EFFECTS OF ETHEPHON ON APOMIXIS IN KENTUCKY BLUEGRASS

By

RYAN M. DADDIO

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And approved by

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ABSTRACT OF THE THESIS

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by RYAN M. DADDIO

Thesis Director:

Dr. William A. Meyer

Apomixis is asexual reproduction via seed. This mode of reproduction can be exploited when an improved genotype is identified, as it can be fixed and reproduced clonally. The problem becomes identifying these novel, improved genotypes. Temporarily disabling apomixis would be an ideal approach to breeding by providing a higher proportion of hybrids to select improved genotypes from. Kentucky bluegrass is a facultative apomict which has led to difficulty in producing improved novel germplasm while retaining a high level of apomictic reproduction, a desired trait in modern cultivars. Through review of literature, it has been hypothesized that ethylene may play a role in the initiation and development of the asexual, apomictic, embryo. To test this hypothesis, 'Rockstar' Kentucky bluegrass clones were pollinized by 'Avalanche' Kentucky bluegrass following a series of treatments with ethephon in a growth chamber. Four timing levels (Early, pre-culm emergence from the boot; Medium, post-culm emergence from the boot; Late, elongation from the boot; and All timings, all three applications timings in series) and two treatment levels (water control and 1000 ppm ethephon) were tested among three replications for a total of 24 experimental units. A sample of 48 progeny from each experimental unit were subjected to analysis with 10 SSR markers to evaluate the

effect of ethephon on reproductive outcomes. No significant difference in the proportion of hybrids was detected as a result of the applications tested.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

Literature Review

INTRODUCTION

Turfgrasses serve many purposes; from utility turfs to prevent erosion and sequester dust within the canopy, to lawn turfs for their ornate qualities and recreational duality, to sports turfs with their recreational focus on sports such as football, baseball, soccer and golf (Turgeon and Kaminski, 2019). It has been estimated that an area of about 163,800 km^2 within the continental United States is cultivated with turfgrasses, three-fold greater than any irrigated crop (Milesi et al, 2005).

Turfgrasses were first used as groundcover of primarily cohabited spaces between livestock grazing and human recreational activities (Turgeon and Kaminski, 2019). One of the earliest records of turfgrass breeding to develop improved cultivars was in the mid-1930s at Rutgers University under H. B. Sprague, who sought to improve velvet bentgrass and ultimately released ‘Raritan’ in 1940 (Funk and Meyer, 2001). Around 1931, Kentucky 31 tall fescue had been released without any explicit breeding effort from a mountain farm in Kentucky. Its total seed production exceeded 80 thousand pounds by 1948, driven by high demand for erosion control (Bailey, 1949). Soon to follow were ‘Merion’ Kentucky bluegrass in 1950 (Wilson and Grau, 1950) and ‘Meyer’ zoysiagrass in 1951 (Grau and Radko, 1951), both cultivars being jointly developed by the United States Golf Association and the United States Department of Agriculture. These early successes paved the way for the development through selective breeding of ‘Manhattan’ perennial ryegrass in 1967, ‘Rebel’ tall fescue in 1980, ‘Banner’ Chewing’s fescue in 1985 and others (Funk and Meyer, 2001).

KENTUCKY BLUEGRASS

Kentucky bluegrass (*Poa pratensis* L.) is one of the most popular cool season turfgrasses, grown primarily in pastures, home lawns and athletic fields throughout much of North America (Bonos et al, 2006). The species is mostly limited to regions north of the transition zone in the United States due to its inadequate heat tolerance. The novel characteristics of Kentucky bluegrass lends itself to a wide range of uses. A key morphological characteristic that stands apart from other popular cool season turfgrasses include a strong rhizomatous growth habit. The rhizomatous growth contributes to its use and predominance in sports turf, with increased sod strength, high mowing quality, resilience, and wear tolerance (Turgeon and Kaminski, 2019).

The ability for Kentucky bluegrass to reproduce asexually via seed, known as apomixis, is another unique characteristic among the cool season turfgrasses, which leads to advantages as well as disadvantages. While uniformity and cultivar perpetuity are enhanced with apomixis, the resulting monocultures lack diversity. The ability of apomixis to fix hybrid vigor also gives the species a pronounced advantage (Bonos et al. 2006), but the lack of genetic recombination limits the ability to obtain such hybrids. Apomixis is believed to have contributed to the species' complicated cytogenetic profile including inconsistent ploidies and deviations from the base chromosome number. These complexities have increased the difficulty of understanding the heritability of traits within the species (Hartung, 1946).

Kentucky bluegrass has a highly variable ploidy level with a base number of 7 chromosomes (n=7). While there is no definitive ploidy range, chromosome counts from 42 to 87 are commonly represented in the literature with an overall range of 28 to 154 having been

observed (Brown,1941; Hartung, 1946; Åkerberg, 1939; Grazi et al., 1961; Muntzing, 1933). Aneuploidy and euploidy are readily fixed as a result of apomixis and both can accumulate through generations (Hartung, 1946). A more recent cultivar analysis utilizing flow cytometry found a range from pentaploid (2n = 5x = 35) to quindecaploid (2n = 15x = 105) with multiple cases of aneuploidy reported among the 22 samples analyzed (Eaton et al., 2004). In summary, ploidy in Kentucky bluegrass can vary from 2n = 4x = 28 to 2n = 22x = 154 with deviations from expected multiples of the base number as a result of aneuploidy.

APOMIXIS

Apomixis is a complex reproductive mechanism lacking a complete genetic understanding. The name is derived from apo- meaning “without”, and -mixis, meaning “mixing”. Some of the challenges in explaining apomixis originates in the variation of apomixis that exists naturally. Forms of apomixis have been classified based on the development timing, location and process. Gametophytic apomixis describes early development of a mitotic, unreduced embryo sac. If this embryo sac is developed in place of the megaspore mother cell (MMC), it is classified as diplospory. Formation of the mitotic unreduced embryo sac elsewhere in the nucellar tissue is termed apospory (Koltunow and Grossniklaus, 2003). Diplospory is functionally unique as it requires the failure of the sexual pathway to develop the apomictic sac (Nogler, 1984; Asker & Jerling, 1992). Yet another classification is adventitious embryony, where the embryo develops directly from the initiating cell and usually occurs later in in ovule development. Combinations of multiple types of apomixis, known as mixed apomixis, has also been known to occur (Koltunow and Grossniklaus, 2003). The apomictic embryo is produced mitotically and bypasses fertilization via the process of parthenogenesis (Nogler, 1984). Facultative apomixis is a process where varying percentages of apomictic progeny allowing for

variable proportions of meiotically derived embryos to develop (Norrmann et al., 1989; Koltunow, 1993; Hojsgaard et al., 2008). While facultative apomixis is more common, obligate apomicts lack variation and reproduce strictly through asexual processes (Kao, 2007; Sorensen et al., 2009). The actual existence of obligate apomicts has been questioned in the literature (Asker & Jerling, 1992; Rebozzio et al., 2011). The gametophytic apomictic system can be summarized in three distinct developmental steps toward asexual reproduction: Apomeiosis, in the forms of diplospory and/or apospory; parthenogenesis; and functional endosperm development, autonomous (without fertilization) or pseudogamous (requiring fertilization) (Nogler, 1984; Asker and Jerling, 1992). These distinct developmental pathways have been shown to be uncoupled (Albertini et al., 2001a; Kaushal et al., 2008; Conner et al., 2013).

