

ABSTRACT

GU, YU. Cut Flower Production and Economic Analysis, Polyploidy Induction in Two Zinnia Cultivars, Zinnia Pollination Mechanisms, and DNA Content of Zinnia Species. (Under the direction of Julia L. Kornegay.)

Cut flower production is a small, but growing, segment of the North Carolina floriculture industries. In summer 2013, two field trials were conducted to help growers make informed decisions about their production practices. In the first study, two cut flower cultivars, zinnia Benary Giant 'Scarlet' and sunflower 'Procut', were evaluated for stem yield (number of stems harvested) and flower quality (stem length and flower diameter) under two planting methods (direct-seeded vs. transplanted) and plant densities (16 vs. 32 plants/plot). In 'Scarlet', stem yield was significantly higher at the higher plant density, but there was no significant effect of planting density and planting method on flower quality. Significantly greater severity of powdery mildew (*Erysiphe cichoracearum*) was observed on zinnia at the higher plant density. Direct-seeded sunflower 'Pro Cut' had significantly longer stem lengths and larger flower diameters compared to the transplanted plants. In this study, the fixed costs of direct-seeded cut flower crops were higher than those of transplanted crops because only limited greenhouse space was used in producing the transplants. Growers are recommended to direct seed zinnia at the higher plant density for increased yield, but for disease management, lower densities can reduce the severity of powdery mildew. In 'Procut' sunflowers, planting method and density can be used to control flower size and stem length depending on market demands.

In the second field study, season-long vs. three successional plantings (mid-season, late-season, and mid- and late-season) of two zinnia cultivars ('Scarlet' and 'Zowie') were evaluated for stem yield and flower quality. Season-long plantings produced greater total stem yields in both 'Scarlet' and 'Zowie', but flower quality of 'Scarlet' was reduced in the season-long planting compared to succession plantings, while no effect on flower quality was observed in 'Zowie', which has small diameter flowers and shorter stems. Lower levels of disease were found in the later succession plantings. Fixed costs increased consistently with succession plantings. Growers are recommended to use one to two successional plantings of zinnia to maintain flower quality and to foliar disease incidence.

Tetraploids were induced in seedlings of *Zinnia violacea* 'Oklahoma White' and *Z. angustifolia* 'Crystal Orange' using oryzalin applied to seedling apical growing points. Oryzalin was an effective mitotic inducer in 'Oklahoma White' when double applications were applied at 50 μ M. Oryzalin was less effective in inducing polyploids in 'Crystal Orange'. Colchicine was effective at inducing polyploids using a 0.33% solution. No correlations were found between the leaf stomata guard cell lengths and 2C DNA values for treated seedlings.

Pollination mechanisms (self-incompatible, partially incompatible, and compatible) were evaluated for 16 cultivars of *Zinnia* from the two subgenera *Diplothrix* and *Zinnia*, and from the two sections *Mendezia* and *Zinnia* in the subgenus *Zinnia*. All species from subgenus *Diplothrix* appeared to be self-incompatible. In section *Mendezia*, *Z. greggii* and *Z. maritima* also did not produced fertile seeds from self-pollination. Other species from section *Zinnia* were partially incompatible, with the exception of *Z. peruviana*, which was

confirmed as a compatible species. The genome size (2C DNA) of cultivars in sections *Mendezia* and *Zinnia*, as measured by flow cytometry analysis, were significantly different. Section *Mendezia* had comparatively smaller and consistent genome size (1.9 to 2.5 pg), while the DNA content in section *Zinnia* varied from 3.3 to 4.8 pg depending on the species and cultivars. *Zinnia tenuifolia* 'Red Spider' had very similar pg values to that of *Z. peruviana*. As the DNA content and other morphological traits of 'Red Spider' were nearly identical those of *Z. peruviana*, it is believed that 'Red Spider' is not a separate species, but is *Z. peruviana*.

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Cut Flower Production and Economic Analysis, Polyploidy Induction in Two Zinnia Cultivars,
Zinnia Pollination Mechanisms, and DNA Content of Zinnia Species

by
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BIOGRAPHY

Yu Gu was born in Dalian, Liaoning, a coastal city in northeast of China. Her interest in plants started in high school, when she was responsible for watering flowers in class. She liked taking care of the flowers and that became her first motivation to pursue a bachelor degree in horticulture science. In 2012, she earned her B.S. at Shenyang Agriculture University under the direction of Dr. Xueling Ye, where she had a chance to take courses in biology, genomics, breeding, plant pathology, and agriculture. She was also fortunate to have some practical experiences like building a high tunnel, and harvesting fruits and vegetables. These experiences aroused her passion in plants and helped her to develop a more comprehensive understanding of horticulture. During her senior year, she had an opportunity to work at a commercial orchid greenhouse where she enjoyed working with flowers. In Fall 2012, she joined the graduate program at North Carolina State University, where she began further research in floriculture under the director of Dr. Julia L. Kornegay.

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TABLE OF CONTENTS

LIST OF TABLES	v
LIST OF FIGURES	vi
CHAPTER 1. LITERATURE REVIEW	1
Cut flower field production.....	1
<i>Zinnia</i>	3
<i>Zinnia</i> germplasm resources.....	4
<i>Zinnia</i> diseases	7
Breeding for disease resistance in <i>Zinnia</i>	9
Self-incompatibility in <i>Zinnia</i>	9
Interspecific compatibility	11
Ploidy induction in <i>Zinnia</i>	11
Flow cytometry.....	13
Summary.....	14
References.....	16
CHAPTER 2. PRODUCTION AND COST ANALYSIS OF DIRECT-SEEDED VS. TRANSPLANTED AND SEASON-LONG VS. SUCCESSION PLANTINGS OF ZINNIA AND OTHER CUT FLOWER FIELD CROPS	21
Abstract.....	21
Introduction.....	22
Materials and Methods.....	24
Results.....	31
Discussion.....	37
References.....	41
CHAPTER 3. POLYPLOIDY INDUCTION IN TWO ZINNIA SPECIES	48
Abstract	48
Introduction.....	48
Materials and Methods.....	50
Results.....	53
Discussion.....	55
References	57
CHAPTER 4. POLLINATION MECHANISMS AND DNA CONTENT OF ZINNIA SPECIES	62
Abstract.....	62
Introduction.....	62
Materials and Methods.....	65
Results.....	68
Discussion.....	69
References.....	71
APPENDIX	75

LIST OF TABLES

Table 2.1	Mean stem length, flower diameter, total harvested stem number, and disease severity of directed-seeded vs. transplanted cut flower crops in two planting densities at HFL, Raleigh and MHCREC, Mills River, NC.....	42
Table 2.2	Mean stem length, flower diameter, and total harvested stem number of season-long vs. succession plantings of two zinnia cultivar grown at HFL, Raleigh and MHCREC, Mills River, NC	43
Table 2.3	Disease severity on two zinnias cultivars planted at three dates at HFL, Raleigh	44
Table 2.4	Cost analysis (Weddington, 2003) of direct-seeded vs. transplanted zinnia at two planting densities (Study 1) and in season-long vs. succession plantings (Study 2) at HFL, Raleigh, NC	45
Table 3.1	Effect of oryzalin and colchicine treatments on polyploid induction and stomatal guard cell length in 'Oklahoma White'	59
Table 3.2	Effect of oryzalin and colchicine treatments on polyploid induction and stomatal guard cell length in 'Crystal Orange'	60
Table 4.1	Results of self- and cross-pollinations and pollen viability test of 16 zinnia cultivars across 12 cultivars.....	73
Table 4.2	DNA content as determined by flow cytometry of 13 <i>Zinnia</i> cultivars across eight species	74

LIST OF FIGURES

Figure 2.1	Groups of season-long and succession plantings at HFL	46
Figure 2.2	Average stem length (cm) of 'Scarlet' in season-long (S-Long), mid-season (Mid-S), late season (Late-S), mid- and late-season (Mid+Late-S) succession plantings at HFL.....	47
Figure 2.3	Average flower diameter (cm) of 'Scarlet' in season-long (S-Long), mid-season (Mid-S), late season (Late-S), mid and late season (Mid+Late-S) succession plantings at HFL	47
Figure 3.1	Relative nuclear DNA content for 'Oklahoma White' and 'Crystal Orange'	61

CHAPTER 1

LITERATURE REVIEW

Cut flower field production

The wholesale value of cut flowers from the top 15 producing states in the United States was \$359 million in 2011 (USDA, 2012). Over 80% of U.S. cut flower production is in specialty cut flowers (Bachmann, 2006). Specialty cut flowers can be defined as flowers that are only available seasonally or for a short time period during the year (Armitage and Laushman, 2003).

In the U.S., three methods of production are used: open field, high tunnel and greenhouses (Whipker and Cavins, 2000; Weddington, 2003). Although some studies have shown that high tunnel production of some specialty cut flowers can result in greater yield and longer stem length of cut flowers due to reduced air movement and irradiance (Ortiz et al., 2012), a recent survey of the NC cut flower industry showed that cut flower growers produce approximately 82% of their crops in open fields and only 6% in high tunnels (Granitz, 2014). For farmers transitioning into cut flower crops, field production start-up costs are generally very low (Whipker and Cavins, 2000). Granitz (2014) found that some NC growers may grow up to 40 cultivars and 23 species of cut flowers, and that annuals comprise 55% of all crops grown, followed by bulbs (20%), herbaceous perennials (14%) and woody perennials (8%).

Because of the wide diversity in crops, cultivars, and production practices, limited research has been done to compare production methods and their effect on cut flower

stem yield, stem length, and flower diameter. Armitage (1987) found that the number of stems in each square meter increased with decreased spacing among five cut flower species. Khazaie et al. (2008) found that thyme (*Thymus vulgaris*) plant biomass and oil production were lower at high planting densities, while hyssop (*Hyssopus officinalis*) plants did not have a significant biomass or oil production change in different densities. Green et al. (2010) reported that there was no significant differences of planting density on yield or stem length on two globe amaranth (*Gomphrena*) species, but that planting density did affect stem yield of two celosia (*Celosia*) species. Research on zinnia planting densities found that closer spacing produced more stems per square foot of production than wider spacing (Stevens et al., 1993).

In a survey of NC cut flower growers, Granitz (2014) found that zinnias, sunflowers, and celosia were grown by 90%, 87%, and 69% of the respondents, respectively. Cut flower zinnias have a long production period and production that can range from early-to-mid summer to the first frost. Schoellhorn et al. (2003) recommended that zinnia be direct seeded in the field, but some growers prefer to plant transplants because they come into flower sooner (Anderson and Schnelle, 2010). For some medium and large flowered zinnias, two or more succession plantings are recommended (Stevens et al., 1993), and that medium flower size cultivars such as 'Pumila', 'Ruffles' and 'Cut-N-Come Again' be planted twice, two to three weeks apart. For large flowered cultivars, like 'State Fair', several successional plantings were recommended, beginning two weeks after the first planting, with each additional plantings a week apart (Stevens et al., 1993).

Zinnia

Zinnia is a member of the *Asteraceae* family and consists of 17 species of annual and perennial herbs or low shrubs that are endemic to scrub and dry grasslands in an area stretching from North to South America (Torres, 1963; Metcalf and Sharma, 1971). *Zinnia violacea* Cav. (syn. *elegans* Jacq.) is the most widely cultivated and one of the most popular garden flowers worldwide due to its wide adaptability, quick flowering, and diverse forms in terms of plant size, shapes, and flower colors. A recent survey by Granitz (2014) found that cut flower zinnias (*Z. violacea*) are grown by over 90% of specialty cut flower growers in North Carolina.

Diseases often reduce the quantity and quality of cut flower zinnias and may cause significant plant mortality. Powdery mildew (*Erysiphe cichoracearum*), bacterial leaf and flower spot (*Xanthomonas campestris* pv. *zinnia*), and alternaria leaf blight (*Alternaria zinnia*) are recurring constraints of cut flower zinnias under both field and greenhouse conditions (Gombert et al, 2001; Hagan, 2009). High levels of disease resistance have been identified in some zinnia species, but only a few species have been successfully utilized by breeders to develop new cultivars. Interspecific crosses between *Z. violacea* and *Z. angustifolia* resulted in a new species of bedding plant, *Z. marylandica*, and cultivars that are highly resistant to powdery mildew (Boyle and Stimart, 1982). However, interspecific crosses have not been successful in the development of cut flower zinnias with long stems, large showy flowers, and disease resistance. Further research is required to better utilize zinnia germplasm in interspecific breeding for the improvement of cut flower types.

Zinnia germplasm resources

Metcalf and Sharma (1971) reviewed the systematics of the genus *Zinnia* and showed that *Zinnia* L. (1759) is comprised of 17 species of annual or perennial herbs or low shrubs. Torres (1963) divided the genus *Zinnia* into subgenera *Diplothrix* and *Zinnia*; furthermore, he divided the subgenus *Zinnia* into sections *Mendezia* and *Zinnia* based on morphological differences.

The distribution of subgenus *Diplothrix* is mainly in the northern part of Mexico, parts of Arizona, and a large area of New Mexico and the eastern part of Texas (Torres, 1963). The species in this subgenus have mat forming, shrub-like growth habits; tiny, linear leaves; and small orange, yellow or white flowers with rays ranging from 9-18 mm long. They prefer growing on shifting dunes, bare rocky slopes, well-drained roadcuts and disturbed habitats in drier environments. The base chromosome number is primarily $n=10$ and both diploid and polyploidy races exist in this subgenus (Torres, 1963; Metcalf and Sharma, 1971). *Zinnia acerosa*, *Z. juniperifolia*, and *Z. oligantha* are primarily diploids, while *Z. acerosa* includes three other chromosome races, $n=11$, 19 and 20, which are morphologically indistinguishable (Metcalf and Sharma, 1971). The tetraploid species within *Diplothrix* include *Z. citrea* ($n=20$), *Z. grandiflora* ($n=21$), and races of *Z. acerosa* ($n=20$). It is thought that the *Z. acerosa* race $n=20$ may be an allotetraploid between *Z. acerosa* ($n=10$) and *Z. oligantha* ($n=10$). The only octoploid species is *Z. anomala* ($n=\pm 42$), which has small ray florets or none at all (Metcalf and Sharma, 1971). Torres (1963) believed that *Z. anomala* is a derivative of *Z. grandiflora*.

