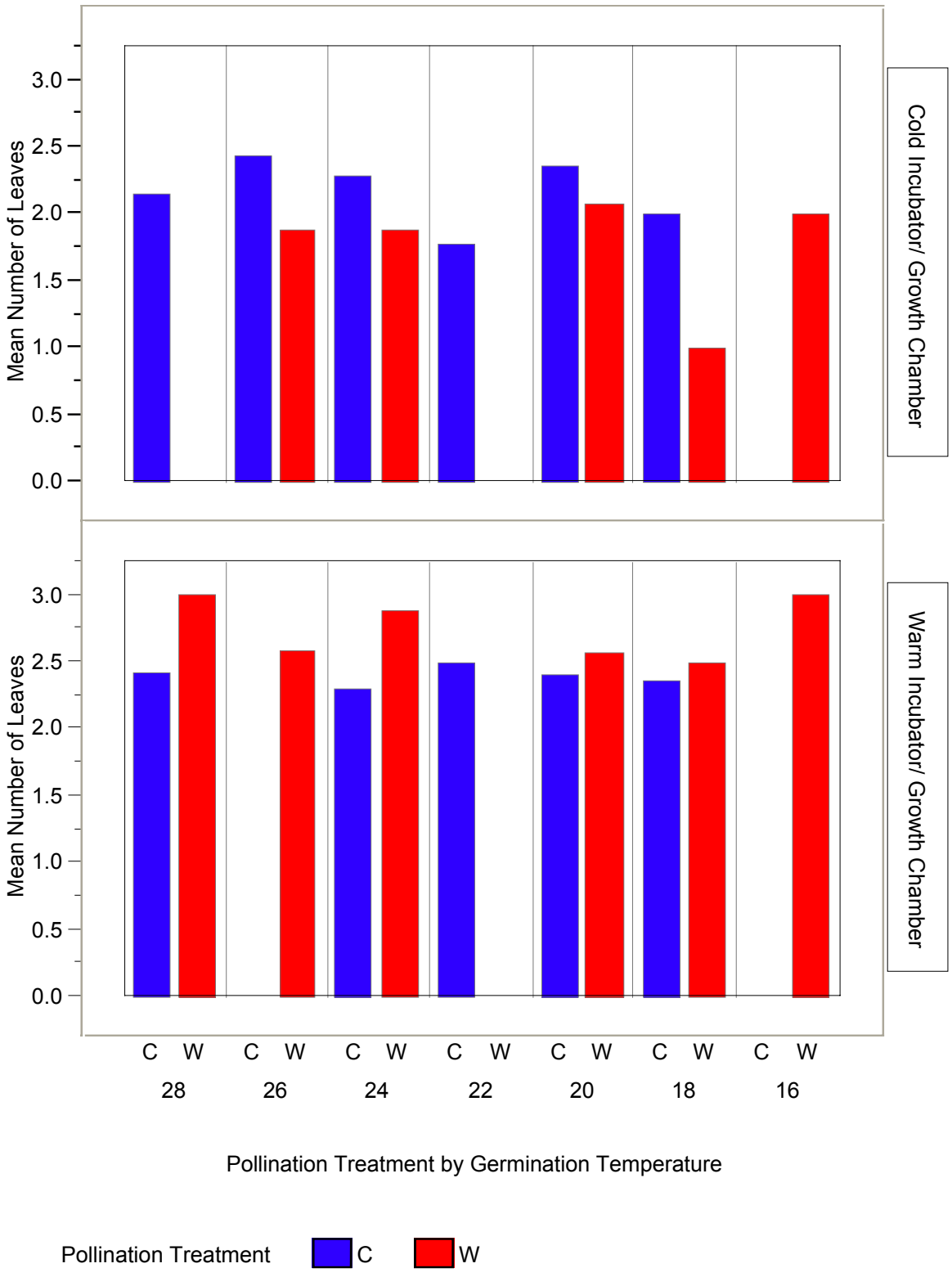

Figure 11: Comparison of the mean leaf number of seedlings measured 1 year after initial plating. Seedlings were derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator/ growth chamber. Germination temperature is in ^{o}C.