Apomixis in Kentucky bluegrass is a facultative aposporous apomict. It has been shown that parthenogenesis in Kentucky bluegrass is not coupled with the initiation of apospory (Albertini et al., 2001b), fertilization of the mitotic unreduced embryo can occur (Tinney, 1940) and co-development of multiple embryos can result in seeds with twin and triplet embryos (Nielsen, 1946). Development of the endosperm requires fertilization, without which, viable seed cannot be produced, a requirement referred to as pseudogamy (Naumova et al., 1993). Kentucky bluegrass, as an aposporous parthenogenic pseudogamous apomict, leads to difficulties in developing improved hybrids in breeding programs.

REPRODUCTIVE DEVELOPMENT IN KENTUCKY BLUEGRASS

Kentucky bluegrass follows the Polygonum type of female gamete development while the apomictic development follows the aposporous Hieracium type (Rutishauser, 1969). Abeln et

al. (1985) provides the most comprehensive and supported description of both sexual development and aposporous development in Kentucky bluegrass and is the basis of the following summary. In a sexual development pathway, the appearance of megaspore mother cells (MMCs) in young hemitropous ovules is first observed. Early development continues with the MMC stretching toward the chalaza from the micropyle end. Meiotic division occurs in the chalazal dyad prior to the micropylar, resulting in four megaspores across the chalaza-mycropylar line. The chalazal megaspore, encapsulated in callose, continues development while the remaining three megaspores degenerate into a cap on the micropylar end. While developing toward the micropyle, the nucleus undergoes mitosis creating an enlarged two nucleate cell. A vacuole enlarges between the nuclei with another developing at the chalazal end. The two nuclei divide to form a four nucleated cell, now separated from the micropyle by only one layer of nucellar tissue. This is referred to as the tetrad stage. A third mitotic division results in eight nuclei and widens the cell. Differentiation of the cell forms the embryo sac containing seven cells with one being a central two nucleated cell. Three cells positioned on the chalazal end become antipodals. The two nucleate central cell fills two thirds of the embryo sac while the antipodals enlarge. At the micropyle end, the egg cell is flanked by two synergids (Tinney, 1940; Åkerberg, 1942; Grazi et al., 1961; Abeln et al., 1985).

In the Kentucky bluegrass apomictic pathway, development of the aposporous embryo sac follows a similar development pattern as the sexual embryo, but with mitosis replacing meiosis. The aposporous initials can be observed developing beginning with the single nucleate megaspore stage of the sexual MMC until the tetrad stage, enlarging more rapidly compared to the sexual embryo (Åkerberg, 1942; Abeln et al., 1985). The presence of aposporous initial development induces changes in the developing sexual embryo sac. These changes include

slowed or reversed callose deposition (Naumova et al., 1993) and the eventual degeneration of the developing sexual embryo sac prior to the enlargement of the antipodals (Tinney, 1940; Grazi et al., 1961; Nygren, 1967; Abeln et al., 1985). The embryo sac development, starting at the chalazal end, contours the sexual embryo sac to reach toward the micropyle (Abeln et al., 1985). Aposporous embryo sacs, with their proximity to the micropyle, may be subject to fertilization and result in recombinant progeny (Grazi et al., 1961). Polyembryony is also possible with the sexual and one or more aposporous embryos surviving to maturity (Huff et al., 2003). More research is needed into the possibilities of progeny outcomes.

Differences between the sexual and apomictic embryo development can be valuable in understanding apomixis further. Callose deposition in the development of the sexual embryo is important to mention as it is one of the few differences, aside from the initiation of the aposporous embryo sac and apo-meiotic nature, between sexual and asexual embryo development. Callose is common in angiosperm MMC development and is hypothesized to contribute to chemical signaling isolation to protect the meiotic process (Rodkiewicz, 1970; Bouman, 1984; Carman et al., 1991; Ünal et al, 2013). This deposition in developing MMC walls is believed to be an artifact from walled spores of ancestral land plants (Graham and Taylor, 1986). Absence of callose in aposporous development, but its presence in sexual embryo development, has been noted in *Eulaliopsis binata* (Li et al., 2011) and in Kentucky bluegrass (Willemse and Naumova, 1992). A complete lack of callose deposition in both sexual and apomictic development has been noted in diplosporous species such as *Elymus rectisetus* (Carman et al., 1991). While more research may be necessary, the consensus appears that callose deposition, in contrast to sexual development, does not occur in the aposporous embryo sac development.

In Kentucky bluegrass, callose deposition occurs in the MMC walls during development through the tetrad stage, at which point, it is rapidly removed via callase (Bouman, 1984). Early aposporous initial development, between the appearance of the MMC and the tetrad stage, correlates with reduced callose deposition closest to the micropyle in the MMC wall (Naumova et al., 1993). This prematurely restricted deposition prior to the tetrad stage in the presence of aposporous initials has been observed in other aposporous species including *Megathyrsus maximus*, *Paspalum squamulatum*, and *Cenchrus ciliaris*, in addition to Kentucky bluegrass, when aposporous initials develop (Peel et al., 1997). Investigations in Tucker et al. (2001) show that apomictic development is not a result of callose deposition, but rather a causal factor of its dissolution.

BREEDING IMPROVED CULTIVARS

Until 1970, cultivars of Kentucky bluegrass were mostly blends of apomictic collections from naturalized stands or older turfgrass stands (Bonos et al, 2000). Ecotype selection and intraspecific breeding dramatically increased the numbers of improved cultivars on the market (Eaton et al., 2004). Kentucky bluegrass breeders have had to incorporate creative approaches to obtain novel germplasm to evaluate for turf and forage applications, attributable to its apomictic breeding system. Early work has explored both intraspecific and interspecific hybridization approaches (Huff et al., 2003). Continuing work to the present has incorporated selective screening techniques to more efficiently extract hybrids from highly apomictic seedling populations. Various techniques have attracted attention from breeders with the use of molecular markers for enhanced hybrid and cultivar identification (Mazzacuto et al., 1995); soma-clonal variation arising from tissue culture techniques (Ke and Lee, 1996), mutagen induced variations

(Powell, 1976) and gene editing (Ha et al., 2001) having been explored. While these methods of incorporating genetic variation would be investigated independent of the apomictic breeding habit of the species, they have more potential in Kentucky bluegrass as a result of its complicated genome and asexual reproductive system. Therefore, approaches of apomixis manipulation would be ideal for the advancement of Kentucky bluegrass breeding and have been explored with no positive results to date.

Hybridization techniques to maximize hybrid seed development using greenhouse isolation was first outlined by Funk and Sang (1967) and refined by Pepin and Funk (1971). Development of novel genotypes through hybridization generally replaced or complimented collection approaches (Curley and Jung, 2004). Intraspecific and interspecific crosses were extensively researched throughout the 20th century with many of the commercial cultivars resulting from intraspecific hybridization. Almost all commercially available cultivars in the 2017 National Turfgrass Evaluation Program (NTEP) Kentucky bluegrass trial were products of intraspecific hybridization (NTEP, 2018). Selective screening is an approach to compliment either hybridization technique, with little published work, that has been applied in the Rutgers breeding program to increase the efficiency of the isolation of potentially improved genotypes for further evaluation.

Intraspecific hybrids, referring to crosses within the Kentucky bluegrass species, have been the most fruitful approach in the development of improved genotypes. The most vigorous selections are typically identified, and flowering times manipulated to match. “Hybrid” seed is harvested from the mother of the cross and grown in various ways to screen the population for phenotypically unique individuals (Pepin and Funk, 1971). Progeny that appears different from the mother are not always hybrids as variation can occur due to non-uniform environmental

conditions in addition to a variety of genetic origins, such as those resulting from unreduced gametes, self-pollination, aneuploidy and unfertilized reduced gametes (Huff and Bara, 1993). Use of sexual mothers has been attempted but was shown to result in less apomictic progeny (Myers, 1943). The use of apomictic, as opposed to sexual, parents has been utilized for intraspecific hybridization to enable the development of highly apomictic progeny, a critical trait for commercialization (Bashaw and Funk, 1987). This comes at the cost of few novel progeny to evaluate (Huff et al., 2003).