Within *Diplothrix*, only *Z. grandiflora* (Lady Bird Wildflower Center Native Plant Database) is grown commercially. Torres (1962) attempted interspecific hybridizations among *Diplothrix* species, but only few produced viable seedlings. Although there is no report that this subgenus has been used in interspecific crosses with the subgenus *Zinnia*, these species may be of interest as sources of disease resistance and drought tolerance in commercial zinnia breeding programs.

The subgenus *Zinnia* (Torres, 1963; Metcalf and Sharma, 1971) is comprised of 11 species, primarily located in the warm temperate and tropical areas of North and Central America, and includes more horticultural forms of flower colors and types than *Diplothrix*. Torres (1963) recognized two sections in the subgenus *Zinnia*: *Mendezia* and *Zinnia*. Section *Mendezia* is mainly distributed in the western part of Mexico, while section *Zinnia* has a wide distribution from northern Mexico to Honduras. One species, *Z. peruviana*, is naturalized in South America and even found in Australia and Hawaii (Torres, 1963).

Section *Mendezia* includes eight species with the base chromosome number $n=11$, 12, 13 (Metcalf and Sharma, 1971). The species may be annual or perennial, erect or procumbent, with small flower heads (≤ 1 cm high and wide). Among these, *Z. angustifolia* ($n=11$) is the most widely grown and several cultivars ('Summer Solstice', 'Cascade Beauty', 'Crystal', 'Star', 'Starbright', and others) are commercially available in white, yellow or orange flower colors. Other species in the section *Mendezia* include *Z. maritima*. ($n=12$), *Z. greggii* ($n=11$), *Z. bicolor* ($n=11$), and others. Seeds of *Z. maritima* are commercially available as the cultivar 'Solcito'. The other species in this section are potential sources of disease

resistance and other traits, and interspecific crossings may be possible as these species are more closely related to *Z. violacea* genetically.

In section *Zinnia*, the flower heads are generally more showy and broad than those of section *Mendezia* with a diversity of ray floret colors and petal forms. Plants are more erect in height, sparsely-branched, with large, ovate to lanceolate leaves. Cultivated forms may have one to several whorls of ray florets. Section *Zinnia* includes three horticulturally important species with the base chromosome number $n=12$: *Z. violacea*, *Z. haageana*, and *Z. peruviana* (Torres, 1963; Metcalf and Sharma, 1971; Terry-Lewandowski et al., 1984). The most widely cultivated zinnia, *Z. violacea*, was introduced to cultivation in Europe in 1796 (Coats, 1968; Metcalf and Sharma, 1971). With its varicolored rays and large showy flower heads, it is the most popular cultivated species and is naturalized in parts of Florida, Central America and South America, and Australia (Torres, 1963). Although there are many cultivars of *Z. violacea* commercially available, most have been derived from a very limited germplasm base (Metcalf and Sharma, 1971). 'State Fair' ($n=24$) is the only tetraploid cultivar that is commercially available. *Zinnia haageana* is the second most important species in section *Zinnia*, with bright orange florets above and dull orange to yellow below (Metcalf and Sharma, 1971). A number of improved cultivars with variegated ray flowers are commercially available, such as 'Persian Carpet', 'Aztec Sunset', and 'Chippendale'. 'Old Mexico' is the only known tetraploid form ($n=24$) of this species (Metcalf and Sharma, 1971). *Zinnia peruviana* has small scarlet, red or yellow ray flowers, and an upright plant growth habit. It is reported to be self-compatible and seeds freely (Torres, 1963). This

species has limited horticultural application (Metcalf and Sharma, 1971). One commercially available cultivar, known as *Z. tenuifolia* 'Red Spider' is not included in the taxonomic studies of Torres (1963) and Metcalf and Sharma (1971), but is morphologically similar to *Z. peruviana*. An accession from the Ornamental Plant Germplasm Center (OPGC) at Ohio State University was listed as *Z. species* 'Red Button', but it has not yet been identified taxonomically.

Zinnia diseases

Zinnias are known to be susceptible mainly to three main diseases: powdery mildew (*Erysiphe cichoracearum*); bacterial leaf spot (*Xanthomonas campestris* pv. *zinnia*); and alternaria blight (*Alternaria zinnia*). Powdery mildew is the most common disease of zinnia. It causes circular white powdery spots on the leaves and, less frequently, on the stems. The symptoms usually appear on the upper surface of the leaves during hot, humid weather, and under high disease pressure, the leaves will turn brown and shrivel (Hagan, 2009). The flowers can also be infected. Gombert et al. (2001) evaluated the disease response of 57 cultivars of *Z. violacea* and found that all were intermediate to fully susceptible to powdery mildew. Torres (1963) also observed that the species in section *Zinnia* are all susceptible to powdery mildew. High levels of resistance to powdery mildew have been identified in *Z. angustifolia*. The cultivars 'Crystal' and 'Star' are known to be highly resistant, even immune, to this disease (Hagan, 2009). *Zinnia* interspecific cultivars such as the 'Profusion' and 'Zahara' series, resulting from the creation of the allotetraploid *Z. marylandica* (*Z. angustifolia* x *Z. violacea*) (Spooner et al., 1991), are highly resistant to powdery mildew

(Hagan, 2009). Fungicides can be used to control powdery mildew when applied at the start of symptom onset, but they may need to be reapplied several times during the growing season (Hagan, 2009).

Bacterial leaf and flower spot causes angular reddish-brown, necrotic leaf spots surrounded by chlorotic halos (Hagan, 2009). Like powdery mildew, the disease starts at the bottom of the leaves and spread up the plant and to the flowers (Hagan, 2009). Heavily diseased leaves will wither and die. The pathogen is spread by rain splashing and overhead irrigation, and can be transmitted through infected seed (Gombert et al., 2001; Hagan, 2009). *Zinnia angustifolia* 'Crystal' and 'Star' series were reported to be highly resistant to immune to bacterial leaf and flower spot (Hagen, 2009), but *Z. angustifolia* 'Pixie Sunshine' was susceptible (Hagan, 2009). The interspecific *Z. marylandica* cultivars also have good levels of resistance, but some spotting on leaves of the lower canopy and occasional leaf death has been observed in the 'Profusion' series (Hagan, 2009). Most *Z. violacea* cultivars are susceptible to the disease, although a few cultivars have expressed intermediate levels of resistance (Gombert et al., 2001). Hagen (2009) reported that *Z. haageana* 'Persian Carpet' and *Z. tenuifolia* 'Red Spider' were both susceptible. No effective chemical control of bacterial leaf and flower spot has been demonstrated in field trials, although copper-containing fungicides/bactericides may provide some protection if applied weekly when the spots first appear (Hagen, 2009)

Alternaria blight causes reddish brown to purple margin spots with a white color center on foliage and flowers (Hagan, 2009). In temperate regions, it generally develops in

late summer and early fall and is favored by overhead watering or frequent rains (Hagan, 2009). *Zinnia angustifolia* 'Crystal' and 'Star' series and *Z. marylandica* 'Profusion' series are highly resistant to alternaria blight. All cultivars of *Z. violacea* are moderately to highly susceptible to this disease. There are effective fungicides that can control alternaria blight, but they need to be reapplied weekly (Hagan, 2009).

Breeding for disease resistance in *zinnia*

Efforts to develop disease resistant cut flower zinnias with long stems and large flowers have not been successful due to the apparent lack of resistance genes in *Z. violacea* germplasm. Interspecific crosses between *Z. angustifolia* and *Z. violacea* resulted in development of fertile allotetraploid *Z. marylandica* with high levels of resistance to powdery mildew, bacterial leaf and flower spot, and Alternaria blight (Boyle and Stimart, 1982; Terry-Lewandowski and Stimart, 1985; Spooner et al., 1991; Hagen, 2009). However, the resulting cultivars had greater resemblance to *Z. angustifolia* than *Z. violacea* (Boyle and Stimart, 1982, 1989). Boyle (1996) found that the stem length for backcross hybrid *Z. marylandica* x autotetraploid *Z. violacea* ($n=24$) was more similar to *Z. violacea*, and had larger capitula and ray flowers than *Z. marylandica*. It is believed that two dominant genes for resistance to powdery mildew were inherited from *Z. angustifolia* and operated in both leaves and ray florets (Terry-Lewandowski and Stimart, 1984).

Self-incompatibility in *zinnia*

Pollination mechanisms are important to breeders since they are a prerequisite to effectively utilize germplasm in breeding programs. Self-incompatibility (SI) is a genetically

controlled mechanism that can prevent fertilization after self-pollination or matings between plants with the same incompatible phenotypes (Samaha et al., 1989). There are three methods currently used to determine SI: (1) embryo or seed counts; (2) visible light microscopy and stains such as acid fuchsin-light green on pollen tubes (Lewis, 1979) or aniline blue in lactophenol (Darlington and La Cour, 1962); and (3) UV epifluorescence microscopy of pollen tubes (Martin 1959; Heslop-Harrison et al., 1973).

Eleven of 17 species of *Zinnia* have been reported as self-incompatible (Pollard, 1939; Torres, 1962, 1963, 1964; Olorode, 1970; Boyle and Stimart, 1986). Torres (1962, 1964) conducted cross- and self-pollination tests as well as pollen staining, in an insect-proof greenhouse (without netting individual flower heads for the self-pollination tests) and reported that *Zinnia* species in the subgenus *Diplothrix* (*Z. juniperifolia*, *Z. acerosa*, *Z. citrea*, *Z. grandiflora*, *Z. oligantha*, and *Z. anomala*) were completely self-incompatible and produced no viable seed, but that their pollen was viable (ranged from 92% to 100%). Olorode (1970) reported that *Z. angustifolia* and *Z. greggii* were also self-incompatible based on selfing and crossing tests, and that the percentage of stainable pollen was 89.5% and 94.5%, respectively. Boyle and Stimart (1986) found that *Z. angustifolia* was largely self-incompatible, but that the intensity of SI varied among clones and lines, and that sporophytic control of SI was present in both *Z. violacea* and *Z. angustifolia*. Olorode (1970) and Pollard (1939) reported that *Z. violacea* to be self-incompatible based on seed set counts.

Interspecific compatibility

Boyle and Stimart (1982) showed that 13 cross combinations of *Z. angustifolia* × *Z. violacea* resulted in 47.2% viable embryos 14 days after reciprocal interspecific pollinations, while six cross combinations of *Z. violacea* × *Z. angustifolia* produced only 4.6% viable embryos. Boyle's 1996 research on the compatibility of backcross hybrids found a higher percentage of stainable pollen in backcrosses between *Z. marylandica* ($2n=46$) and autotetraploid *Z. angustifolia* ($2n=44$) and *Z. violacea* ($2n=48$) than with the diploid species, while crosses between *Z. marylandica* and *Z. angustifolia* ($2n=44$) had a higher percentage of stainable pollen than crosses between *Z. marylandica* and *Z. violacea* ($2n=48$). However BC_1 between *Z. marylandica* and autotetraploid *Z. violacea* may be a good source of bedding plants due to novel color combinations and large flower capitula.

Ploidy induction in *zinnia*

A polyploid plant is the one that has multiple sets of chromosomes and it is relatively common in plants. Harlan et al. (1975) introduced three types of polyploids: Class I polyploids occur in the first generation through the union of gametes with one or both of which are unreduced. Class II polyploids occur in the second generation when an offspring of a hybrid with meiotic disturbances lead to unreduced gametes. Class III polyploids occur when the chromosomes of somatic tissue doubles due to somatic mutation, which is a mitotic disturbance. Autopolyploids may be infertile due to multiple homologous chromosomes which could cause false pairing, unpaired chromosomes, and gametes with unbalanced chromosome numbers (Stebbins 1950; Parisod et al., 2010; Ranney, 2006).

Allopolyploids are the fusion of chromosomes from two species. In allopolyploids, the chromosomes from one species generally do not pair with another species during meiosis, but chromosomes pair with their homologous partner and results in fertile germ cells. As a result, allopolyploids are typically fertile or semi-fertile (Chen and Ni, 2006; Ranney, 2006)

Polyploidy is considered a major pathway for abrupt speciation and reproductive isolation in plants (Ramsey and Schemske, 1998; Wendel, 2000; Soltis et al., 2004) and that ploidy levels can influence crossability, fertility of hybrids, plant vigor, and gene expression (Ranney, 2006). Since allopolyploids can have a greater degree of heterozygosity than autopolyploids, this may contribute to greater heterosis. Ranney (2006) introduced five breeding advantages for polyploids: 1) overcoming barriers to hybridization, 2) developing sterile cultivars from invasive species, 3) restoring fertility in wide hybrids, 4) enhancing stress tolerance, and 5) enhancing plant vigor.

Colchicine (N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide) is a mitotic inhibitor that can prevent mitosis at the anaphase stage. Other mitotic inhibitors include the herbicides oryzalin (4-(Dipropylamino)-3,5-dinitrobenzenesulfonamide); trifluralin (2,6-Dinitro-N,N-dipropyl-4-(trifluoromethyl) aniline; amiprofos-methyl; and N₂O gas (Taylor et al., 1976; Van Tuyl et al., 1992; Bouvier et al., 1994). These mitotic inhibitors are now used as chromosome doubling agents.