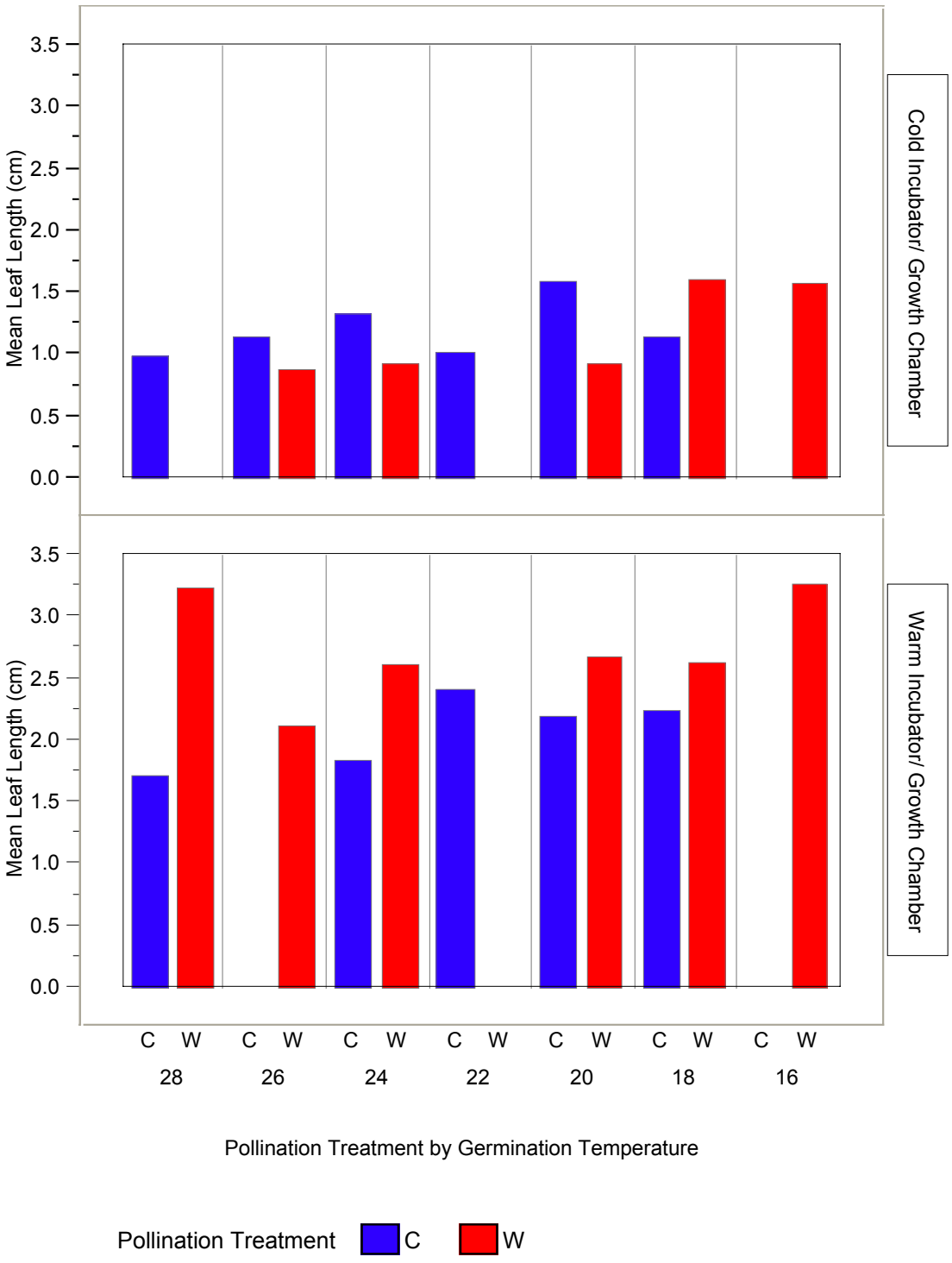

Figure 12: Comparison of the mean leaf length (cm) of seedlings measured 1 year after initial plating. Seedlings were derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator/ growth chamber. Germination temperature is in ^{o}C.