Interspecific hybrids include progeny from any other compatible *Poa* species crossed with Kentucky bluegrass. Pepin and Funk (1971) conclude that improved selections from interspecific crosses were readily identified in field evaluations and likely a product of ploidy increase. While Kentucky bluegrass can be used as the mother in such crosses, apomixis limits the number of hybrid progeny and therefore is likely to be used as the pollen donor. Texas Bluegrass (*Poa arachnifera*) has been the most common and persistent candidate for producing interspecific hybrids with Kentucky bluegrass due to its improved heat and drought tolerance and dioecious breeding system. While research has found Texas bluegrass incorporation has improved heat and drought tolerance in the hybrids (Abraham et al., 2008), some research provided mixed results (Abraham et al., 2004). This approach has had limited application in the development of commercially available cultivars. This limitation is likely due to the limited seed production capacity of the interspecific hybrids. While many other *Poa* species are compatible with Kentucky bluegrass, such as *Poa compressa* (Dale et al., 1975), *Poa ampla* (Grun, 1954), *Poa scabrella* (Grun, 1954), and *Poa alpina* (Akerberg, 1942), they have failed to be incorporated into any commercially available turfgrass cultivars to date.

Breeding outcomes in Kentucky bluegrass are complicated by variable ploidy in combination with the apomictic reproductive process. Progeny type can be classified by the following: Apomict, resulting from an unreduced maternal gamete and no external paternal gamete contribution; B_I, or polyhaploid, from a reduced maternal gamete and no external paternal gamete contributions; B_{II} hybrid, from the fusion of a reduced maternal gamete and reduced paternal gamete; B_{III}^M hybrid, from the fusion of an unreduced maternal gamete and reduced paternal gamete; B_{III}^P hybrid, from the fusion of a reduced maternal gamete and unreduced paternal gamete; B_{IV} hybrid, from the fusion of unreduced maternal and paternal gametes (Huff et al., 2003; Bushman et al., 2018). Identification of progeny classification can be conducted through the combination of molecular markers and flow cytometry (Huff et al., 2003; Bushman et al., 2018).

Considerations in the ploidy levels of offspring should be established as meiosis has been shown to be highly irregular in Kentucky bluegrass (Müntzing, 1932; Flovik, 1938; Åkerberg, 1942). Plants have been shown to produce viable pollen and successfully reproduce with ploidy levels as low as 2n = 45 to 50 (Grazi et al., 1961) with ploidies of 2n = 18 having been reported (Åkerberg and Bingefors, 1953). This irregularity suggests dramatic fluctuations in progeny ploidy levels.

Assessing aberrants, hybrid or other, to determine rates of apomixis is crucial in a breeding program. Field evaluation is a straightforward approach, relying on phenotypic identification of progeny aberrants to determine apomictic levels of a plant at anthesis. The phenotyping method has the strength that visual observations are in essence the most important correlation with turfgrass uniformity, but the weakness of lengthy time and space requirements. Alternative lab-based procedures have been developed as well. Flow Cytometric Seed Screen

(FCSS) utilizes flow cytometry to evaluate nucleus sizes in seed embryo and endosperm to discern apomictic seed from aberrant seed (Matzk et al., 2000), but the tested seeds are concomitantly destroyed in the process. Molecular markers are able to show 'bands' or alleles from both parents, but not able to detect ploidy changes in progeny. The auxin test involves an auxin treatment at anthesis to create a morphological difference, observable with a light microscope, between the developed apomictic seed and aberrant seed (Matzk, 1991). While destructive sampling is useful in progeny testing of new genotypes for apomictic rates, any aberrants detected with these methodologies cannot be recovered and further evaluated.

PROGENY IDENTIFICATION AND CHARACTERIZATION

The broad concept of a methodology to rapidly classify organisms is not new. Phenotypic classification has been explored in some depth while genotypic classification methodologies have come to light more recently. Phenotypic approaches have included morphology (Bonos et al., 2000; Shortell et al., 2009) as well as protein polymorphisms, or isozymes (Weeden et al, 1985). These have been difficult to utilize with influence from environmental factors, limited numbers of identifiable polymorphisms (Lombard et al., 2000) and scalability (Shortell et al., 2009) being of major concern.

There is a general demand for high throughput identification of cultivars and accessions (Cooke and Reeves, 2003). Morphological systems can be utilized and have been investigated resulting in the Pedigree, Turf Performance and Morphological (PTM) Kentucky bluegrass classification system (Bonos et al., 2000; Shortell et al., 2009; Honig et al., 2010). Molecular markers such as Randomly Amplified Polymorphic DNA (RAPD) were quickly improved to be equally reliable to isozymes or morphology at identifying apomictic rates of progeny (Mazzucato et al., 1995). The PTM Kentucky bluegrass classification system was subsequently improved

through SSR marker analysis, in association with pedigree data (Honig et al., 2010). Microsatellite markers such as Simple Sequence Repeats (SSRs) are codominant and highly polymorphic PCR based markers which have been utilized in Kentucky bluegrass (Honig et al., 2010; Honig et al., 2012; Bushman et al., 2013; Raggi et al., 2015; Honig et al., 2018; Xiaojun et al., 2018). Other PCR based marker types used in Kentucky bluegrass include the arbitrary-primed RAPDs (Mazzacuto et al., 1995; Barcaccia et al., 1997; Arnholdt-Schmitt, 2000; Huff, 2001; Johnson et al., 2002; Curley and Jung, 2004; Ning et al., 2005; Fard et al., 2012; Szenejko and Rogalski, 2015; Szenejko et al., 2016; Rodrigo et al., 2017) and AFLPs, Amplified Fragment Length Polymorphisms (Van Treuren, 2008; Hojsgaard et al., 2013; Rebozzio et al., 2011) while sequence tagged site markers include ISSRs, Inter-simple Sequence Repeats (Goldman, 2008; Szenejko et al., 2016); SCARs, Sequence Characterized Amplified Regions (Albertini et al., 2001); and SRAPs, Sequence Related Amplified Polymorphisms (Xiaojun et al., 2018).

Molecular markers have applications beyond “fingerprint” classification including diversity studies, genetic mapping, QTL analysis and marker assisted selection (Zhang et al., 2006). RFLPs, Random Fragment Length Polymorphisms, are reproduceable and codominant but as hybridization-based markers, the process is slow and requires large amounts of high-quality DNA (Zhang et al., 2006). The following are PCR-based markers, enabling higher throughput and requiring lower quantities of DNA. RAPDs are less reproducible and dominant, but high levels of polymorphisms, readily available primers, and low price make them highly appealing (Curley and Jung, 2004; Zhang et al., 2006). AFLPs are comparable to RAPDs with a key difference being a requirement for enzymatic digestions, with improved reliability at the cost of limited comparability between studies due to their population specific nature (Zhang et al., 2006). These random primer-based markers needed to be converted to other PCR based markers

for applications such as marker assisted selection (Paran and Michelmore, 1993). ISSRs, comparable to SSRs, outperform in comparison as they do not require often costly and limited prior sequence knowledge (Goldman, 2008). SCARs are targeted markers utilizing longer primers to flank polymorphic DNA fragments (Paran and Michelmore, 1993). SRAPs are codominant and highly polymorphic markers that preferentially amplify ORFs (Open Reading Frames) and are utilized for diversity, QTL and gene tagging studies (Xiaojun et al., 2018). SNPs, Single Nucleotide Polymorphisms, are sequencing based markers that refer to polymorphisms of single nucleotides, are the most common type of markers identified in more recent genotyping-by-sequencing protocols, and possess the most potential for applications such as functional markers (Zhang et al., 2006).