Colchicine is the most commonly used chemical to induce polyploidy in plants and it has been successfully used to induce autotetraploids in *Z. violacea*, *Z. angustifolia*, and

allotetraploids in their interspecific hybrids (Gupta and Koak, 1976; Terry-Lewandowski et al., 1984; Boyle, 1996). Because of concerns about colchicine toxicity, oryzalin, with lower toxicity, effectiveness at lower concentrations, and tendency to produce plants without deformed tissue or abnormal growth, is being used by many researchers to induce chromosome doubling (Van Tuyl et al., 1992), especially in ornamental and woody species. Jones et al. (2008) successfully induced polyploidy in rhododendron seedlings with oryzalin. Contreras et al. (2012) were able to double chromosomes in three species of *Cupressaceae*. Oates et al. (2012) successfully induced tetraploids in rudbeckia. The importance and prevalence of polyploids is also evident in zinnias; however, there are no published reports on the use of oryzalin in zinnia polyploid induction.

Flow cytometry

There are many methods to verify polyploidy levels. Traditionally, these have included conducting cytological counts of chromosome numbers of leaf, root tip, and anther tissue (Ruzin, 1999); measurements of pollen size; counts of chloroplast numbers in guard cells (Solov'eva, 1990); and measurement of stomata guard cell lengths (Ranney, 2006).

Flow cytometry (Dolezel, 1991) is also used to determine ploidy levels. Flow cytometry was originally developed as a method for rapid counting and analysis in blood cells, but it has become a useful analytical tool in many areas of biological research, including the determination of DNA content in tissue. A flow cytometer is an instrument which analyzes optical parameters of particles in flow, moving with respect to the point of measures. The flow cytometry assay has some important advantages over chromosome

counting. It is convenient, rapid, and does not require dividing cells. It is also non-destructive and can detect mixoploids (the presence of two different ploidy levels one genome) (Anantasaran et al., 2007). Flow cytometry is now broadly used for determining ploidy levels in plant breeding. An analysis of *Hydrangea macrophylla* DNA content with flow cytometry (Jones et al., 2007) showed that the measurement of total nuclear DNA identified both diploids and triploids among the 75 cultivars tested in the study. Palmer et al. (2009) used flow cytometry in analysis of *Rudbeckia* species and hybrids.

Summary

Growers use a wide variety of field production practices to produce field grown cut flowers. Limited research has been done to determine the effect of these practices on stem yield, stem length, flower diameter, and other traits, as well as the cost of different production practices. Two field studies were conducted to compare planting methods and plant densities and season-long vs. succession plantings of cut flower crops that are commonly grown in North Carolina, with the aim of providing growers with information that may be used to increase the production and lower the costs of their farming practices.

Zinnia is an important cut flower crop in many areas, but the cut flower species *Z. violacea* is susceptible to diseases. Resistant cut flower zinnias should be achievable through interspecific breeding methods, as resistance to the primary diseases is available in other zinnias species. The purpose of this research was to evaluate zinnia species to determine their pollination mechanisms and DNA content using flow cytometry. Different

solutions and applications of oryzalin were evaluated as a means to obtain a safer method for chromosome doubling in interspecific zinnia hybrids than colchicine.

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CHAPTER TWO

PRODUCTION AND COST ANALYSIS OF DIRECT-SEEDED VS. TRANSPLANTED AND SEASON-LONG VS. SUCCESSION PLANTINGS OF ZINNIA AND OTHER CUT FLOWER FIELD CROPS.

Abstract

Cut flower production is a small, but growing, segment of the North Carolina floriculture industries; however, little research information is available to help growers make informed decisions about their production practices. Two studies were conducted at the Horticultural Field Lab (HFL) in Raleigh and the Mountain Horticultural Research and Extension Center (MHCREC) in Mills River, NC to determine the effect of production practices on flower quality (average stem length and flower diameter) and stem yield (total number of harvested stems).

Study one compared direct-seeded vs. transplanted specialty cut flower crops at two planting densities (16 or 32 plants per plot). No significant differences in stem length and flower diameter were observed in the direct-seeded vs. transplanted 'Scarlet', but higher stem yields were obtained at the higher plant density. However, at the higher plant density greater disease severity of powdery mildew (*Erysiphe cichoracearum*) was observed in direct-seeded plants. Direct-seeded 'Pro Cut' had significantly longer stem length and larger flower diameters compared to plants in the transplanted plots. At a second location, no differences were observed between planting densities and direct-seeded vs. transplanted

crops for stem length, flower diameter, or stem yield in 'Scarlet' or 'Pro Cut'. Fixed costs of the direct-seeded cut flower crops were higher than those of transplanted crops.

Study two evaluated two zinnia cultivars, 'Scarlet' and 'Zowie Yellow Flame' ('Zowie') in season-long (S-Long) vs. three succession plantings: mid-season (Mid-S), late-season (Late-S), and mid- plus late-season (Mid+Late-S). At MCHREC, zinnia 'Scarlet' and celosia 'Chief Fire' were planted. 'Scarlet' had the longest average stem length and largest flower diameter when two succession plantings (Mid+Late-S) were made, compared to the season-long (S-Long) planting at HFL. No differences in stem length and flower diameter were observed in 'Zowie' among plantings. Higher total stem yield for 'Scarlet' and 'Zowie' were obtained in the S-Long planting. Both zinnia cultivars had lower levels of powdery mildew and bacterial leaf and flower spot in the later succession plantings. Fixed costs increased consistently with succession plantings.

Introduction

North Carolina is the second largest producer of specialty cut flowers on the East Coast of the United States with over \$6.8 million in annual sales (USDA, 2013). Many small-scale farmers have added cut flower production to their on-going farming operations as an effective way to increase farm diversification and income (Whipker and Cavins, 2000; Granitz, 2014). Although studies have shown that some cut flower crops may produce greater stem numbers and longer stem lengths when grown in greenhouses and high tunnels (Ortiz et al., 2012), a recent survey of the NC cut flower industry showed that 82% of cut flower production was in open fields, with only 16% in greenhouses or high tunnels

(Granitz, 2014). Installation of field grown cut flowers generally requires little or no additional equipment on established farms as compared to the cost of building and operating greenhouses and high tunnels (Whipker and Cavins, 2000).

Granitz (2014) found that NC cut flower growers may produce up to 40 cultivars of cut flowers each year from over 75 different species. About 55% of the cut flower crops are annuals and nearly 40% of the acreage is direct seeded. The most commonly grown cut flower crops are zinnias (90% of growers), sunflowers (87%), and celosia (69%) (Granitz, 2014). Some growers prefer to direct seed their annual crops, while others may grow seedlings in the greenhouse to transplant into the field.

There have been few studies conducted on the effect of plant densities, planting practices (transplants vs. direct-seeded), and succession plantings on annual cut flower crop yield (stem number) and flower quality (stem length and flower diameter). Armitage (1987) found a linear relationship between perennial cut flower crop plant density and stem number per unit area. A positive effect of higher plant densities on stem yield in globe amaranth (*Gomphrena*), celosia (*Celosia*) and zinnia (*Zinnia*) have also been reported (Stevens et al., 1993; Green et al., 2010). Stevens et al. (1993) at Kansas State University (KSU) recommended that zinnias be started from transplants instead of direct-seeded to have an early harvest advantage. They noted, however, that growers generally prefer to direct seed their crops.

Succession planting (also called sequential cropping) is a planting method used by growers to increase crop availability during a growing season by following one crop with

another to make efficient use of space and time. Stevens et al. (1993) recommended that medium-size zinnia cultivars such as 'Pumila', 'Ruffles' and 'Cut-N-Come Again' may require two plantings, two to three weeks apart, to maintain consistent production throughout the growing season. For a larger flowered cultivar like 'State Fair', several succession plantings may be required, with the second planting made two weeks after the first, and each additional planting made a week apart. The later plantings were advantageous in having fewer diseases late in the season (Stevens et al., 1993).

The objectives of these studies were to evaluate the effects of planting practices (transplants vs. direct-seeded), plant density, and succession plantings on harvested stem number, stem length, flower diameter, disease severity, and production costs of zinnia and other cut flower crops at two research stations in NC. These results will benefit cut flower growers by helping them evaluate the impact of production practices on cut flower production.

Materials and Methods

Study one: Production and cost of production of directed-seeded vs. transplanted cut flower crops in two planting densities.

Summer field trials were conducted during 2013 at the NC State Horticulture Field Lab (HFL) in Raleigh and at the Mountain Horticultural Research and Extension Center (MHCREC) in Mills River, NC. Two specialty cut flower crops, zinnia 'Benary Giant Scarlet' ('Scarlet') and sunflower 'Pro Cut Orange' ('Pro Cut'), were evaluated. Seed for 'Scarlet' and 'Pro Cut' were sourced from Germania Seed Company, Chicago, IL.

At HFL, prior to planting, the field plots were amended with composted leaves to increase organic matter content. A split-split plot RCB design with five replications was used, with crops (zinnia or sunflower) as main plots, plant densities (16 or 32 plants per plot) as subplots, and planting methods (direct-seeded or transplanted) as sub-sub plots. Each plot was 1.2 m². Each plot was dibbled at either 15 or 30 cm apart for a density of 16 or 32 plants per plot, respectively. In the direct-seeded plots, two to three seeds of a cultivar were planted in each dibbled opening and covered with approximate 3 cm of soil. The plots were hand water as needed and the seedlings thinned to one seedling per space. At the fourth leaf stage, the zinnias were pinched back to encourage branching.

Seeds for transplant production were planted in 48 cell trays filled with Farfard[®] 4P Mix and placed in a greenhouse at 21°C to germinate on the same day as direct-seeding in the field. The seedlings were watered and fertilized with a constant liquid feed of J. R. Peters[®] 20N-4.4P-16.6K fertilizer. When the plants were at the fourth leaf stage, they were pinched back and moved outside under a 50% shade cloth for one week to hardening off. The seedlings were then transplanted into field into the dibbled plots at one of the two plant densities, and hand watered until established.

At seeding or transplanting, ammonium nitrate was applied at 3 g per 30 cm². The plots also received supplemental fertilization through the drip irrigation system with a liquid feed of 250 ppm 20N-10P-20K every other week. The plots were mulched with an inch layer of composted leaves and hand weeded as needed.

At MHCREC, the experimental design, plot size and planting procedures followed the

same protocols as at HFL. After plant establishment, the plants were fertigated twice during the growing season. Due to the cool and wet growing conditions during the growing season, no additional irrigations were applied (Appendix 1). Weeds were controlled by hand weeding; no mulch was applied to the plots.

At HFL and MHCREC, the flowers were cut at the first branching node when the flowers were fully open in the field. Flowers having a minimum stem length of 12 cm or longer were cut and counted three days a week and stem number recorded. Flowers less than 12 cm in length were discarded. Stem length of all cut flowers and flower diameter of five sampled flowers were recorded once a week. Because of the environmental conditions in MHCREC, direct-seeded zinnia 'Scarlet' and sunflower 'Pro Cut Orange' had low germination rates and uneven plant stands. As a result, only the data from 'Scarlet' and 'Pro Cut' transplanted plots at two densities were collected.

Estimations of average disease severity per plot were conducted four times during growing season at HFL using a disease severity rating of 1 to 9, where 1 represents plants with no disease and 9 represents plants that were severely diseased or dead. The primary diseases present in at HFL were powdery mildew (PM) (*Erysiphe cichoracearum*) and bacterial leaf and flower spot (BLFS) (*Xanthomonas campestris* pv. *zinniae*).

Mean stem length and flower diameter, and total stem number (yield), were calculated for each plot. The HFL data was analyzed using analysis of variance (SAS Institute, Cary, NC) for a RCB split-split plot design and means separation across treatments was

conducted using PROC GLM. At the MHCREC, the data from the transplanted plots at two densities was analyzed as a RCB design with one treatment effect.

The fixed cost of zinnia cut flower production was calculated using the retail space adjusted method developed by Weddington (2003), based on financial data obtained from an economic survey of floricultural firms in NC and the southeastern United States. Expenses were categorized as either fixed or variable. Variable costs consisted of costs associated with plants and seeds, and materials and supplies; while labor, energy, facilities, vehicles, equipment, travel, taxes and licenses were included in fixed costs. In this study, to determine the average annual fixed cost per week, Weddington's (2003) method for estimating retail outdoor field and greenhouse production cost was used, the calculated $\$/\text{ft}^2/\text{yr}$ was divided by 52 weeks to estimate the fixed cost of production of zinnia planted as direct-seed or transplants.

Study two: Production and cost of production of season-long planting vs. succession plantings.

Field trials were conducted in summer 2013 at HFL and MHCREC. Two zinnia cut flower cultivars 'Benary Giant Scarlet' ('Scarlet') and 'Zowie Yellow Flame' ('Zowie') were evaluated at HFL and zinnia 'Scarlet' and celosia 'Chief Fire' ('Chief Fire') were evaluated at MHCREC. Seeds of 'Scarlet' and 'Chief Fire' were sourced from Germania Seed Co. and seed of 'Zowie' were from Grimes Horticulture, Concord, OH. All crops were planted as transplants. Field preparation and the production of transplants in the greenhouse followed the methodologies described in study one.