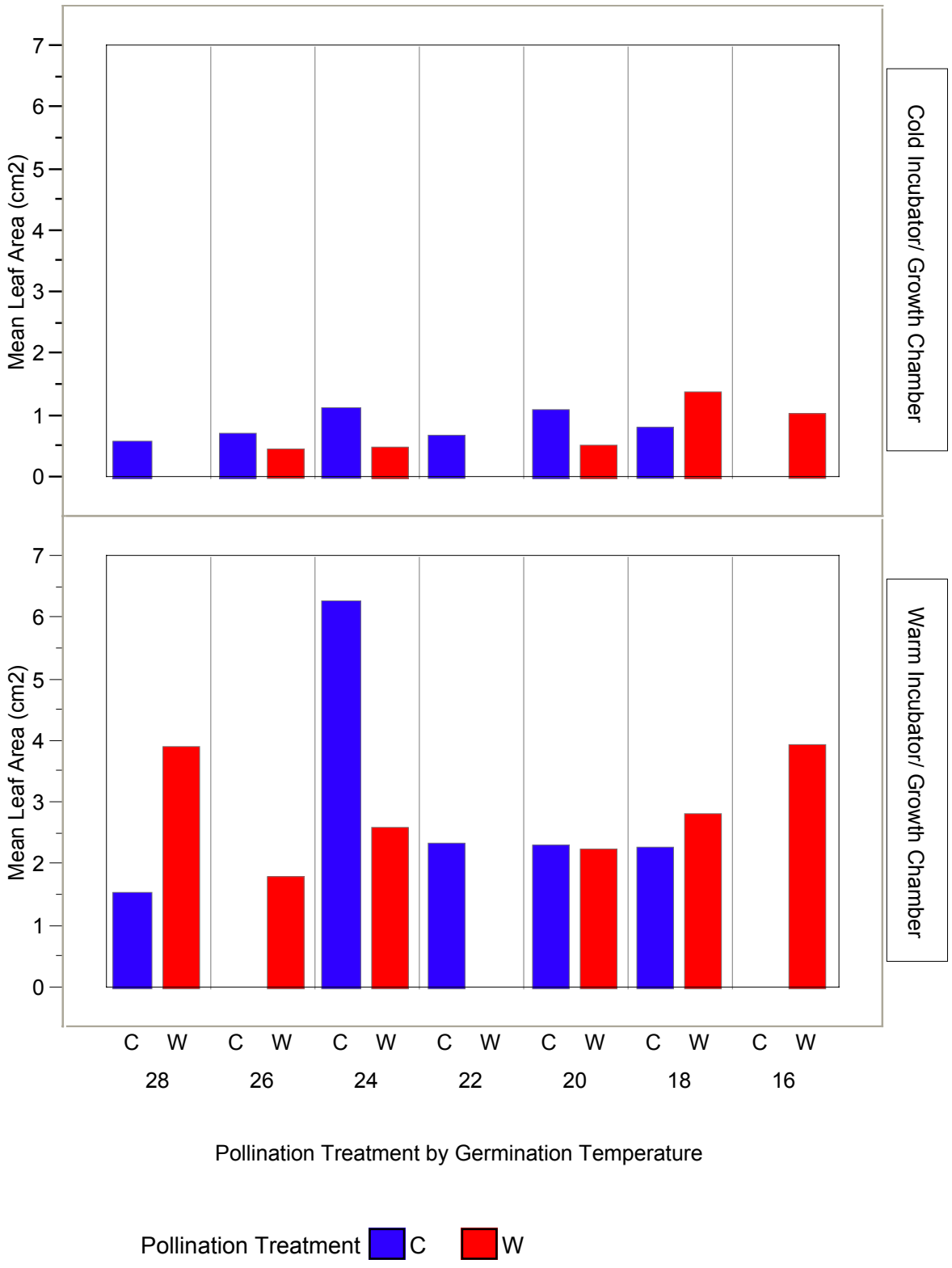

Figure 13: Comparison of mean leaf area (cm^2) of seedlings measured 1 year after initial plating. Seedlings were derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator/ growth chamber. Table position indicates germination temperature in ^{o}C.