Polyploidy increases the difficulty of almost all genomic analyses, from molecular markers to genotyping-by-sequencing through complex and repetitive genomes. Co-dominant markers such as SSRs in polyploids, including Kentucky bluegrass, result in phenotypic banding patterns rather than allele calls (Kosman and Leonard, 2005) and are commonly scored as individual presence or absence binary data as a result (Honig et al., 2012; Bushman et al., 2013; Raggi et al., 2015; Zhao et al., 2015; Honig et al., 2018). This is because scoring can be complicated by limitations in assigning alleles and determining allelic dosage (Kosman and Leonard, 2005; George et al., 2006; Bushman et al., 2013).

MANIPULATING APOMIXIS

No major seed crop is apomictic, but the benefits of incorporating apomixis would be revolutionary (Hand and Koltunow, 2014). From a breeding perspective, the need to switch

between sexual and apomictic reproduction is paramount in apomictic species (Kumar, 2017; Kaushal et al., 2019). The underlying control of apomixis has been proposed in many directions from qualitative inheritance (Ozias-Akins and van Dijk, 2007) to quantitative inheritance (Matzk et al., 2005) and the further complications involving epigenetic control (Kumar, 2017). Investigations into the genetic components of apomixis have been conducted across many apomictic species such as *Panicum maximum* (Ebina et al., 2005); *Paspalum simplex* (Calderini et al., 2006); *Hypericum perforatum* (Schallau et al., 2010); *Hieracium, Pennisetum,* and *Cenchrus* (Okada et al., 2011); and *Boechera* (Corral et al., 2013). Investigation into the genetic control of parthenogenesis (Conner et al., 2015) and endosperm development (Siena et al., 2016; Henderson et al., 2017) have been investigated to a lesser extent. Approaches utilizing chemical or environmental influences on existing apomictic systems have been evaluated (Rebozzio et al, 2011; Takahara et al., 2014; Takahara et al., 2016; Rodrigo et al., 2017) and will be discussed further, along with chemical induction of apomixis in otherwise sexual species (Chen et al., 2018; Gaafer et al., 2018) with no success. The ability to create artificial apomictic phenotypes has recently been demonstrated (Khanday et al., 2019) and will be discussed in more detail.

A variety of genetic components involved in apomixis have been rapidly identified in a number of model aposporous species including members of *Brachiaria, Pennisetum, Crenchrus, Hieracium, Paspalum* and *Poa,* with many additional diplosporous species (Kaushal et al., 2019). While ploidy level, due to its correlation, has been hypothesized to cause or increase levels of apomixis, genetic contributions have ultimately been credited (Matzk et al., 2005; Voigt-Zielinski et al., 2012; Schinkel et al., 2016; Kaushal et al., 2018). In *Pennisetum ssp.* and *Crenchrus ciliaris* an entire region associated with apospory has been identified and named Apospory Specific Genome Region (ASGR) (Akiyama et al., 2005; Conner

et al., 2008). Other proposed genetic components to apomixis include: Apomixis Controlling Locus (ACL) in *Paspalum simplex* (Calderini et al., 2006); Somatic Embryogenesis Receptor-like Kinase (SERK) and APOSTART in *Poa pratensis* (Albertini et al., 2005); Loss of Apomeiosis (LOA) in *Hieracium* subgenus *pilosella* (Okada et al., 2011); Apospory (Apo) locus in *Panicum maximum* (Ebina et al., 2005; Takahara et al., 2014); Apomixis Linked Locus (APOLLO) in *Boechera spp.* (Corral et al., 2013); and HAPPY and HpARI genes in *Hieracium perforatum* (Schallau et al., 2010). For the parthenogenesis step in the apomictic pathway the Apospory Specific Genomic Region-Baby Boom (ASGR-BBML) in *Pennisetum squamulatum* (Conner et al., 2015), also known as the PsASGR-BBML gene in *Pennisetum glaucum* (Conner et al., 2017) has been shown to be a major genetic component. The Baby Boom (BBM1) ectopic expression in the ovule has been shown to induce parthenogenesis in *Oryza sativa* (Khanday et al., 2019). When BBM1 is paired with Mitosis instead of Meiosis (MiMe) (d'Erfurth et al., 2009), asexual reproduction via seeds is attained (Khanday et al., 2019), the first report of artificial apomixis in an otherwise sexual species.

Genes related to endosperm development involved in apomictic development are less prolific but include Origin Recognition Complex (PsORC3a) in *Paspalum simplex* (Siena et al., 2016) and AED (AutE) in *Hieracium* (Henderson et al., 2017). The autonomous endosperm fill could be a highly desirable component with regards to maximizing seed yield. These findings bring the apomixis cassette containing components that would lead to apomeiosis, parthenogenesis and autonomous endosperm development proposed by Conner and Ozias-Akins (2017) to reality. More research into the application and morphology of the embryo sac development in the artificial apomixis system is necessary, but the identification of artificial apomixis processes are clearly underway.

In Kentucky bluegrass there has been limited research into the genetic control of apomixis. It has been suggested that parthenogenesis is unlinked from apomixis (Albertini et al., 2001a). Parthenogenesis has been shown to be preferentially expressed in the aposporous embryo but can be expressed in the sexual embryo as well, including those of non-apomictic genotypes (Baraccia et al., 2001). Markers have also been developed to discern apomictic germplasm (Albertini et al., 2001b). *Pp*SERK and APOSTART, identified in Kentucky bluegrass, differ in expression between sexual and apomictic genotypes. The role of these genes involves hormone signaling and cell-to-cell interactions (Albertini et al., 2005). Albertini et al. (2005) further propose *Pp*SERK as the switch that redirects embryo sac development gene products to alternative cells when activated, suggesting its responsibility for the preferential development of the apomictic embryo sac.

Epigenetic control has more recently been considered a significant contributing factor (Bocchini et al., 2018). Expression alterations in ovules of apomicts has resulted in further confidence and interpretation of epigenetic factors (Polegri et al., 2010; Sharbel et al., 2010; Baroux et al., 2011; Pupilli & Barcaccia, 2012). Disturbances in the timing of the sexual development pathway allowing for apomixis has been proposed and demonstrated (Grimanelli et al., 2003; Sharbel et al., 2010; Koltunow et al., 2011; Hojsgaard et al., 2012; Tucker et al., 2013). Epigenetic control of apomixis is further supported by the development of an artificial apomict via ectopic expression of BBM1 in the egg cell (Khanday et al., 2019) in addition to studies regarding environmental effects on apomictic development including salt stress (Gournaris et al., 1991) and drought stress (Carman et al., 2015; Kumar, 2017; Rodrigo et al., 2017). Cyto-embryological investigation shows increased sexual embryo dominance over apomictic embryo development under stress. Unfortunately, this does not result in more sexual progeny,

presumably due to abortion of embryos as a result of the very stress that inhibited the apomictic dominance (Gournaris et al., 1991; Carman et al., 2015; Kumar, 2017; Rodrigo et al., 2017). It has been suggested that apomixis is a consequence of suppressed expression of sexual reproduction genes that can be modified through epigenetic changes triggered by environmental influences (Rodrigo et al., 2017). The rate of sexual reproduction in Kentucky bluegrass has been shown to be higher in greenhouse conditions and with hot water shock treatment of florets but remained unaffected by position of floret, daylength, light intensity, or nitrogen fertility (Han, 1970).