The trials were planted in a RCB split-plot design with three planting times as main plots and two crops as subplots with five replications. Each plot was 1.2 x 1.2 m². At approximately five weeks after sowing, the transplants were planted in the field at 30 cm apart with 16 plants per plot. At the time of planting, each plot was fertilized with ammonium nitrate at 3 g per 30 cm². The transplants were hand watered until established and then drip irrigated as needed. Liquid fertilizer at 250 ppm 20N-10P-20K was applied every other week through the drip irrigation system.

Three field plantings, representing possible succession planting dates for NC cut flower growers, were made (Fig. 2.1). At HFL, the first planting (Season-Long Planting) was planted on April 24, 2013 (week 1). The second planting (Mid-Season Succession) was made on July 1 (week 11) and the third planting (Late-Season Succession) was made on August 1 (week 15).

All flowers were cut at the first branching node when the flowers were fully open. Because 'Zowie' has short internodes, to be consistent between crops, all flowers with stem lengths of 12 cm or longer were cut for both 'Zowie' and 'Scarlet'. Flowers less than 12 cm were discarded. The first planting started flowering at week 8 and continued through week 23 when the trial ended (16 weeks total). The second planting flowered at week 14 and continued to the end of the trial ended (10 weeks total). The third planting flowered at week 18 through week 23 (6 weeks total). Flowers were cut and stem number recorded three times per week. Stem length (cm) of the cut flowers and flower diameter (cm) of five sampled flowers were recorded once a week. The third planting had lower flower

production per plot as compared to the first and second plantings. Therefore, to ensure an adequate sample size from the third planting, stem length and flower diameter data were collected twice a week, while stem number continued to be collected three times per week. Total stem number and mean stem length and flower diameter were calculated for each plot. Disease evaluations were conducted four times during growing season following the methodology described in study one.

To determine the effect of succession plantings on total harvested stem number, mean stem length and flower diameter, from the start of the growing season to the end of summer, the data from the plantings were grouped by weeks, with each group having 16 weeks of data.

Season-Long Planting (S-Long) – includes data from the first planting only (weeks 8-23). This group represents growers who plant a crop in the late spring and harvest from that crop all season with no succession plantings.

Mid-Season Succession (Mid-S) – includes data from the first planting (weeks 8-15) and the second planting (weeks 16-23). This group represents growers who plant a succession crop during mid-season and once that starts flowering, they eliminate the first planting.

Late-Season Succession (Late-S) – includes data taken from the first planting (weeks 8-19) and the third planting (weeks 20-23). This group represents growers who plant a succession crop late in the season.

Mid- and Late-Season Succession (Mid+Late-S) – includes data taken from the first planting (weeks 8-15), second planting (weeks 16-19), and the third planting (weeks 20-23). This group represents growers who plant more than one succession crop throughout the growing season.

Estimated fixed cost for the S-Long and the three succession plantings were calculated using the same formulas as in study one. The costs for the three plantings were estimated separately and then used to calculate the cost of each succession planting.

At MHCREC, the experimental design, plot size and layout was the same as at HFL. The transplants were planted on June 5, July 2 and August 2. After plant establishment, drip irrigation was applied as needed. The plants were fertigated twice during the growing season. Weeds were controlled by hand weeding. At MCHREC, to determine the effect of succession plantings on total harvested stem number and mean stem length and flower diameter, from the start of flowering to the end of the season, the data from the plantings were grouped by weeks, with each group having 12 weeks of data:

Season-Long Planting (S-Long) – included data from the first planting only (weeks 6-17).

Mid-Season Succession (Mid-S) – included data from the first planting (weeks 6-11) and the second planting (weeks 12-17).

Late-Season Succession (Late-S) – included data taken from the first planting (weeks 6-13) and the third planting (weeks 14-17).

Mid- and Late-Season Succession (Mid+Late-S) – included data taken from the first planting (weeks 6-11), second planting (weeks 12-14), and the third planting (weeks 15-17).

Like study one, the wet and cool environmental conditions at MHCREC during the summer (Appendix 1) resulted in uneven plant stands and stunting of celosia ‘Chief Fire’ transplants. As a result, only the data from the zinnia ‘Benary Scarlet’ plantings was used in the statistical analysis.

At HFL, mean stem length and flower diameter, and total stem number per plot were calculated for each planting group. The data was analyzed using analysis of variance (SAS Institute, Cary, NC) for a RCB split plot design and means separation across treatments was conducted using PROC GLM. At the MHCREC, the data from the ‘Scarlet’ plots was analyzed as a RCB design with four groups of comparison.

Results

Study One: Production and cost of production of directed-seeded vs. transplanted cut flower crops in two planting densities

Stem length

At HFL, main plot effects (crops ‘Scarlet’ and ‘Pro Cut’) were significantly different ($p < 0.01$) for stem length as would be expected in the comparison of two different cut flower crops. ‘Pro Cut’ had an average stem length of 87.7 cm while ‘Scarlet’ was 26.7 cm (Table 1). Sub plot effects (planting densities 16 and 32 plants/plot) and sub-sub plot effects (direct-seeded and transplant) were also significantly different ($P < 0.01$) for stem length, primarily

due to the significant effects of the main plots. The interaction between crops and densities was also significant ($P < 0.01$). 'Pro Cut' produced longer stem lengths (94.2 cm) at the higher density than at the lower density (81.1 cm), while there were no effect of planting density on stem length in 'Scarlet' (Table 1). The interaction between crop and planting method was significant ($P < 0.01$). 'Pro Cut' produced significant longer stems in the direct-seeded plots (103.7 cm) than in the transplanted plots (71.7 cm), while planting method did not significantly affect stem length in 'Scarlet'. These results show that 'Pro Cut' produced longer stems when the crop was directed seeded at higher densities. Stem length in 'Scarlet', however, was not impacted by planting method or density.

At MHCREC, the only significant differences observed in stem length were due to the main effects between crops ($P < 0.01$) (Table 1). No differences were observed between planting densities in either crop. Under the cool, wet conditions at MHCREC in 2013, 'Scarlet' tended to have longer stems compared to HFL, while 'Pro Cut' was shorter.

Flower diameter

At HFL, as expected, significant differences in flower diameter ($P < 0.01$) were found between crops. However, no differences were found for flower diameter in 'Scarlet' due to planting density or method. In 'Pro Cut', significantly larger flower diameter was found in direct-seeded (13.8 cm) than transplant (9.0 cm) plots, while no differences were observed in planting densities. These results showed that the sunflower 'Pro Cut' produced larger flower diameters when direct-seeded at either plant density. At MHCREC, the only

difference in flower diameter was between crops ($P < 0.01$) (Table 1). There was no effect of planting density or method.

Stem number

Differences in total stem number (yield) over the growing season at HFL were significant ($P < 0.01$) between crops (Table 1). 'Pro Cut' is a single harvest sunflower cultivar with most plants producing only one marketable stem. 'Scarlet' is a multi-branched cut flower zinnia that continues to branch and produce more flowers after each harvest. At the higher plant density, 'Scarlet' had an average total yield of 458.6 stems/plot, which was significantly more ($P < 0.01$) than 348.8 stems/plot at the low density. There was no significant effect of planting density and planting method on the stem number of 'Pro Cut', although transplanted plots had higher total yields (24.1 stems/plot) than did the direct-seeded plots (19.0 stems/plot), which had lower plant establishment due to uneven seed germination and plant establishment.

At MHCREC, no significance differences were observed between crop yields. Transplanted 'Pro Cut' had somewhat higher yields at the higher plant density than the lower, but the differences were not significant. Similar response was also seen in 'Scarlet'.

Disease evaluations

Powdery mildew disease ratings on 'Scarlet' were significantly higher ($P < 0.05$) in the high density plots (6.8) than in the low density plots (5.9), but no differences were found between direct-seeded and transplanted plots (Table 1). Bacteria leaf and flower spot

(BLFS) was slower to develop in the field and no significant difference were observed on 'Scarlet' for planting densities or planting methods (Table1).

Economic Analysis

The space adjusted method of Weddington (2003) to estimate fixed production costs for retail cut flowers were calculated to be \$0.061/ft²/wk for greenhouse and \$0.015/ft²/wk for field production. The planting environment (greenhouse and field) and time (weeks) of zinnias grown in each environment were applied to a general cost formula for retail cut flower production (Weddington, 2003):

$$\text{Cost}(\$) = (\$0.061/\text{ft}^2/\text{wk} * \text{Week}_1 * \text{Area}_1^2) + (\$0.015/\text{ft}^2/\text{wk} * \text{Week}_2 * \text{Area}_2^2)$$

Where: Week₁ and Area₁² are the weeks and planting area for greenhouse transplant production and Week₂ and Area₂² are for field production.

Study showed that the fixed production costs of direct-seeded zinnias grown in 80 ft² (7.4 m²) of field land for 19 weeks was \$22.80 for either planting densities. Lower production costs were obtained when the zinnias were planted as transplants at 6 inches (15 cm) or 12 inches (30 cm) in the same area and number of weeks (Table 4). In this study only 6.12 ft² (0.6 m²) of greenhouse bench was utilized to produce sufficient transplants for 80 ft² (7.4 m²) at the higher plant density and 3.06 ft² (0.3 m²) at the lower plant density, as a result, field production costs are higher than greenhouse costs. As each grower has different cultivars, growers may need to increase the greenhouse area when seeding for transplants. In practice, growers could use the above equation to calculate their fixed costs and choose different methods based on those estimates before planting. These calculations

do not include variable costs such as the cost of seeds or fertilizers. If high seeding rates of expensive seeds are needed to obtain an adequate plant stand in the field, then producing transplants in the greenhouse to ensure high germination rates and seedling growth may be more economical.

Study 2: Production and cost of production of season-long planting vs. succession plantings of zinnia

Stem length.

At HFL, 'Scarlet' produced significantly ($P < 0.01$) longer stems (25.8 cm) than 'Zowie' (17.5 cm) across all plantings (Table 2.2). 'Scarlet' stem lengths increased with succession plantings with the longest average stem length (27.3 cm) obtained in the Mid+Late-S plantings and the shortest stem length (24.2 cm) was in S-Long planting (Table 2.2; Figure 2.2). In this cultivar, succession plantings were beneficial to obtain plants with longer stems, although the timing of the first succession planting (Mid-S or Late-S) did not have an effect on stem length (Table 2.2; Figure 2.2). The stem length of 'Zowie' was not affected by succession plantings (Table 2.2).

At MHCREC, only 'Scarlet' plantings were compared (Table 2.2). Although, no significant differences among groups were found for stem length, the cool conditions at MHCREC produced stems that were on average about 7 cm longer than those obtained for 'Scarlet' planted at HFL. This may be due to the increase of sugar storage resulted by low temperatures.

Flower diameter

'Scarlet' had significantly ($P < 0.05$) larger flower diameters (6.8 cm) than 'Zowie' (5.1 cm) at HFL (Table 2.2). 'Scarlet' flower diameter increased slightly over succession plantings, with the Mid+Late-S plantings having significantly larger flowers (8.8 cm) than the S-Long (6.0 cm) or Mid-S and Late-S plantings (6.2 cm and 6.3, respectively) (Table 2.2; Figure 2.3). As seen with stem length, succession plantings produced larger flowers, but that the timing of the first succession plantings (Mid-S or Late-S) was less important than having two succession planting (Mid+Late-S) (Table 2.2; Figure 2.3). Within 'Zowie', no significant difference in flower diameter were observed, although there was a slightly tendency for increasing diameter when more succession plantings were conducted. At MHCRC, although the Late-S succession produced the largest flowers, these very small differences in flower diameter would not be important commercially (Table 2.2). The mean flower diameter of 'Scarlet' at MHCRC was similar to that at HFL.

Stem number

'Zowie' is a productive cut flower cultivar that continued to increase in production over the growing season at HFL. Succession plantings (Mid-S, Late-S, and Mid+Late-S) produced significantly ($P < 0.01$) fewer stems (yield) than the S-Long planting (Table 2.2). Similarly, 'Scarlet' had higher stem yields in the S-Long planting; significantly higher than the Mid+Late-S planting. At MHCRC, the highest yields were obtained in the Mid-S plantings, followed by the S-Long planting. The difference in stem yield at MHCRC may reflect the effect of uneven, wet and chilly weather during the growing season, but it also supports the results seen in HFL where the highest yields tended to be obtained in the season-long

plantings or Mid-S plantings. On average, stem yield of 'Scarlet' at MHCREC was about one-third less than the stem yield obtained at HFL.

Disease evaluation

Powdery mildew ratings were significantly different ($P < 0.01$) in the three 'Zowie' plantings, with the third planting having the lowest of disease (2.4), while the first planting (S-long) had the high levels of disease (7.4). Similar results were found with 'Scarlet' (Table 2.3). Disease ratings for bacteria leaf and flower spot were similar to powdery mildew, with the third planting having lower disease scores than the first and second plantings (Table 2.3).

Economic analysis

Estimated fixed costs for the S-Long and Mid+Late-S were \$16.72 and \$18.96, respectively, while both Mid-S and Late-S costs were \$17.84 (Table 2.4). Although the differences in fixed costs among plantings were not significant due to the small area of greenhouse and field space needed for this trial; with an increase in number of succession plantings, fixed costs increased. Thus, growers should conduct a fixed cost evaluation analysis before implementing multiple succession plantings to maximize flower yield and quality while controlling costs

Discussion

Cut flower growers use a variety of production practices depending on the crop and personal experience (Whipker and Cavins, 2000; Granitz, 2014). However, little research has been done to help growers optimize flower quality and stem yield through comparative

studies of production practices. Our studies evaluated zinnia and other annual cut flowers at two locations in North Carolina. No differences were found between planting methods (direct-seeded vs. transplants) on stem length, flower diameter, total number of harvested stems, and disease (powdery mildew and bacterial leaf and flower spot) ratings in field grown zinnia 'Scarlet' at HFL. However, planting densities (16 vs. 32 plants/plot) did impact total stem number, with higher plant densities producing more flowering stems than lower densities. These results confirm the positive linear relationship of cut flower density and stem number in perennials reported by Armitage (1987).