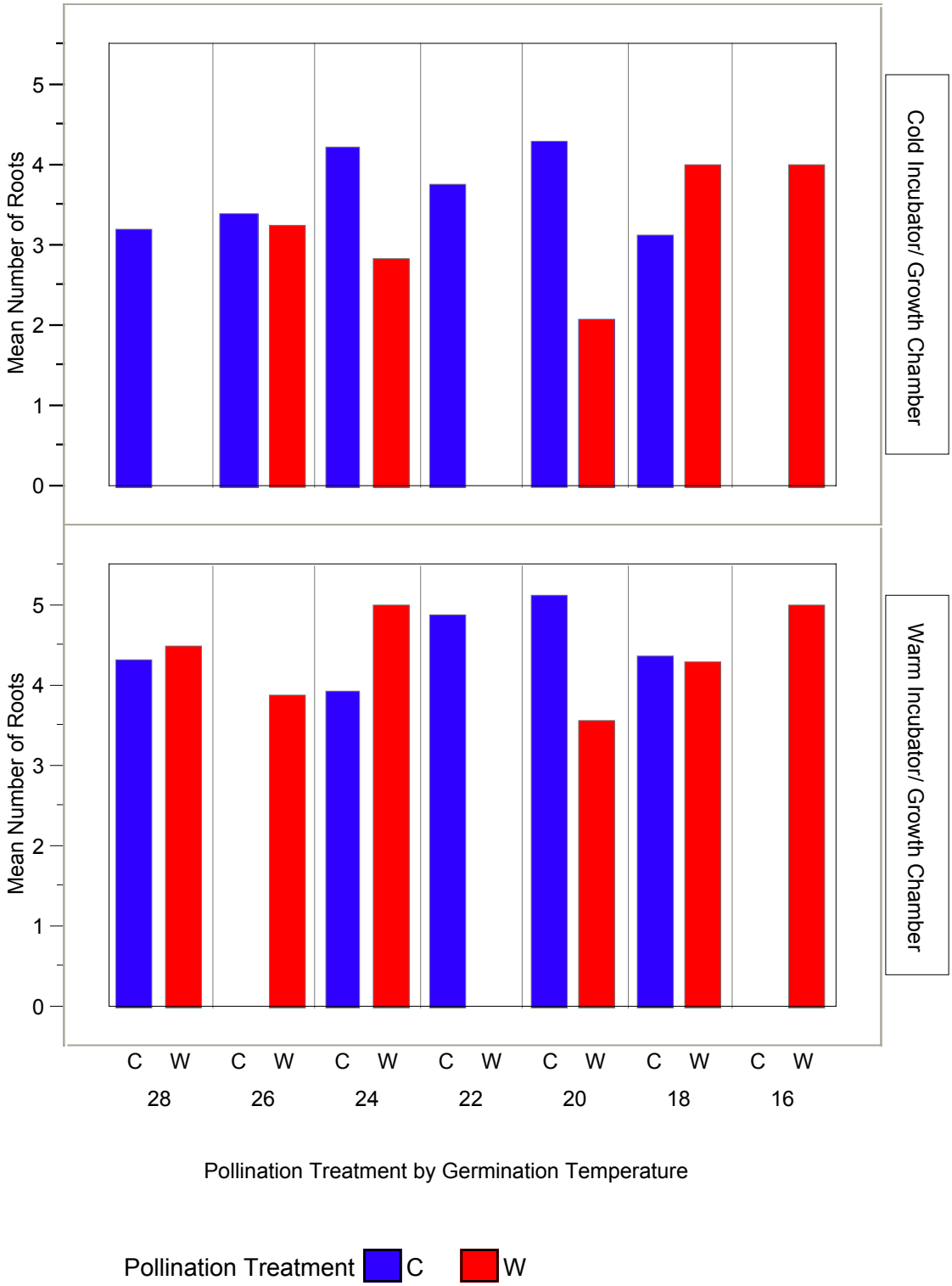

Figure 14: Comparison of the mean root number of seedlings measured 1 year after initial plating. Seedlings were derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator/ growth chamber. Germination temperature is in ^{o}C.