PLANT GROWTH REGULATORS

Many exogenous applications to manipulate apomixis involved plant growth regulators including auxins, cytokinins and gibberellins have been investigated with little to no positive results (Han, 1970; Matzk, 1991). Many plant growth regulators are readily available with documented effects on plant growth and development with respect to exogenous applications and endogenous presence, which would be desirable as a simple, inexpensive and reproducible treatments for breeding programs. The suggestion that stress would inhibit initiation of apomixis may provide an additional operational window for exogenous plant growth regulators to manipulate levels of apomixis.

Plant growth regulators (PGRs) include organic compounds that modify growth or development in plants without providing nutrition. This includes naturally occurring phytohormones and synthetic analogs (Basra, 2000). While they do not typically result in phytotoxicity (Rademacher, 2015), some synthetic auxins have proven to be highly valued selective herbicides (Grossmann, 2010). The most prevalent phytohormones include auxins, gibberellins, cytokinins, abscisic acid and ethylene with more recent additions including

oligosaccarins, brassinosteroids, jasmonates, salicylates and polyamines (Basra, 2000). The number of identified phytohormones has grown dramatically from the discovery of the first plant growth regulator, ethylene, which was observed inhibiting elongation and radial growth in peas (Neljubow, 1901). Plant growth regulators, such as ethylene, have been in use long before that first discovery, with wounding and gassing applied to hasten fruit ripening. While phytohormones manipulate almost every aspect of plant growth and development, their utility can be limited by complex interactions that can be difficult to dissect and costly to research (Rademacher, 2015).

Auxins have been shown to promote cell elongation and cell division owing to its contribution to phototropic and gravitropic responses as well as apical dominance (Cline, 1991). It can inhibit the very growth it promotes if concentrations become too high and is responsible for the attraction of assimilates in tissues. Auxins are applied in plant propagation techniques including tissue culture and rooting cuttings. They are also used for flower and fruit thinning at high doses as well as fruit retention at lower doses and synthetics have found applications as herbicides (Basra, 2000; Rademacher, 2015).

Cytokinins are responsible for the stimulation of cell division in meristematic tissues and attraction of assimilates into sinks leading to effects such as the opposition of auxin induced apical dominance and the delaying of leaf senescence. Applications of cytokinens include delaying onset of drought symptoms (Liu and Huang, 2002), promotion of leaf drop and early fruit thinning (Basra, 2000; Rademacher, 2015). Abscisic acid is produced in association with abiotic stressors and induces stomatal closure, cold hardiness and inhibition of precocious seed germination. Abscisic acid has limited applications due to high costs in manufacturing (Basra, 2000; Rademacher, 2015). Gibberellins induce longitudinal growth, bolting in long day plants,

hydrolytic enzyme induction in germinating seeds and plays a role in fruit setting and development. Application of gibberellins are limited, but giberillin inhibitors enjoy a wide range of use as plant growth regulators due to internode shortening, and fungicides due to overlap in biosynthetic pathways (Basra, 2000; Rademacher, 2015).Ethylene can stimulate or inhibit growth (depending on concentration), induce flower initiation and tissue senescence, promote fruit ripening, and aid in the development of abscission layers. Ethylene production can be enhanced by both auxins and cytokinins. Applications in flowering initiation, lodging prevention, defoliation and increasing latex flow in rubber trees are made simple through exogenous ethylene application. Ethylene inhibitors, on the other hand, are used to delay senescence, prolong cut flower life, and enhance drought and heat resistance (Basra, 2000; Rademacher, 2015).

SOMATIC EMBRYOGENESIS

Somatic embryogenesis is the development of embryos from somatic tissue *in vitro*. One of the critical stages of apomixis include the development of an apomictic initial cell that then develops into an embryo sac (aposporous in the case of Kentucky bluegrass) bearing a clonal embryo that arises from somatic tissue. Links between somatic embryogenesis and apomixis can be observed in a variety of processes. Enrichment of spermidine metabolism related genes associated with apomixis in *Boechera gunnisoniana* (Schmidt et al., 2014) is comparable to the elevated spermidine levels associated with somatic embryogenesis in many species including *Panax ginseng* (Monteiro et al., 2002), *Solanum melongena* (Sharma et al., 1995), and *Pinus radiate* (Minocha et al., 1999). Expression of *Pp*SERK in Kentucky bluegrass apomicts (Albertini et al., 2005) can be compared to increases in SERK expression during initiation of somatic embryogenesis that has been documented in many plant species including *Oryza sativa*

(Hu et al., 2005), *Helianthus annuus* (Thomas et al., 2004), and *Solanum tuberosum* (Sharma et al., 2008). SERK expression has also been associated with apomixis in other species such as *Paspalum notatum* (Podio et al., 2014). BBM1 ectopic expression has been shown to induce somatic embryogenesis in cotyledons of Arabidopsis (Horstman et al., 2017) while the expression of BBM1 in combination with MiMe to induce artificial apomixis in rice (Khanday et al., 2019) further strengthens the developmental overlap of apomictic development and somatic embryogenesis. Detailed hormonal research into *in vivo* apomictic embryo development is extremely limited, however, somatic embryogenesis in tissue culture is not. Much of the research involves sources of tissue but more importantly, plant growth regulator composition within the culture media (Zimmerman, 1993). The following explores the potential of plant growth regulators to influence the development of the apomictic initial or embryo initiation.

Auxins and cytokinins are necessary components to almost all successful somatic embryogenic media compositions (Jiménez, 2005). Cytokinins have been shown to be more involved in the growth of cultures rather than their aid in the initiation of embryogenesis (Jiménez and Bangerth, 2000). In a transcriptome analysis of diplosporous *Boechera gunnisoniana*, a cytokinin degradation upregulation in apomictic development contrasts the cytokinin modification genes upregulated in the egg cell (Schmidt et al., 2014), which indicate that cytokinins may play a role in the development of the apomictic embryo sac. Cytokinin levels have been correlated with reduced somatic embryogenesis in leaves of eggplant (Gleddie et al, 1983) and orchardgrass (Wenck et al, 1988) with conclusions of pH being the causative factor. While cytokinins appear to inhibit somatic embryogenesis, auxin consistently enhances the process (Jiménez, 2005). The transcriptome analysis of *Boechera gunnisoniana* also found auxin

signal transduction gene expression not found in the megaspore mother cell (Schmidt et al., 2014).

Endogenous abscisic acid has been found in many species to be critical in the initiation of somatic embryogenesis (Jiménez, 2005), but have been found to be detrimental in others such as alfalfa (Ivanova et al., 1994). Addition of abscisic acid has been shown to increase success of somatic embryogenesis in coniferous species (Pullman et al., 2003), *Daucus carota* (Kamada and Harada, 1981; Nishiwaki et al, 2000; Kikuchi et al., 2006) and *Cocos nucifera* (Fernando and Gamage, 2000). Abscisic acid inhibits ethylene production in the successful indirect somatic embryogenesis of alfalfa (Kępczyńska et al., 2009a).

Analysis of endogenous levels of gibberellins during somatic embryogenesis have yielded no consistent results of their importance (Jiménez, 2005). However, exogenous gibberellins have been shown to promote somatic embryogenesis while giberillin inhibitors, such as paclobutrazole, prevent the process in *Medicago sativa* (Ruduś et al., 2002). Endogenous gibberellins have been found to be involved in *in vitro* somatic embryogenesis with differential presence of polar and less-polar GA types in successful embryo development (Noma et al., 1982).