The studies showed that higher yields (number of harvested stems) of 'Scarlet' and 'Zowie' were obtained in the season-long plantings, while two succession plantings resulted in lower yields. In later succession plantings, cut flower zinnias may not have enough time to fully develop highly productive, branching growth habits needed to produce multiple flowering stems. In the season-long plantings, however, longer stem length and larger flower diameter of 'Scarlet' were reduced over time, while in the succession plantings these flower quality traits were maintained. Not all zinnia cultivars reacted the same to succession plantings. 'Zowie' stem length and flower size was not affected by succession planting, but only yield was reduced. However, flower size of 'Zowie' is genetically smaller than 'Scarlet', which had greater capacity to decrease in size.

In the season-long planting, the disease severity of PM and BLFS increased in both zinnia cultivars. 'Scarlet' was intermediate to susceptible to PM and BLFS and 'Zowie' was

susceptible to both diseases. Higher planting densities and direct seeding of 'Scarlet' slightly increased PM disease severity, but not BLFS. These results support recommendations that have shown that higher planting densities can result in higher levels of PM in zinnia (Stevens et al., 1993). Late succession plantings of zinnias had significantly less disease than earlier plantings for both 'Scarlet' and 'Zowie'. These results confirm that succession plantings can be an effective means of reducing disease in zinnia (Stevens et al., 1993). This study did not examine the impact of disease on yield, but by the end of the season, we observed that disease symptoms were also present on some flowers in the season-long plantings, thereby reducing their quality.

'Pro Cut' is a single-stem cultivar that generally produces only one harvestable flower per stem. 'Pro Cut' had longer stems and larger flower diameter when it was direct-seeded into the field rather than transplanted. As documented in other crops (Ortiz et al., 2012), it is possible that 'Pro Cut' may experience some transplant shock after being planted into the field, which would explain its shorter stem lengths and flower diameter. While long stems and large flowers may be good for home gardens and specific floral displays, short stems and smaller flowers may be optimal for bouquets. As a result, growers can use high planting densities or transplants to reduce flower size or, conversely, use lower planting densities and direct-seeding when larger flower diameters are needed.

Based on the economic analysis, late- and mid-season succession plantings have higher fixed costs than a season-long planting. If growers have large planting areas, the cost

difference could be a factor to consider. However, the data suggested that growers have one late succession planting of 'Scarlet' to maintain flower quality and to reduce foliar disease incidence. In these studies, the data was not collected after the end of September; however, late season succession plantings of zinnia may continue to produce until the first frost. Growers could reduce expenditures related to labor and greenhouse operations by direct-seeding 'Scarlet' in the field rather than using transplants. Other costs (i.e., plants, seeds, materials and supplies) are also important factors in a grower's production decision. For example, a grower may prefer to produce transplants in the greenhouse to ensure high seed germination and plant establishment, rather than sow expensive seed in the field and risk poor seed germination or the added labor of removing excess seedlings. The trials at HFL and MHCREC showed that temperature and precipitation have large effects on flower quality and yield. Further studies are needed to confirm these results in multiple environments and with other annual cut flower crops.

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Table 2.1 Mean stem length, flower diameter, total harvested stem number, and disease severity of directed-seeded vs. transplanted cut flower crops in two planting densities at HFL, Raleigh and MHCREC, Mills River, NC.

Crop	Planting density	Planting method	HFL					MHCREC		
			Stem length (cm)	Flower diameter (cm)	Total stem no.	PM (1-9) ^z	BLFS (1-9) ^z	Stem length (cm)	Flower diameter (cm)	Total stem no.
'Scarlet' zinnia	16/plot	Direct-seeded	26.9 e	6.2 c	339.6 b	6.1 ab	4.7 a	--	--	--
		Transplant	26.7 e	6.0 c	358.0 b	5.7 b	4.6 a	30.8 b	6.1 b	101.2 a
	32/plot	Direct-seeded	26.7 e	6.0 c	464.4 a	6.9 a	5.0 a	--	--	--
		Transplant	26.6 e	5.8 c	452.8 a	6.7 ab	4.8 a	30.6 b	6.0 b	114.4 a
'ProCut' sunflower	16/plot	Direct-seeded	94.6 b	13.4 a	13.2 c	--	--	--	--	--
		Transplant	67.6 d	8.9 b	15.0 c	--	--	58.6 a	9.9 a	20.2 b
	32/plot	Direct-seeded	112.8 a	14.1 a	24.8 c	--	--	--	--	--
		Transplant	75.7 c	9.1 b	33.2 c	--	--	60.2 a	9.7 a	36.2 b

Means with different letters in the same column are significantly different at P < 0.05.

^zDisease rating scale of 1 to 9 for powdery mildew (PM) and bacterial leaf and flower spot (BLFS), where 1 = no disease present and 9 = plants severely diseased or dead.

Table 2.2 Mean stem length, flower diameter, and total harvested stem number of season-long vs. succession plantings of two zinnia cultivars grown at HFL, Raleigh and MHCREC, Mills River, NC

Zinnia cultivar	Planting ^z	HFL			MHCREC		
		Stem length (cm)	Flower diameter (cm)	Total stem no.	Stem length (cm)	Flower diameter (cm)	Total stem no.
'Scarlet'	S-Long	24.2 c	6.0 b	620.8 d	32.8 ab	6.2 b	148.8 b
	Mid-S	25.9 b	6.2 b	608.6 de	33.5 a	6.2 b	173.4 a
	Late-S	25.9 b	6.3 b	589.2 de	32.3 b	6.4 a	124.4 c
	Mid-Late-S	27.3 a	8.8 a	548.0 e	32.9 ab	6.3 ab	141.8 bc
'Zowie'	S-Long	17.5 d	5.0 b	1105.0 a	–	–	–
	Mid-S	17.5 d	5.1 b	924.6 b	–	–	–
	Late-S	17.8 d	5.2 b	979.2 b	–	–	–
	Mid-Late-S	17.2 d	5.3 b	850.6 c	–	–	–

^zPlantings: Season-long (S-Long); Mid-season succession (Mid-S); Late-season succession (Late-S); Two successions (Mid+Late-S)
Means with the same letter in the same column are not significantly different at P < 0.05.

Table 2.3 Disease severity ratings on two zinnias cultivars planted at three dates at HFL, Raleigh

Zinnia cultivar	Plantings dates	Powdery Mildew (1-9) ^z	Bacteria Leaf and Flower Spot (1-9) ^z
'Scarlet'	First	6.6 ab	5.1 b
	Second	6.1 ab	4.2 b
	Third	2.5 c	1.8 c
'Zowie'	First	7.4 a	6.8 a
	Second	5.4 b	5.3 b
	Third	2.4 c	2.3 c

^zDisease rating scale of 1 to 9 for powdery mildew and bacterial leaf and flower spot, where 1 = no disease present and 9 = plants severely diseased or dead.

Means with the same letter in the same column are not significantly different at P < 0.05.

Table 2.4 Cost analysis (Weddington, 2003) of direct-seeded vs. transplanted zinnia at two planting densities (Study 1) and in season-long vs. succession plantings (Study 2) at HFL, Raleigh, NC

	Plantings	Greenhouse		Field		Cost(\$) ^y
		Weeks ₁ ^z	Area ₁ (ft ²)	Weeks ₂	Area ₂ (ft ²)	
Study 1	Direct-seeded 16/plot	0	0	19	80	22.80
	Transplanted 16/plot	6	3.06	13	80	16.72
	Direct-seeded 32/plot	0	0	19	80	22.80
	Transplanted 32/plot	6	6.12	13	80	17.84
Study 2	S-Long	6	3.06	13	80	16.72
	Mid-S	12	3.06	13	80	17.84
	Late-S	12	3.06	13	80	17.84
	Mid+Late-S	18	3.06	13	80	18.96

^zWeek₁ and Area₁² are the weeks and planting area for greenhouse; Week₂ and Area₂² are the weeks and planting area for open field.

^yCost of production formula for retail cut flower growers:

$$\text{Cost}(\$) = (\$0.061/\text{ft}^2/\text{wk} * \text{Weeks}_1 * \text{ft}_1^2) + (\$0.015/\text{ft}^2/\text{wk} * \text{Weeks}_2 * \text{ft}_2^2)$$

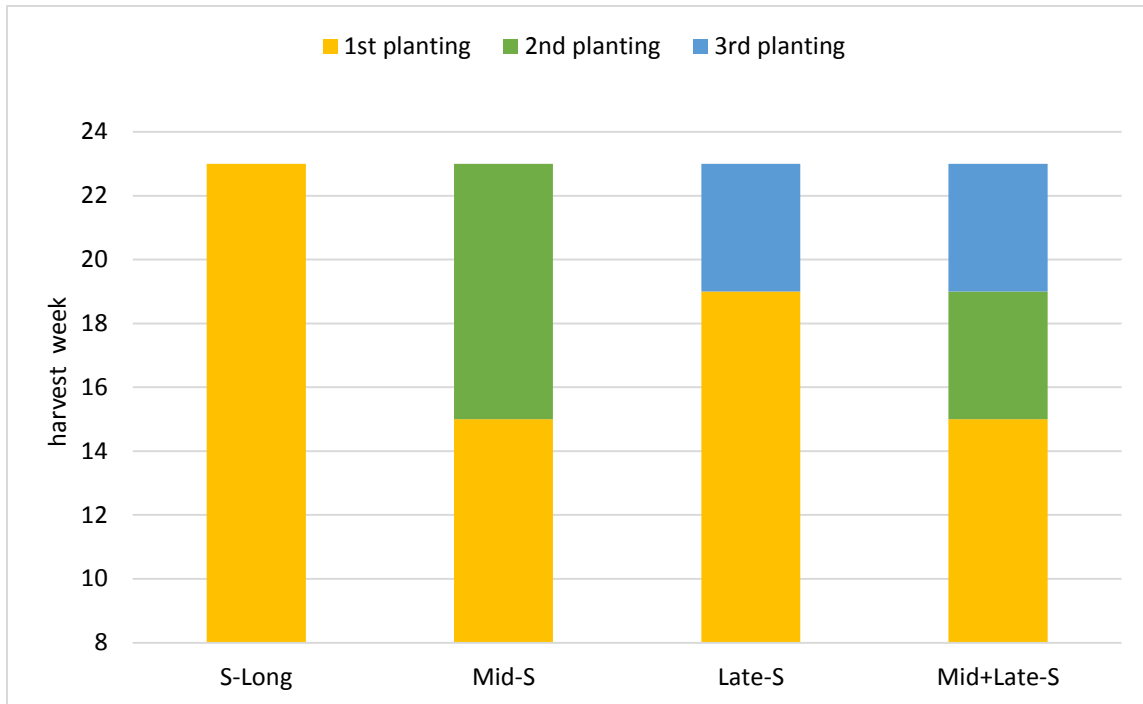


Fig.2.1: Groups of season-long and succession plantings at HFL, where:
 S-Long = 1st planting (week 8 to 23)
 Mid-S = 1st planting (week 8 to 15) + 2nd planting (week 15 to 23)
 Late-S = 1st planting (week 8 to 19) + 3rd planting (week 19 to 23)
 Mid + Late-S = 1st planting (week 8 to 15) + 2nd planting (week 15-19) + 3rd planting (week 19 + 23)

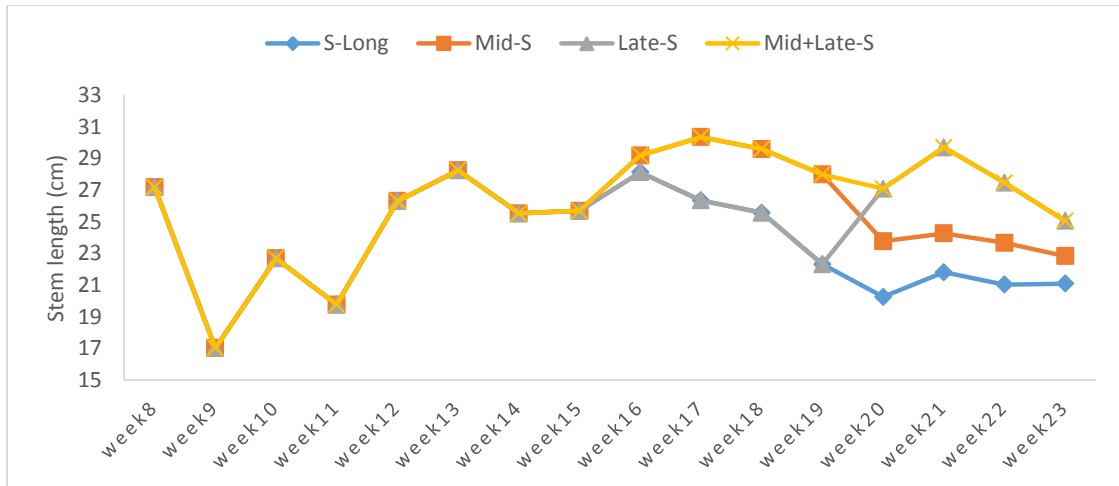


Fig. 2.2. Average stem length (cm) of 'Scarlet' in season-long (S-Long), mid-season (Mid-S), late season (Late-S), mid- and late-season (Mid+Late-S) succession plantings at HFL.