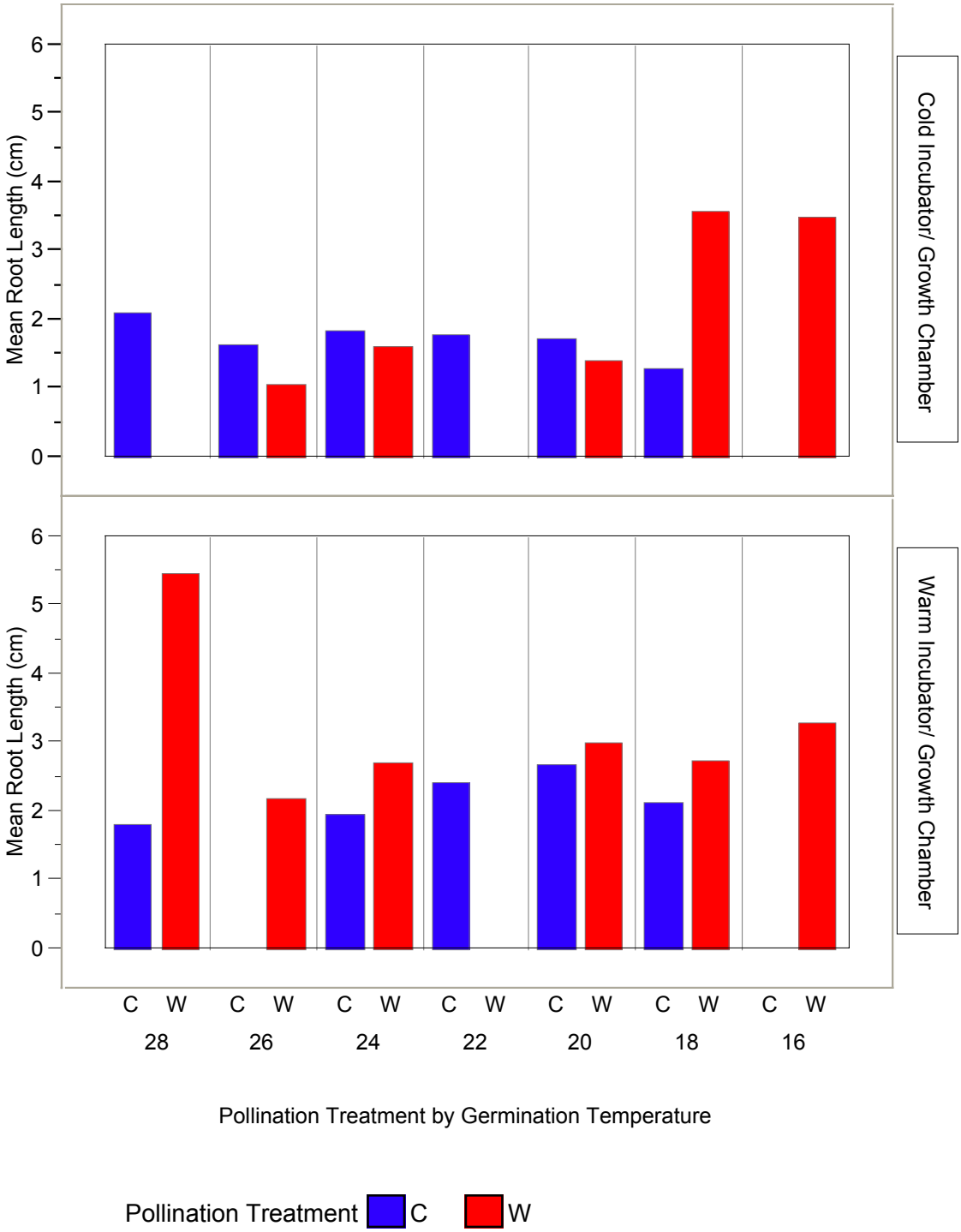

Figure 15: Comparison of the mean root length (cm) of seedlings measured 1 year after initial plating. Seedlings were derived from the same *Phalaenopsis* hybridization but pollinated under cold (C) or warm (W) conditions until evidence of fruit set was obvious and then germinated under different temperatures and incubated in either a cold or a warm incubator/growth chamber. Germination temperature is in ^{o}C.