Ethylene has been shown to restrict embryogenic callus initiation in corn (Vain et al., 1989). Low levels of ethylene and its biosynthetic precursors were shown to stimulate embryogenesis while higher levels proved to be inhibitory (Nissen, 1994). Elevated levels of endogenous ethylene have been correlated to non-embryogenic tissue, compared to embryogenic tissue (Jiménez, 2005), with exogenous ethylene resulting in consistent inhibition of somatic embryogenesis at multiple stages (Kong and Yeung, 1994). Inhibitors of endogenous ethylene have been demonstrated to improve rates of somatic embryogenesis (Roustan, 1989). A QTL for

apomixis in citrus was traced back to an ethylene receptor (Garcia et al., 1999). Ethylene has also been shown to inhibit embryogenesis of nucellar tissue *in vitro* (Tisserat and Murishige, 1977). The BBM gene family involvement in apomixis in *Paspalum squamulatum* where BBM-like *Ps*ASGR-BBML is expressed in apomictic egg cells prior to double fertilization and expression results in parthenogenesis (Conner et al., 2017). This gene family is known to encode members of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) (León-Martínez and Vielle-Clazada, 2019) and further strengthens the connection between ethylene and apomixis. It has also been hypothesized that nucellar receptors for ethylene may prevent the initiation of nucellar embryos (Asins et al., 2002).

Brassinosteroids have been shown to increase embryogenic tissue initiation rates, via somatic embryogenesis, in conifers, *Oryza sativa* (Pullman et al., 2003b), *Cocos nucifera* (Azpeitia et al., 2003) and *Gossypium* (Aydin et al., 2006). SERK1 and SERK3 involvement in the brassinosteroid signaling pathway (Karlova et al., 2006) observed in Arabidopsis roots and hypocotyls (Albrecht et al., 2008) reinforces the contribution of brassinosteroids to somatic embryogenesis. It is important to note that brassinosteroids are not required for somatic embryogenesis, but rather compliment auxin in the process (Azpeitia et al., 2003).

Jasmonic acid (jasmonate) has been shown to have a similar, though weaker, effect on somatic embryogenesis compared to abscisic acid (Ruduś et al., 2006). Jasmonate leads to the stimulation of auxin (Mira et al., 2016), which may be the cause of the observed effects in somatic embryogenesis (Ruduś et al., 2009).

SUMMARY AND OBJECTIVE

A candidate hormone to manipulate apomixis in Kentucky bluegrass would need to replicate a stress response while allowing the sexual embryos to develop, to avoid previously defined complications with stress induced sexuality (Rodrigo et al., 2017). The negative results of tested hormones further narrow the potential options by eliminating Auxins, Cytokinins and Giberellins (Matzk, 1991). According to the research conducted thus far, the stress hormone abscisic acid has only been shown to promote somatic embryogenesis in most cases (Kamada and Harada, 1981; Nishiwaki et al, 2000; Fernando and Gamage, 2000; Pullman et al., 2003; Jimenez, 2005; Kikuchi et al., 2006) while they have been found to be detrimental in alfalfa (Ivanova et al., 1994), orchard grass (Wenck et al., 1988), and eggplant (Gleddie et al., 1983). Limited experimentation with jasmonic acid, brassinosteroids and other stress related hormones *in vitro* (Jiménez et al., 2005) diminishes the ability to hypothesize their potential as a switch to apomixis.

Ethylene, however, results in several observed effects in somatic embryogenesis that may enable a greater level of sexual reproduction in aposporous species. Reduced endogenous levels of ethylene have been found to be involved in the indirect somatic embryogenesis of Medicago sativa (Kępczyńska et al., 2009). Though endogenous ethylene is typical in somatic embryogenesis (Nissen, 1994), possibly owing to weak production resulting from the presence of auxin and cytokinin (Rademacher, 2015), artificial elevation of the concentration results in inhibition (Nissen, 1994).

The sexual germline, buffered from the external ovule environment by callose, may offer protection to the developing sexual embryo(Rodkiewicz, 1970; Bouman, 1984; Carman et al., 1991; Ünal et al, 2013), while apomictic initials would be subjected to the inhibitory effects of

high levels of ethylene. In this research, it is hypothesized that targeted applications of exogenous ethylene, via ethephon, will result in higher rates of sexual progeny. Timing would be paramount in this approach with precise applications before anthesis being critical to target the initiation of the apomictic initials or their subsequent embryo sacs. Progeny assessment using molecular markers (SSR) can provide us with valuable information regarding rates of apomictic and hybridization events resulting in live progeny. These data can be utilized to assess the treatment effects on reproductive behavior among these categories.

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CHAPTER II

Evaluating Effects of Ethephon in Kentucky Bluegrass

INTRODUCTION

Ethylene has a relatively simple biosynthesis *in planta* involving only three steps. L-methionine is the initial substrate and is converted into S-adenosylmethionine (AdoMet) through the addition of ATP. The second step is catalyzed by ACC synthase, converting AdoMet into 1-aminocyclopropane-1-carboxylic acid (ACC) with MTA being a side product. The activity of ACC synthase is readily inhibited in the presence of aminoethoxyvinylglycine (AVG) among other compounds. ACC is the direct precursor to ethylene with ethylene-forming enzyme (EFE) facilitating the final step in the presence of oxygen. ACC can also react with malonyl CoA, which is converted, via malonyl transferase, into n-malonyl ACC (MACC). This deviation from ethylene synthesis only occurs when ACC is present in excess or EFE activity is limited (Abeles et al., 2012).

Ethylene's role in flower development has been defined in a few plant systems. Ethylene related mutants exposed the role of the phytohormone on flowering and anthesis timing (Ibqal et al., 2017). Complete inhibition of floral development has also been demonstrated in both *Arabidopsis thaliana* (Achard et al., 2007) and *Oryza sativa* (Wang et al., 2013). A similar effect has been observed and exploited in *Poa annua* for turfgrass uses, where a properly timed application of ethylene promoting compounds have been shown to inhibit seed head formation (Eggens et al., 1989). The opposite effect has been observed and broadly utilized for the induction of flowering in bromeliads such as *Ananas comosus* (Han et al., 1989).

Timing of the applications for the current research should ensure ethylene presence in nucellar tissue prior to development of aposporous initials and remain present until the tetrad stage of the sexual embryo sac development. The approach targets the dedifferentiation event of the somatic tissue and embryo initiation, comparable to somatic embryogenesis (Albertini et al., 2005; Schmidt et al., 2014) as discussed in the previous chapter. The tetrad stage of the sexual embryo sac development has been observed as the latest stage of apomictic initial appearance in cyto-embryological investigations in Kentucky bluegrass (Åkerberg, 1942; Abeln et al., 1985). Application timing should allow for ethylene levels to begin to approach 'normal' after this tetrad stage to avoid the potential for damage to the sexual embryo when the protective callose layer (Rodkiewicz, 1970; Bouman, 1984; Carman et al., 1991; Ünal et al, 2013) begins to break down (Bouman, 1984; Tucker et al., 2001).

Concentration of ethylene treatments should be sufficiently elevated to avoid the low levels of ethylene that have been shown to promote somatic embryogenesis (Nissen, 1994). High levels of ethylene, elevated beyond typical *in vivo* concentrations, have consistently inhibited somatic embryogenesis *in vitro* (Tisserat and Murishige, 1977; Kong and Yeung, 1994; Jiménez, 2005), which will be preferred in these experiments. Application concentrations leading to excessively elevated levels of ethylene, however, may result in negative effects to the reproductive development (Campbell et al., 2001) and yield (Ethrel Label, Bayer Corp.).

Ethylene has been utilized to prevent lodging in cereal crops (Basra, 2000; Rademacher, 2015). This application exploits the rapid inhibitory effect of ethylene on cell elongation (Abeles et al., 2012). This is done through applications of ethylene promoting compounds, such as ethephon, prior to inflorescence extension (Ethrel Label, Bayer Corp., Leverkusen, Germany). These applications result in shorter stems at time of seed development, reducing lodging.