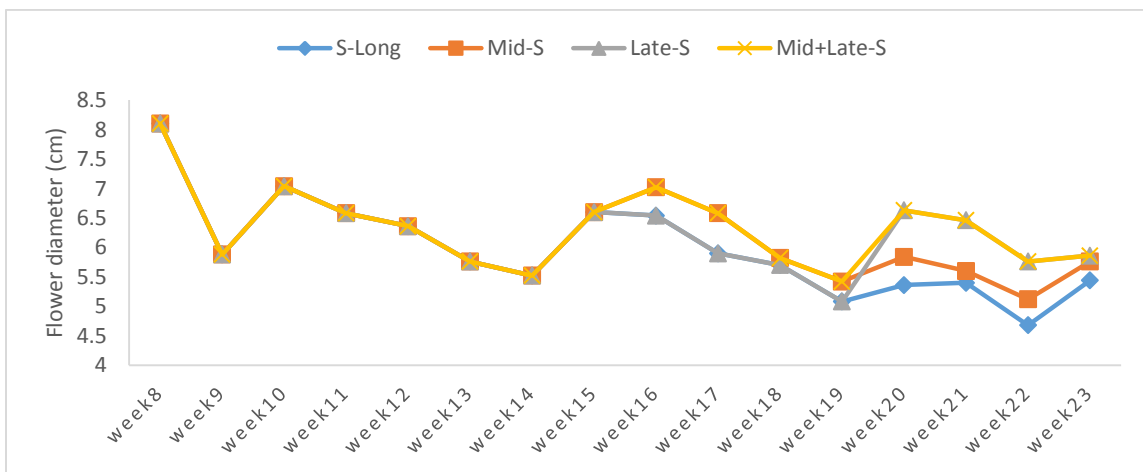


Fig. 2.3. Average flower diameter (cm) of 'Scarlet' in season-long (S-Long), mid-season (Mid-S), late season (Late-S), mid and late season (Mid+Late-S) succession plantings at HFL.

CHAPTER THREE

POLYPLOIDY INDUCTION IN TWO ZINNIA SPECIES

Abstract

Oryzalin was an effective mitotic inducer in 'Oklahoma White' at the 50 μM concentrations when applied 7 d after germination and 5 d later again. Five tetraploids were identified: four were obtained from the oryzalin treatments and one from colchicine (0.33%), which was applied at 7 d after germination, again at 2 and 4 d later. Oryzalin was not effective in inducing polyploids in 'Crystal Orange', with most treated plants remaining diploid, only one tetraploid was obtained. One tetraploid was induced in the 50 μM Oryzalin treatment and two tetraploids were obtained from the colchicine treatment. No correlation between the guard cell lengths and 2C DNA values for treated seedlings were found. Colchicine and oryzalin concentrations will need to be tested on other species to determine optimal efficiency of polyploidization in zinnia. Successful induction of autotetraploids would enhance the genetic diversity within zinnia species and provide parental materials for interspecific crossing.

Introduction

A polyploid plant is one that has multiple sets of chromosomes. Ploidy levels can influence cross pollination, fertility of hybrids, plant vigor, and gene expression, as well as provide a wider germplasm base for plant breeding efforts (Blakeslee and Avery, 1937; Jones et al., 2008; Ranney, 2006). An autopolyploid is a polyploid plant whose chromosomes are derived from a single species, while an allopolyploid is a polyploid plant

whose chromosomes are derived from different species. As a result, allopolyploids have a greater degree of heterozygosity which may contribute to increased heterosis (Ranney, 2006).

Blakeslee and Avery (1937) first induced polyploids in plants using colchicine, an inhibitor of mitosis; other inhibitors include oryzalin, trifluralin, amiprofos-methyl and N₂O gas (Bouvier et al., 1994; Taylor et al., 1976; Van Tuyl et al., 1992). In zinnia, colchicine has been used to induce tetraploids in *Z. violacea*, *Z. angustifolia*, and their interspecific hybrids (Boyle, 1996; Gupta and Koak, 1976; Terry-Lewandowski et al., 1984). There are concerns, however, about the safety to users of colchicine used in ploidy induction (Finkelstein et al., 2010; Folpini and Furfori, 1995), and oryzalin is increasingly being used as a mitotic inhibitor due to its lower toxicity and tendency to produce less deformed tissue or growth (Van Tuyl et al., 1992). However, there are no reports on the effectiveness of oryzalin in zinnia ploidy induction.

Zinnia violacea is the most widely cultivated and commercially important zinnia and is widely grown as a specialty cut flower and bedding plant. It is a diploid species ($2x=24$) and only one tetraploid ($4x=48$) cultivar, 'State Fair', is commercially available. Efforts to develop disease resistant cut flower zinnias with long stems and large flowers have not been successful due to the lack of resistant genes in *Z. violacea* germplasm. *Zinnia angustifolia* ($2x=22$) is highly resistant to three major zinnia diseases: powdery mildew, bacterial leaf and flower spot, and *Alternaria* blight. Boyle and Stimart (1982) intercrossed *Z. angustifolia* and *Z. violacea* and treated the F₁ seedlings with 0.33% colchicine to produce

a partially, self-fertile allotetraploid with high levels of disease resistance, and the resulting offspring were named *Z. marylandica* ($4x=46$) (Boyle and Stimart, 1982; Hagan, 2009; Spooner et al., 1991; Terry-Lewandowski et al., 1985). The objective of this study was to evaluate different concentrations of oryzalin for induction of polyploids in *Z. angustifolia* and *Z. violacea*. The induction of polyploids using oryzalin would provide plant breeders with an alternative to colchicine in the development of interspecific crosses.

Materials and Methods

Ploidy induction. A 50 mL stock solution of oryzalin at 10,000 uM concentration was prepared using Surflan herbicide (United Phosphorus, Inc.) containing 484.8 g/L (1.3997 moles/L) oryzalin. From the stock solution, three working solutions with concentrations of 50, 100, and 150 μ M oryzalin were prepared by adding 0.125, 0.25, and 0.375 mL of stock to 24.875, 24.75, and 24.625 mL, respectively, to DI water containing 0.00275 g/mL agar, to make 25 ml oryzalin solutions. The distilled water + agar solution was prepared by adding 0.55 g powder agar (Caisson Lab Inc.) into 100 ml DI water, heated until the agar dissolved and the solution became transparent, then cooled to room temperature. A fourth treatment of 25 ml DI water + agar solution was used as the control to maintain the diploid status of the cultivar. The oryzalin treatments were also compared to a 0.33% colchicine solution made by dissolving 0.17g colchicine (97% colchicine, Fisher Scientific, ACROS Organics) into 49.83ml DI water.

Seeds of *Z. violacea* 'Oklahoma White' and *Z. angustifolia* 'Crystal Orange' were

sourced from Germania Seed Company, Chicago, IL. Two seeds of each cultivar were planted in 18 cell trays (21"x 10.5"x 2.25") filled with Farfard® 4P Mix and covered a layer of vermiculite. The trays were placed on a bench in the NC State Horticultural Field Lab greenhouse at approximately 21°C and watered. After the seeds germinated, the seedlings were thinned to leave one seedlings in each cell. When the first true leaf appeared (Day 1), 36 seedlings (two trays) of each cultivar were treated with either 50, 100, or 150 µM oryzalin/ agar solution or 0.33% colchicine solution, and 18 seedlings per cultivar were treated with the control solution. One drop of a solution was applied to the apical growing point of the plant using a disposable pipette. A new pipette was used for each treatment application. The cell trays were placed in a shade-cloth covered, humidity chamber in the greenhouse for 24 h to slow the solution evaporation, and each cell was carefully watered to avoid wetting the apical growing points. Five days after the first treatment, 18 of the 36 plants were retreated with 50, 100, 150 µM oryzalin/agar solutions and placed in the humidity chamber for 24 h. The 0.33% colchicine solution was applied to the growing points of 18 seedlings of both cultivars at three days (Days 1, 3, and 5). After treatment, the plant trays were placed in the humidity chamber for 24 h, after which they were placed on a greenhouse bench.

Flow cytometry. Two standards were used in the flow cytometry analysis to separate from samples. *Raphanus sativus* cv. 'Saxa' (2C DNA = 1.11 pg) was used as the DNA standard for 'Oklahoma White' and *Pisum sativum* 'Ctirad' (2C DNA = 8.75 pg) was used for 'Crystal

Orange' (Dolezel, 1991; Dolezel et al., 2007; Galbraith, 2009). Seeds of the standards were planted in cell trays in the greenhouse following the same procedure as the zinnias. Zinnia and standard plant nuclei were extracted and nuclear DNA was stained using the Partec CyStain PI Absolute P reagent kit (Partec Co., Germany). From each treated plant, approximately 0.5 cm² piece of young leaf tissue was placed in a 55 mm plastic Petri dish along with a similar-sized piece of tissue from the standard. The samples were chopped into small pieces with a razor blade for about 30 seconds in 400 µl extraction buffer to extract the nuclei. The resulting extract was filtered through a 50 µm nylon filter into a flow cytometry test tube, and 1.0 ml staining solution (with PI and Rnase) was added. The tubes were wrapped in aluminum foil to avoid light and kept in a refrigerator at 4°C.

The DNA quantity of the samples were tested using a Becton-Dickinson FACSCalibur flow cytometer. All analyses were made using peak height detection. For each sample, approximately 1000 total events nuclei were collected. Seedlings that appeared to be mixoploids or putative tetraploids were retested following the same method as above. The DNA content of the sample was calculated using the following formula:

$$2C \text{ DNA (pg)} = (\text{sample mean G1 peak}/\text{std. mean G1 peak}) * \text{std. 2C DNA content}$$

Stomatal guard cell observations. Leaves from each 'Oklahoma White' treated seedlings (above the point of chemical treatment) were collected in small plastic, sealable bags and taken to the lab. 'Crystal Orange' leaves were collected from the control, 50 µM oryzalin, and 0.33% colchicine treatments. A thin layer of transparent nail polish was

brushed on the upper surface of the leaf. After 5 min, the dried nail polish, with an imprint of the leaf epidermal layer, was carefully lifted from the leaf, placed on a microscope slide and the sample and slide were covered with clear tape. Using a Motic BA210 Type 102M microscope (Hong Kong, China) at 40X magnification, the length (μm) of 20 stomatal guard cells on each leaf were measured using an eyepiece reticle that had been calibrated using a stage micrometer. To determine whether stomatal guard cell length was a reliable measure of ploidy induction, mean leaf stomata length was compared to the mean 2C DNA value for that plant. Spearman correlation coefficient of stomata length and 2C DNA value were analyzed using JMP 11 (SAS Institute, Cary, NC)

Results

The number of 'Oklahoma White' seedlings that died or were highly deformed from the oryzalin and colchicine treatments ranged from 11.1% to 38.9%, respectively (Table 3.1). All concentrations and applications of oryzalin had a similar effect on seedling growth and development: chlorosis and blanching of the plant tissue at the application site, some twisting and narrowing of the leaves, and in some cases, necrosis and death of the growing point. The colchicine application caused varying degrees of stunting, leaf cupping and other deformities, and an overall thickening and scabbing of the leaf tissue which caused the death of the growing point in some seedlings. There was little to no effect of oryzalin on 'Crystal Orange' at any concentrations and applications method used in this study (Table 3.2). Colchicine application to 'Crystal Orange' resulted in similar symptoms as those on 'Oklahoma White' and resulted in the death of some seedlings (Tables 3.2).

Figure 3.1 shows a sample result of the flow cytometric histogram obtained for a diploid (Figure 3.1a, 3.1b), tetraploid (Figure 3.1c, 3.1d), and mixoploid (Figure 3.1e, 3.1f) DNA contents in both cultivars. The non-treated diploid 'Oklahoma White' had a mean 2C DNA pg value of 2.28. The oryzalin-treated seedlings of 'Oklahoma White' had both diploid and mixoploid DNA content (Table 3.1). The DNA content ranged from 2.28 to 2.45 pg in the diploids and 2.33 to 4.90 pg in the mixoploids. No stable tetraploid plants were found in oryzalin treatment. The double application of 50 μ M oryzalin produced the largest number (6) of mixoploids. Both the single and double applications of 150 μ M oryzalin produced the lowest percentage of mixoploids. The 0.33% colchicine treatment produced the highest number (11) of mixoploids and one tetraploid (Table 3.1).

The non-treated diploid 'Crystal Orange' had a mean 2C DNA pg value of 2.00. All oryzalin-treated seedlings of 'Crystal Orange' were diploids (Table 3.2), with the exception of two mixoploids obtained in the 50 μ M treatment. Unlike 'Oklahoma White', oryzalin had little to no mitotic effect on this cultivar. The 0.33% colchicine treatment had a greater effect, with three mixoploids and two tetraploids identified (Table 3.2).

Selected mixoploid and tetraploid plants from 'Oklahoma White' and 'Crystal Orange' were retested to confirm ploidy levels. Mixoploids may be unstable and expressed chimerically in different plant tissues and may revert to diploids as plants grow, but they also have the potential to form stable tetraploids and may breed as tetraploids. Among the 12 'Oklahoma White' plants retested, four reverted to diploids, three remained mixoploids, one was tested as an octoploid, and four tetraploids were identified (data not shown). The

retest results for 'Crystal Orange' showed that one plant reverted to diploid, five remained mixoploid, one of the mixoploid formed a tetraploid, and two colchicine tetraploids identified in the first test, were reconfirmed as tetraploids (Table 3.2).