Vita

Leslie A. Blischak
Department of Horticulture
Virginia Polytechnic Institute & State University
Blacksburg, VA 24061
Email: lblischa@vt.edu

EDUCATION

VIRGINIA POLYTECHNIC INSTITUTE & STATE UNIVERSITY, Blacksburg, Virginia
M.S. in Horticulture, expected degree completion July, 2005

VIRGINIA POLYTECHNIC INSTITUTE & STATE UNIVERSITY, Blacksburg, Virginia
B.S., Horticulture Science, Minor: Biology and German – August, 1998 - December 2001

EXPERIENCE

VIRGINIA BIOINFORMATICS INSTITUTE

April 2005 - June 2005

Lab Assistant
Continued work on strawberry genomics project. Duties include screening of T1 seedlings for positive GFP expression and mutations, as well as transplantation, and transfer to greenhouse facilities. Supervise one undergraduate research assistant.

INSTITUTE of ADVANCED LEARNING and RESEARCH (IALR), Danville, VA

September 2004 – January 2005

Research Assistant at VPI and SU
Prepared protocols for the development of orchids as a high value horticulture crop for the IALR and the Virginia Tech Horticulture Department cooperative venture in order to invigorate Southside Virginia's economy. Trained IALR Lab Technician in orchid tissue culture technique. Developed plant material for transfer to new lab facility in Danville, VA. Cultivated future customers of lab services by providing services from facilities at Virginia Tech.

VIRGINIA BIOINFORMATICS INSTITUTE, Blacksburg, VA

May 2004 - August 2004

Lab Assistant
Worked on the strawberry genomics project. Proliferated transgenic diploid strawberry callus, selected GFP positive callus, as well as transferred and maintained callus until shoot and root development. Transferred regenerated transgenic strawberries to growth chambers as well as maintained lab facilities.

GRADUATE TEACHING ASSISTANT, VPI & SU, Blacksburg, VA

August 2003 - May 2004

Indoor Plants (Hort 2144)
Prepared and presented all lectures for the 9-10:45 am section. Conducted lab exercises. Assisted in composition of the multiple choice portion of exam as well as individually prepared short answer portion of exam.

GRADUATE TEACHING ASSISTANT, VPI & SU, Blacksburg, VA

January 2003- May 2003

Plant Tissue Culture (Hort 5404)
Prepared media, plant materials and equipment for lab portion of class. Developed lab procedure using orchids for classroom use.

PLANT PATHOLOGY LAB, VPI & SU, Blacksburg, VA

September 2000 - December 2000

Undergraduate Lab Assistant
Used laboratory equipment to make media, tubes, stains, stock solutions, and plate plant pathogens. Maintained plant pathogen inventory. Responsible for greenhouse, and completed research project on a

possible biological control of Buttonweed (*Diodea virginiana*).

May 2000 - August 2000

SMITHSONIAN INSTITUTE, Washington, D.C.

Interior Plant Program Intern, Horticulture Department

Worked on Orchid exhibit, cataloged Bromeliad collection, and created database on specimen plants to be used for public education. Also performed general plant care and green house management.

PRESENTATIONS to Professional Audiences

- Presented research poster at the American Society of Horticultural Science's 2004 National Conference in Austin, TX.
- Presented seminar to VPI & SU Horticulture Department
- Presented research poster at the 21st Annual Graduate Research Symposium at VPI & SU
- Scheduled for oral presentation at the American Society of Horticultural Science's 2005 National Conference in Las Vegas, NV

ACCOMPLISHMENTS

- Horticulture Graduate Student Association President
- Horticulture Department Representative to Graduate Honor System
- Graduate Student Representative to Horticulture Faculty
- Member of Gamma Beta Phi Honor Society
- Member of Phi Kappa Phi Honor Society
- Virginia 4-H All-star