Applications prior to emergence from the boot are not expected to greatly influence inflorescence height as this is prior to the label instructed timing for lodging prevention in wheat (Dahnous et al., 1982; Ethrel Label, Bayer Corp., Leverkusen, Germany). Evaluating the penetrance and bioactivity of the ethylene treatments through height measurements would be an effective proof of ethylene presence in the inflorescence tissue.

Genomic tools such as molecular markers, such as micro satellites, are important in population genetics and breeding efforts alike. A consequence of complex ploidy, such as that in Kentucky bluegrass, has been partially overcome by using codominant marker data as 'allele phenotypes' (Becher et al., 2000). The inability to determine allele dosage using SSR markers (Bushman et al., 2013) is a limitation on determining the gamete origin of progeny from a controlled cross. Complications in classification of progeny can arise from errors in sampling, PCR (enzymes, cocktails, or thermalcycler protocols), fragment sizing, band homoplasy, and allele calling. When identifying clones using microsatellites, these errors have been shown to culminate in splitting errors, where clonal samples are identified as different due to the errors listed above on a high allele resolution instrument. Another error type, merging errors, where different samples being identified as clonal, typically occur as a result of allele redundancy or a low allele resolution (Pfeiffer et al., 2011).

The allele phenotype (Becher et al., 2000) approach to the codominant microsatellites has been successfully used in Kentucky bluegrass population studies for the classification of cultivars (Honig et al., 2010; Honig et al., 2018), determination of progeny to aid breeding efforts (Bushman et al., 2013; Bushman et al., 2018), and evaluation of population spatial distribution (Dennhardt et al., 2016). While specific breeding outcomes such as B_I, B_{II}, B_{III}^M, B_{III}^P, and B_{IV} can be discerned from apomictic progeny, differential classification between the

non-apomictic progeny outcomes requires flow cytometry or chromosome counts to supplement microsatellite data (Bushman et al., 2018) (Table 3.1). The hypothesis being tested requires the evaluation of rates of hybrid progeny, with the goal of providing a tool for enhanced Kentucky bluegrass breeding efficiency. No hypothesized effect on ploidy levels of the progeny, including differential rates of self-pollination from poly-haploid (B_I progeny) development or sexual hybrids (B_{II} progeny) from sexual hybrids resulting from unreduced gametes (B_{III}^M and B_{III}^P progeny), has been made. The focus of this evaluation is on the inhibition of the development of the aposporous initials without the elimination of the sexual embryo to increase the proportion of sexual hybrids for breeding applications. In this study, SSRs will be used to evaluate the effects of the treatments on the rate of sexual progeny. To these ends, SSR marker analysis to detect paternal alleles in progeny is expected to provide conclusive results.

The anticipated effects of ethephon on seed head height will be utilized in this study to confirm the bioactivity of ethylene *in vivo*. Treatments at the high label rate of Ethrel will be applied at various stages of inflorescence development. Progeny samples from technical replicates will be established for analysis via SSR markers and subsequent classification for analysis to discern the effects of the tested ethephon treatments on rates of apomixis.

MATERIALS AND METHODS

Plant Material: ‘Rockstar’ Kentucky bluegrass, a ‘Shamrock’ derivative, was selected as the mother due to its common use in the Rutgers breeding program and known ability to hybridize (Personal Communication, Dr. William A. Meyer). Rockstar is estimated to produce about 85 to 90% apomictic offspring from phenotypically evaluated field trials. ‘Avalanche’ Kentucky bluegrass, a Washington type, was selected as the pollen donor, due to its prolonged

use as a pollen donor in the Rutgers breeding program, and its genetic distance from ‘Rockstar’ (Honig et al., 2010).

Forty-eight plants of each cultivar, ‘Rockstar’ and ‘Avalanche’, were clonally propagated from a single true-to-type plant identified from a seeded spaced-plant nursery in Freehold, NJ at the Adelphia Plant Biology Research and Extension Farm. Clonal propagules, consisting of three tillers, were established in ProMix BX Mycorise Fomulation (Premier Tech Inc., Riviere-du-Loup, QC, Canada) in 48 cell flats with cells measuring 3.2 x 5 cm. Established plants were transplanted to the field October 2017 to vernalize overwinter. On 11 April 2018, twenty four clones from each cultivar, of equal size, were extracted from the field using a 7.6cm circular plugger to a depth of 12.7cm. Plants were potted in 15cm diameter round nursery pots using Sungro Professional Growing Mix (Sun Gro Horticulture, Agawam, MA) and watered thoroughly before being moved to a walk-in cooler (Mr. Winter, Hialeah, Florida), with 8/16hr (Day/Night) fluorescent lighting at 120um photons/s. The cooler was set to 4°C and used to slow development and match flowering times between cultivars. Plants were watered to avoid foliar stress throughout the experiment. Avalanche clones were moved into a growth chamber 24 May 2018 to begin flower development. The walk-in growth chamber (Environmental Growth Chambers, Chagrin Falls, Ohio) was maintained at 23/18C (Day/Night) with 14/10hr fluorescent and incandescent lighting at 300um photons/s. ‘Rockstar’ clones were moved into the growth chamber 1 June 2018. ‘Avalanche’ clones were returned to the cooler 5 June 2018 for the duration of the applications prior to pollination.

Ethephon Treatment Applications

The ‘Rockstar’ clones were each randomly assigned an application timing and treatment combination of one of the following: early control, early ethephon, medium control, medium

ethephon, late control, late ethephon, all timings control, and all timings ethephon. Early refers to the developmental stage where the inflorescence is just about to emerge from the boot (Figure 2.1). Medium refers to the developmental stage where the inflorescence is about halfway emerged from the boot (Figure 2.2). Late refers to the developmental stage where the inflorescence has completely emerged from the boot (Figure 2.3). All timings refer to applications at all three aforementioned timings. Treatments included a control water-only treatment of 24ml, and an ethephon treatment of 24ml of a 1000ppm ethephon (Ethrel, high label rate) in water solution. Every application timing and treatment combination was administered to three different clones (3 technical replicates).

Prior to the first application, each ‘Rockstar’ clone was randomly assigned to a 30.5 x 30.5 x 61cm clear polyvinyl isolation chamber within the growth chamber. Applications were made with a handheld spray bottle adjusted to a coarse droplet size and calibrated to 6ml per spray prior to applications; and confirmed after applications to ensure applications were uniform. The application was directed at the developing inflorescences from approximately 30 cm and completed in four sprays, one from each direction, to ensure coverage. Applications were performed in two separate polyvinyl isolation chambers, for the two respective treatment levels. Growth chamber air circulation and lights were disabled for applications until the applied solution dried, which took approximately 4 hours. The plants were then returned to their chambers and normal growth chamber function was resumed. For each application timing, six ‘Rockstar’ clones were treated with a water control and six were treated with ethephon (three timing specific replicates and the three all timing replicates per treatment). Applications were made 6 June 2018 (early timing); 8 June 2018 (medium timing); and 10 June 2018 (late timing). After the conclusion of all treatments, 11 June 2018, ‘Avalanche’ clones were removed from the

cooler and placed back into the growth chamber elevated above the ‘Rockstar’ clones within the chambers on inverted pots for pollination.