For stomatal guard cell length, there was no significant correlation between guard cell length and pg values of the control and ploidy induction treatments in 'Oklahoma White' (Spearman's ranked correlation coefficient = 0.0615), although there was a tendency for control (diploid) 'Oklahoma White' to have a smaller average guard cell length (8.2 μm) and the double 100 μM oryzalin treatment to have the average longest length (11.8 μm), which was similar to the colchicine treatment (11.6 μm) (Table 3.1). For 'Crystal Orange', the colchicine treatment had an average guard cell length of 7.3 μm , which was smaller than the diploid control. These results suggest that stomata guard cell length is not a reliable measurement to use as an indicator of ploidy level in ploidy induction studies.

Discussion

The results confirmed those of Boyle and Stimart (1982) which showed that ploidy induction in zinnia was possible using a 0.33% colchicine solution. Oryzalin was successful in inducing mixoploids and tetraploids in one cultivar of *Z. violacea*, but was much less effective in one cultivar of *Z. angustifolia*. Based on these results, it is recommended that double applications of oryzalin at the 50 to 150 μM concentrations may be used to induce polyploids in *Z. violacea* cut flower types, but further testing with other zinnia cut flower cultivars is needed. For *Z. angustifolia* 'Crystal Orange', colchicine was more effective at ploidy induction than oryzalin. Although one tetraploid was obtained with the 50 μM

oryzalin application, higher concentrations and double applications of oryzalin may be needed to effect ploidy induction in this species. In addition, an increase in the proportion of agar in the solution may assist in stabilizing the treatment solution on the growing tips of zinnia species, such as *Z. angustifolia*, with small apical growing points. Further research is needed to identify a more effective oryzalin ploidy induction method in small leafed zinnia species as well as to see if these findings can be replicated in other cultivars within these species and with other species of zinnia. To identify stable tetraploids, multiple flow cytometry tests on different parts of a plant are imperative to confirm ploidy levels as mixoploid chimeras can revert to diploids. Flow cytometry was an effective means of separating diploid from polyploidy plants.

Anantasaran et al. (2007) found that guard cell length, number of chloroplasts, and flower diameter in zinnia were positively correlated with DNA content and could be used to classify different species. However, in this study, the instability of the mixoploids in both cultivars contributed to greater variation in guard cell length, and a positive or negative correlation between guard cell length and ploidy induction levels could not be detected. However, once a stable polyploid zinnia is obtained, guard cell length may be a useful method to distinguish diploids from tetraploids.

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Table 3.1 Effect of oryzalin and colchicine treatments on polyploid induction and stomatal guard cell length in 'Oklahoma White'.

Oryzalin ploidy induction treatment (μM)	No. (%) dead or highly deformed plants	Diploid (2x)		Mixoploid (2x+4x)		Tetraploid (4x)		Stomatal guard cell length (μm) mean \pm SE	Re-test
		No. plants (%)	2C DNA (pg) mean \pm SE	No. plants (%)	2C DNA (pg) mean \pm SE	No. plants (%)	2C DNA (pg) mean \pm SE		Tetraploid no.
0	0	6 (100)	2.28 \pm 0.07	0	-	0	-	8.2 \pm 0.9	-
50	4 (22.2)	11 (78.6)	2.33 \pm 0.03	3 (21.4)	2.33 \pm 0.08 / 4.69 \pm 0.04	0	-	10.5 \pm 2.4	-
100	7 (38.9)	7 (63.6)	2.40 \pm 0.03	4 (36.4)	2.44 \pm 0.01 / 4.81 \pm 0.03	0	-	10.6 \pm 2.6	-
150	6 (33.3)	10 (83.3)	2.42 \pm 0.06	2 (16.7)	2.41 \pm 0.02 / 4.79 \pm 0.05	0	-	9.9 \pm 2.2	-
50-50 ²	4 (22.2)	8 (57.1)	2.38 \pm 0.02	6 (42.9)	2.32 \pm 0.08 / 4.64 \pm 0.17	0	-	11.3 \pm 2.0	1
100-100	2 (11.1)	12 (75)	2.38 \pm 0.04	4 (25)	2.35 \pm 0.04 / 4.68 \pm 0.13	0	-	11.8 \pm 3.1	1
150-150	3 (16.7)	13 (86.7)	2.45 \pm 0.05	2 (13.3)	2.43 \pm 0.06 / 4.90 \pm 0.04	0	-	10.9 \pm 3.1	1
0.33% Colchicine	6 (33.3)	0	-	11 (91.7)	2.50 \pm 0.15 / 4.79 \pm 0.08	1 (8.3)	4.87	11.6 \pm 1.7	1

²Oryzalin treatments of 50, 100, 150 μM solutions with agar were made twice, five days apart.

Table 3.2 Effect of oryzalin and colchicine treatments on polyploid induction and stomatal guard cell length in 'Crystal Orange'.

Oryzalin ploid induction treatments (μM)	No. dead or highly deformed plants	Diploid (2x)		Mixoploid (2x+4x)		Tetraploid (4x)		Stomatal guard cell length (μm) mean \pm SE	Re-test
		No. plants (%)	2C DNA (pg) mean \pm SE	No. plants (%)	2C DNA (pg) mean \pm SE	No. plants (%)	2C DNA (pg) mean \pm SE		Tetraploid no.
0	0	5 (100)	2.00 \pm 0.07	0	-	0	-	8.00 \pm 1.3	-
50	0	16 (88.9)	1.97 \pm 0.05	2 (11.1)	1.95 \pm 0.02 / 3.95 \pm 0.08	0	-	8.82 \pm 2.9	1
100	0	18 (100)	1.99 \pm 0.07	0	-	0	-	-	-
150	0	18 (100)	2.01 \pm 0.09	0	-	0	-	-	-
50-50 ²	4	18 (100)	1.94 \pm 0.05	0	-	0	-	-	-
100-100	0	14 (100)	1.98 \pm 0.04	0	-	0	-	-	-
150-150	5	18 (100)	1.96 \pm 0.06	0	-	0	-	-	-
0.33% Colchicine	3	8 (61.5)	1.94 \pm 0.05	3 (23.1)	2.00 \pm 0.03 / 4.10 \pm 0.05	2 (15.4)	4.10 \pm 0.04	7.30 \pm 2.49	2

²Oryzalin treatments of 50, 100, 150 μM solutions with agar were made twice, five days apart.

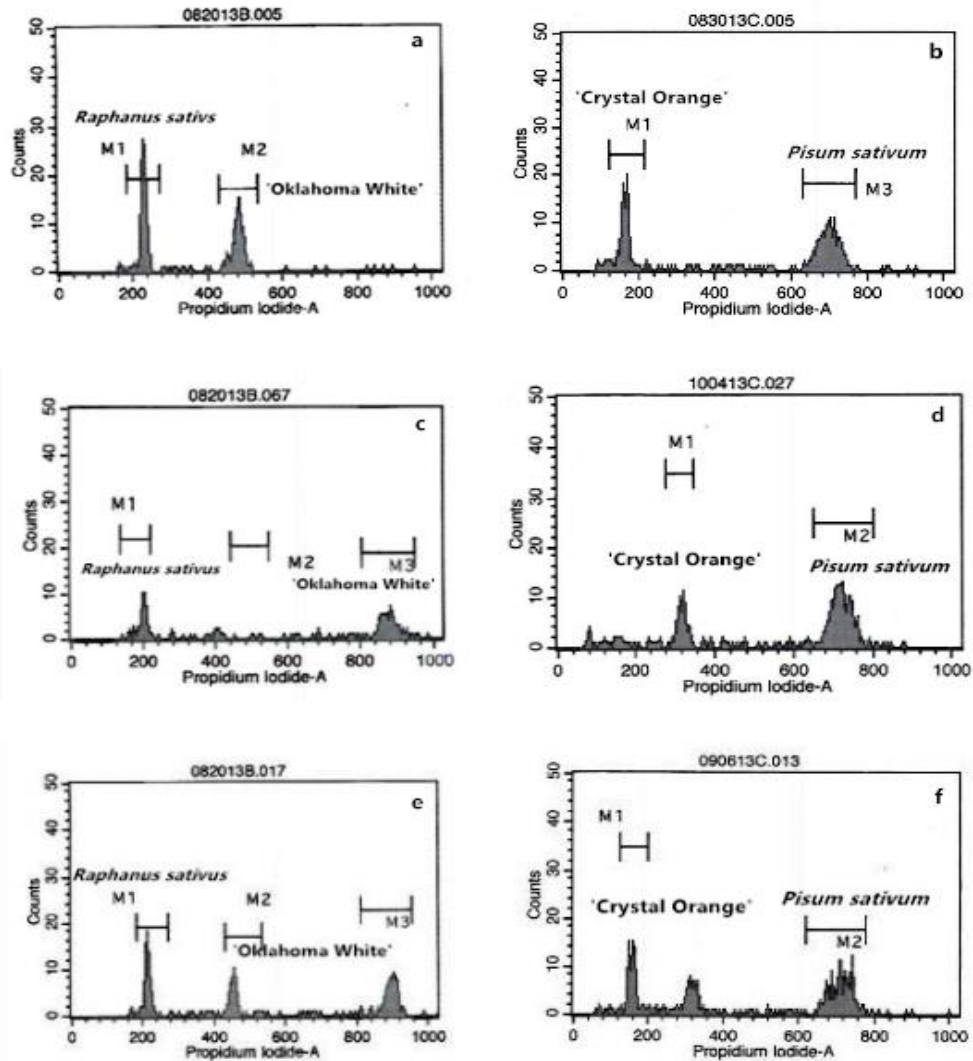


Fig. 3.1 Relative nuclear DNA content for 'Oklahoma White' and 'Crystal Orange'. Internal standards: *Raphanus sativus* cv. 'Saxa' (2C=1.11 pg); *Pisum sativum* cv. 'Ctirad' (2C=8.75 pg). Figure 3.1a, 3.1b are diploid (2x), Figure 3.1c, 3.1d are tetraploids (4x), and Figure 3.1e, 3.1f are mixoploids (2x+4x) DNA contents in both varieties

CHAPTER FOUR

POLLINATION MECHANISM AND DNA CONTENT OF ZINNIA SPECIES

Abstract

Self- and cross-pollination tests were conducted for 16 zinnia cultivars from 12 species. All species from subgenus *Diplothrix* appeared to be self-incompatible, as well as *Z. greggii* and *Z. maritima* from subgenus *Zinnia*, section *Mendezia*. Species from subgenus *Zinnia*, section *Zinnia* were partially self-incompatible, with the exception of *Z. peruviana* which was confirmed as a compatible species. Pollen was viable for all cultivar tested. Sections *Mendezia* and *Zinnia* were significantly different in genome size as determined by flower cytometry. Section *Mendezia* had comparatively smaller and consistent genome size, while the DNA content in section *Zinnia* ranged from 3.3 to 4.8 pg. 'Old Mexico', a tetraploid cultivar, had a 6.2 pg value. The pg values and other morphological traits for *Z. tenuifolia* 'Red Spider' and *Z. peruviana* were nearly identical, which indicates that 'Red Spider' is not a separate species, but should be considered a taxon of *Z. peruviana*. Although self-incompatibility is common in *Diplothrix* and *Mendezia* species, partial self-incompatibility to compatibility occurs in section *Zinnia* species. In a breeding program, removal of male disk florets will be required in these species to prevent the development of self-pollinated seed.

Introduction

The genus *Zinnia* L. is comprised of approximately 17 species of annual or perennial herbs or low shrubs in family *Asteraceae* (Torres, 1963; Metcalf and Sharma, 1971). The genus is divided into two subgenera: *Diplothrix* and *Zinnia*. The species in the subgenus

Diplothrix are perennial caespitose shrubs or subshrubs from the temperate regions of the southwestern United States and northern Mexico (Torres, 1963). Taxa within *Diplothrix* have a base chromosome number of $n=10$ and both diploid and tetraploid species have been reported (Torres, 1963; Metcalf and Sharma, 1971). Among these, *Z. juniperifolia* and *Z. oligantha* are diploids, *Z. citrea* and *Z. grandiflora* are tetraploids, and *Z. acerosa* has both diploid and tetraploid forms (Torres, 1963; Metcalf and Sharma, 1971). The only octoploid species is *Z. anomala*, which has small ray florets, or none at all, and may be a derivative of the tetraploid *Z. grandiflora* (Torres, 1963). There are no known improved horticultural forms within this subgenus (Metcalf and Sharma, 1971), although seeds of *Z. grandiflora* may be obtained commercially, primarily for use in native plantings in the southwestern United States. Torres (1962; 1964) attempted to make interspecific crosses among several of the species in *Diplothrix*; however, only a few of them survived as plants. Other crosses failed because the seeds were infertile and failed to germinate or died as seedlings.

The subgenus *Zinnia* includes two sections, *Mendezia* and *Zinnia*, and is comprised of 11 species that are native to the warm temperate and tropical regions of North and Central America, and one species native to South America (Torres, 1963; Metcalf and Sharma, 1971). Section *Mendezia* includes eight species with a base chromosome number $n=11$, 12 (Metcalf and Sharma, 1971). Among these, only *Z. angustifolia* has been successfully used by plant breeders in interspecific crosses for its resistance to diseases and compact growth habit. Other species in this section, such as *Z. greggii* and *Z. maritima*, also are potential sources of disease resistance and other traits. Section *Zinnia* includes three horticulturally

important species with a base chromosome number $n=12$. Seeds of the most widely cultivated zinnia, *Z. violacea*, were introduced into Europe in the 1790s from Mexico (Coats, 1968; McVaugh, 1984). Since then, this species has naturalized in parts of Florida, Central and South America, and Australia. *Zinnia haageana* is the second most important commercial species and is grown for its ease of cultivation and variegated ray flowers. It was introduced from Mexico to France in 1825 (Beeks, 1954). *Zinnia peruviana* is the only known species in the genus to be self-compatible (Torres, 1963) and it was introduced from Peru to France in the early 1700s (Beeks, 1954).

With the exception of *Z. peruviana*, *Zinnia* species are generally thought to be primarily or partially self-incompatible for seed production (Pollard, 1939; Torres, 1962; Torres, 1963; Olorode, 1970; Boyle and Stimart, 1986; Samaha et al., 1989). Torres (1962) found that the five species in *Diplothrix* were completely self-incompatible based on seed set. However, Boyle and Stimart (1986) observed pollen germination and pollen tube growth in self-pollinated and outcrossed *Z. violacea* and *Z. angustifolia*, and found that self-incompatibility and compatibility varied among selections made within the species. To effectively use *Zinnia* species in a breeding program, a better understanding of their pollination mechanisms for individual cultivars and clones is needed.

The early taxonomic studies of *Zinnia* were based on extensive and time consuming cytological observations to obtain basic chromosome numbers for each species. Today, flow cytometry is a useful tool to rapidly analyze the DNA content of plant cells (Dolezel and

Bartos, 2005; Anantaran et al., 2007). The advantage of using flow cytometry to analyze DNA content is the high speed of analysis allowing many samples to be tested in a short amount of time, and that the particles are randomly selected from the test sample without bias (Anantaran et al., 2007). The relative fluorescence of samples were measured by flow cytometry, the estimation of relative DNA content of tested samples can be estimated by standard DNA content in pictogram (pg) or basepair (bp). Dolezel et al. (2003) showed that 1pg DNA is equal to 0.987×10^9 basepairs, assuming the ratio of AT:CG is 1:1 and ignoring modified nucleotides. Flow cytometry is now routinely used to analyze DNA content in plant species, polyploidy inductions, and in interspecific plant breeding programs (Robinson, 2006; Anantaran et al., 2007; Galbraith, 2009).

The objective of these studies were to confirm the pollination mechanism (self-incompatible, partially incompatible, or compatible) of 12 zinnia species. The DNA content of selected zinnia species and cultivars was determined using flow cytometry. This information will be useful for future interspecific crosses and polyploidy-induction studies.

Materials and Methods

Pollination mechanisms: Seed of 16 accessions/ cultivars from 12 *Zinnia* species (Table 4.1) were obtained from the Ornamental Germplasm Center (The Ohio State University, Columbus, OH 43210) or commercial seed companies. Seed of each cultivar were sown in individual cells in an 18 cell tray filled with Fafard® 4P Mix and the trays were placed in a greenhouse at approximately 21°C to germinate. The trays were watered as

needed and fertilized weekly with J.R. Peters® 20N-4.4P-16.6K fertilizer. At approximately three weeks after sowing, five seedlings of each cultivar were transplanted into 15 cm pots, watered, and fertilized with 10 g of Osmocote® slow release fertilizer (14N-14P-14K). Each pot was connected to a drip irrigation system and liquid fertilizer was applied once a week. After flower initiation and over a six week period prior to anthesis, five or more flowers per plant were bagged using insect-exclusion net bags to evaluate self-compatibility by obliging self-pollination. To evaluate cross-pollination, ray and disk florets of six or more newly opened flowers/plant were cross pollinated using a pollen mixture obtained from the disk florets of other plants within the same cultivar. Approximately 28 days after bagging or cross-pollination, the flowers were harvested and the seeds were cleaned, dried, and stored under cool, dry conditions. Seed germination tests were conducted over a six month period. A maximum of 30 seeds from each flower head were sown in one or more cells filled with Fafard® 4P Mix and lightly covered with vermiculite. Fifteen commercially produced seeds of *Z. violacea* Benary Giant 'Scarlet', obtained from Grimes Horticulture Inc., were planted in one cell of each tray as a control for seed germination. The cell trays were placed in a greenhouse mist system to germinate and temperatures were maintained at approximately 21°C. The number of germinated seed were recorded for each cultivar.

Pollen viability: One anther from two flowers from each cultivar was placed on a glass slide in a drop of 1% acetocarmine and squashed under a cover slip. The slide was heated for several seconds on a hot plate to speed pollen staining. The slides were then

examined under the microscope using a green filter and the number of viable pollen grains was counted. Viable pollen had a round shape and were stained a magenta color, while non-viable pollen were irregular in shape and not stained. 300 pollen grains were observed for each cultivar.

Flow cytometry: Seeds of 13 zinnia cultivars from eight species in section *Mendezia* and *Zinnia* (Table 4.2) were sown in the greenhouse for flow cytometry analysis of their nuclear DNA content in 2014 and 2015. For each cultivar, approximately 0.5 cm² leaf tissue and 0.5 cm² of an internal standard were placed in a plastic petri dish (55 mm) with 400 µl extraction buffer (Flow cytometry kit, Partec CyStain PI absolute P, Partec 05-5022, Germany). The leaf tissue was finely chopped with a razor blade for about 30 seconds to extract the nuclei. The nuclei suspension was passed through a 50 µm filter into a flow cytometry tube and 1 ml of staining solution (with PI and Rnase) was added. The tubes were wrapped in aluminum foil for light exclusion and kept on ice in a cooler. The DNA content was analyzed using two flow cytometers (Becton-Dickinson FACSCalibur in 2014 and Becton-Dickinson LSR II in 2015) and 1000 total events nuclei were collected. All samples were analyzed with the internal standard (*Pisum sativum* L. 'Ctirad'; 2C=8.67 pg) to correct for peak shifting and to verify the interpretation of peak location. The DNA content of each sample was calculated using the following formula:

$$2C \text{ DNA (pg)} = (\text{sample mean G1 peak} / \text{standard mean G1 peak}) * 2C \text{ DNA content of the standard}$$

Data analysis: Percent seed germination was calculated for the self-pollinated and cross-pollinated flowers, and the control cultivar 'Scarlet'. The percentage of stained pollen was calculated to determine pollen viability. DNA content (pg) data were subjected to analysis of variance and means were separated using Tukey's honestly significant difference procedure.

Results

Pollen viability and pollination mechanisms. As indicated by a high level of acetocarmine-stained pollen grains (86.9% to 99.2%), pollen viability was high in all cultivars tested (Table 4.1). These results indicate that in a greenhouse environment, pollen viability would not be a barrier to successful pollination in *Zinnia* species (Table 4.1).

In the subgenus *Diplothrix* none of the self-pollinated (SP) and few of the cross-pollinated (CP) seed germinated (Table 4.1). However, seed of the control cultivar also had germination rates as low as 26.7%, which suggests that the environmental conditions in the greenhouse at that time may have negatively impacted seed germination. cultivars in section *Mendezia* also had low SP and CP seed germination rates, and no SP seed of *Z. greggii* and *Z. maritima* germinated (Table 4.1). *Zinnia angustifolia* had the highest SP seed germination rate (12.7%), an indication that *Z. angustifolia* was partially incompatible.

In the subgenus *Zinnia*, section *Zinnia*, the two *Z. peruviana* cultivars (PI 410405 and PI 442427) and *Z. tenuifolia* (Red Spider) had high levels of self-pollinated seed (66.3%, 69.3%, and 48.1%, respectively). These results confirm those of Torres (1962, 1963) who

reported that *Z. peruviana* was self-compatible. The other cultivars in the section *Zinnia* showed high to moderate levels of self-incompatibility in SP crosses (7.8% to 38.8% seed germination) (Table 4.1).

The results of the flow cytometry tests found significant differences in 2C DNA content among species in sections *Mendezia* and *Zinnia* (Table 4.2). Section *Mendezia* had comparatively smaller and consistent genome size, while genome size in section *Zinnia* ranged from 3.3 to 4.8 pg. 'Old Mexico', a tetraploid cultivar, had a 6.2 pg value. The pg values for *Z. tenuifolia* and *Z. peruviana* were nearly identical. This data and morphological evaluations (data not shown) indicates that 'Red Spider' is not a separate species, but is a cultivar of *Z. peruviana*.

Discussion

The results of our pollination mechanism study supports the findings of Torres (1962, 1964) that species from subgenus *Diplothrix* are self-incompatible. However, as both the self-pollinated and cross-pollinated seeds in this subgenus had low germination rates, further studies are needed to confirm these results. There was a range of pollination mechanisms from self-incompatible, to partial incompatible, to compatible in the subgenus *Zinnia*. Boyle and Stimart (1986) reported that *Z. angustifolia* was largely self-incompatible and that sporophytic control of self-incompatibility was present in both *Z. violacea* and *Z. angustifolia*. *Zinnia* breeding programs may need to remove male disk florets to prevent the occurrence of self-pollinated seed in hybrid crosses.

Although 'Red Spider' is called *Z. tenuifolia* in the commercial sector (e.g., Parks Seed: <http://parkseed.com/red-spider-zinnia-seeds/p/02233-PK-P1/>), the study indicated that this is an error in nomenclature. Torres (1963) and other taxonomist do not include *Z. tenuifolia* in their taxonomic descriptions. The current *Z. tenuifolia* was very similar to *Z. peruviana* for both DNA content and morphological traits, and should not be considered a separate monotypic species.

Flow cytometry data showed significant differences in DNA content between sections in the subgenus *Zinnia*, which agrees with the cytological and morphological studies by Torres (1962, 1963). Anantasaran et al. (2007), using flow cytometry, found that the DNA content for zinnia species ranged from 1.0 pg (*Z. angustifolia*) up to 3.85 pg (*Z. violacea*). In the current study, with *Pisum* 'Ctirad' as the standard, consistent genome size results were obtained over multiple analyzes, using two flow cytometers, and that the pg values for 'Starbright' were 2.0 pg, while the pg values for *Z. violacea* (range of 3.3 to 4.6 pg) were similar to the findings of Torres. Flow cytometry was a useful tool to distinguish between cytotypes within the sections *Mendezia* and *Zinnia*, but was not precise enough to distinguish between species within a section.

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Table 4.1 Results of self- and cross-pollinations and pollen viability test of 16 zinnia cultivars.

Zinnia cultivars	Controlled pollination	Seed tested (no.)	Seed germ. (%)	Control germ. (%)	Viable pollen (%)
Subgenus <i>Diplothrix</i>					
<i>Z. acerosa</i> Ames 21564	SP ^z	537	0	60.0	87.5
	CP ^y	515	3.3		
<i>Z. citrea</i> Ames 21582	SP	524	0	40.0	91.0
	CP	309	3.2		
<i>Z. juniperifolia</i> Ames 21580	SP	212	0	26.7	90.2
	CP	317	1.6		
Subgenus <i>Zinnia</i> , Section <i>Mendezia</i>					
<i>Z. angustifolia</i> 'Summer Solstice'	SP	638	12.7	53.3	92.1
	CP	667	35.1		
<i>Z. bicolor</i> PI 613039	SP	320	0.3	13.3	89.8
	CP	407	6.4		
<i>Z. greggii</i> PI 278179	SP	660	0	40.0	96.0
	CP	703	4.4		
<i>Z. maritima</i> 'Solcito'	SP	870	0	53.3	89.7
	CP	894	1.6		
Subgenus <i>Zinnia</i> , Section <i>Zinnia</i>					
<i>Z. haageana</i> PI 596404	SP	112	17.0	93.3	94.3
	CP	273	61.2		
<i>Z. haageana</i> 'Old Mexico'	SP	230	7.8	100.0	97.7
	CP	308	42.5		
<i>Z. peruviana</i> PI 410405	SP	719	66.3	60.0	94.6
	CP	605	30.9		
<i>Z. peruviana</i> PI 442427	SP	332	69.3	60.0	97.0
	CP	505	51.1		
<i>Z. tenuifolia</i> 'Red Spider'	SP	416	48.1	80.0	87.2
	CP	350	78.6		
<i>Z. species.</i> 'Red Button'	SP	498	22.5	40.0	86.9
	CP	409	51.1		
<i>Z. violacea</i> 'Thumbelina Mix'	SP	615	27.2	100.0	96.3
	CP	335	54.9		
<i>Z. violacea</i> 'Magellan Salmon'	SP	363	38.8	86.7	99.2
	CP	187	58.8		
<i>Z. violacea</i> 'State Fair Mix'	SP	430	15.1	33.3	99.0
	CP	363	24.0		

^zSP=self-pollinated flowers that were bagged prior to anthesis

^yCP=cross-pollinated flower that were pollinated using a mixture of pollen from multiple plants within the variety.

Table 4.2 DNA content as determined by flow cytometry of 13 *Zinnia* cultivars across eight species.

Species	Variety	Mean 2C DNA content (pg±SE)
Section <i>Mendezia</i>		
<i>Z. angustifolia</i>	'Crystal orange'	2.2 ² ± 0.04 f
	'Crystal white'	2.0 ± 0.07 f
	'Starbright'	2.0 ± 0.07 f
<i>Z. bicolor</i>	PI 613039	2.5 ± 0.04 f
<i>Z. greggii</i>	PI 278179	1.9 ± 0.07 f
Section <i>Zinnia</i>		
<i>Z. haageana</i>	'Chippendale'	3.6 ± 0.13 de
	'Old Mexico'	6.2 ± 0.05 a
<i>Z. peruviana</i>	PI 410405	4.3 ± 0.11 cd
	PI 442436	4.2 ± 0.08 cd
<i>Z. tenuifolia</i>	'Red spider'	4.3 ± 0.09 cd
<i>Z. species</i>	'Red button'	4.8 ± 0.13 b
<i>Z. violacea</i>	'Jazzy'	3.3 ± 0.13 e
	'Senora'	4.6 ± 0.04 bc

²Means followed by the same letter are not significantly different (P ≤ 0.05).

APPENDIX

APPENDIX

Temperature and precipitation monthly data for HFL and MHCREC during May – September, 2013.