Pollination and Harvest

After the initiation of anthesis on 20 June 2018, air circulation at night was disabled, to prevent undesired pollen dispersion during dehiscing. ‘Avalanche’ clone seed heads were carefully disturbed at least two times per night, without disturbing the ‘Rockstar’ clones below, to release the pollen onto the ‘Rockstar’ clones. Following the conclusion of pollination on 28 June 2018, normal growth chamber function was restored, ‘Avalanche’ clones were removed from the isolation chambers and the ‘Rockstar’ clones were spaced evenly within the growth chamber. At the time of harvest, 27 July 2018, heights from the surface of the soil to the tip of the tallest seed head were recorded to evaluate the introduction of ethylene in the inflorescence tissue. Height data was subjected to ANOVA using the GLM procedure. The data analysis was generated using SAS software, Version 9.4 of the SAS System for Windows (Copyright 2013 SAS Institute Inc.).

Progeny Establishment

Samples of about 150 seeds from each ‘Rockstar’ mother were sown in 10.2 cm square pots with ProMix BX Mycorrhizae potting mix (Premier Tech Inc., Riviere-du-Loup, QC, Canada) on 14 September 2018. Forty-eight seedlings were transplanted from each pot into trays of 48 cells to represent the progeny population. Established plants were sampled for SSR Marker analysis.

Microsatellite Marker Selection

SSR primer pairs (Markers) were selected from Honig et al. (2010) based on polymorphic bands between the parents, ‘Rockstar’ and ‘Avalanche’, using data from Honig et al., 2018. The markers selected were: GA9, GA125, GA446, GA749, GA1071, GA1119, GA1153, GA9307, GT135, and GT9434. Previous data suggests that there are 94 polymorphic alleles between the 10 selected markers (Tables 2.2 and 2.3).

DNA extraction

Tissue samples of about 100 mg from forty-eight established progeny from each Rockstar mother were cut into roughly 5 mm long leaf segments and places into 2 ml sampling tubes with a small tungsten tissue lysing bead and held on ice for transportation from the greenhouse. Sampling tubes were flash frozen in liquid nitrogen and treated for 120 seconds in a Qiagen Tissue Lyser II (Qiagen Inc. Hilden, Germany) to grind tissue into a fine powder. DNA was extracted from the tissue powder using the Qiagen Plant DNA Extraction Kit (Qiagen Inc. Hilden, Germany) according to the manufacturer’s instructions.

SSR Marker Protocol

Polymerase chain reactions (PCRs) were performed in 13 ul reactions containing approximately 5.1 ng Kentucky bluegrass DNA, 6 µl master mix (550.8 µl H_2O, 408 µl dNTP, 255 µl 10x Ramp-Taq Buffer, 102 µl of 50 mM $MgCl_2$, and 10.2 µl Ramp-Taq DNA Polymerase), 0.5 pmol forward primer with attached 18 bp M13(-21) 5’-end (Schuelke, 2000), 1 pmol reverse primer, and 1 pmol 18bp M13(-21) primer labeled with florescent dye (FAM, NED, PET, or VIC). PCR was conducted on GeneAmp PCR System 9700 thermocycler (Applied Biosystems Inc., Foster City, CA, USA) with the following cycling parameters: 94°C for 5 min.; 30 cycles of 94°C for 30s, 55°C for 45s, 72°C for 45s; and 20 cycles of 94°C for 30s,

53°C for 45s, 72°C for 45s; and finished with 72°C for 10 min before cooling to 4°C and holding. PCR products were diluted at 1.5 pmol, FAM and VIC labelled, or 2 pmol, NED and PET labelled, in RNAse-free H_2O. A sample of 2 µl of PCR dilute was combined with 9 µl HiDi Mix (from a 1 ml HiDi and 12.5 µl LIZ 600 mastermix) before a three-minute soak at 95°C followed by a snap-cool on ice for ten minutes. The processed PCR products were analyzed using an ABI 3500xl capillary electrophoresis genetic analyzer (Applied Biosystems Inc., Foster City, CA, USA). Sizing was done using Genemapper 5.0 (Applied Biosystems Inc., Foster City, CA, USA) and LIZ 600 size standard.

Progeny Classification

The semi-auto band selection approach (Pfeiffer et al., 2011) was used with a more stringent 100 rfu threshold in the allele calling procedure using Genemapper 5.0. While it has been shown that tolerance of up to four fragment deviations for clonal classification can minimize splitting errors (Pfeiffer et al., 2011), a threshold to discern clonal and other aberrant groups was selected based on the data to eliminate splitting errors. Monomorphic alleles, bearing no valuable information for classification of progeny were excluded in addition to ambiguous alleles resulting from +A, stutter, or inconsistent presence in re-runs. Allele phenotypes were used to classify progeny as clonal, hybrid or other aberrant. The progeny classified as clonal are expected to exhibit an identical allele phenotype as the ‘Rockstar’ maternal parent, with a minimal number of allowed allele differences to eliminate splitting errors. The progeny classified as hybrid exhibit allele contributions from both parents in their allele phenotype. The progeny classified as other aberrant exhibit no allele contribution from the ‘Avalanche’ parent in addition to the absence of numerous alleles from the expected ‘Rockstar’ allele phenotype. Clones, hybrids and other aberrants were manually identified and scored accordingly (Table 2.4).

Proportions of classified progeny resulting from the treatment and timing combinations were subjected to analysis via the GLIMMIX procedure using a beta logistic regression and Bonferoni adjustment for multiple comparisons (Westfall et al., 2011). The data analysis was generated using SAS software, Version 9.4 of the SAS System for Windows (Copyright 2013 SAS Institute Inc.).

RESULTS

There were significant effects on harvest height from application timing and treatment as well as significant interactions between application timing and treatment (Figure 2.4). Mean harvest heights for all ethephon treated clones, regardless of application timing, were less than controls (Table 2.1 and Figure 2.4). While the mean height of the medium control was greater than the medium ethephon, this difference was not significant ($p = 0.4121$). All other ethephon treatments were significantly shorter than their respective water treated controls ($\alpha = 0.001$). The plants treated with ethephon at all application timings resulted in the shortest harvest heights ($p = <0.0001$). The plants treated with ethephon at the late application timing resulted in the next shortest harvest heights ($p = <0.0001$). The complete summary of least square means for effect of application timing by treatment comparisons can be found in table 2.1.

Of the expected 94 polymorphic alleles (Honig et al., 2010), 67 alleles were used among 10 markers (Table 2.2) to classify progeny (Table 2.3). Nine of the expected allele bands belonging to two of the ten markers were not observed in this study. Alleles excluded from the classification include 14 monomorphic alleles; 8 band stutter ambiguous alleles; 3 alleles with +A behavior; 6 weak ambiguous alleles; and 1 run dependent allele. The addition of clones into the genotyping allowed for the estimation of an error-rate. The distribution of the data showed a minimum at two allele differences from clonal, appearing to be errors in analysis. The allowance

of two allele differences in the data result in the elimination of splitting errors, this allowance of two deviations from clonal is less than the suggested four allele differences in Pfeiffer et al. (2011). This allowance of two allele differences from the clonal allele phenotype incorporated 49 and 7 samples with one and two deviations, respectively, (for a total of 56) as additional clonal progeny. A total of 908 progeny shared an identical allele phenotype with 'Rockstar'. In this study, 964 progenies were scored as clonal out of 1152 (Table 2.5). Progeny were successfully classified based on their frequency of parental alleles (Table 2.6 and Figure 2.5).

The percent of clonal progeny observed as a result of the water treated controls were 90.1, 84.7, 81.9, and 80.6 for the early, medium, late, and all timings application timings, respectively. The percent of clonal progeny observed as a result of the ethephon treatment were 84.7, 84.7, 76.4, and 85.4 for the early, medium, late, and all timings application timings, respectively (Figure 2.6). The percent of hybrid progeny observed as a result of the water treated controls were 1.4, 1.4, 1.4 and 2.8 for the early, medium, late, and all timings application timings. The percent of clonal progeny observed as a result of the ethephon treatment were 2.8, 2.1, 7.6 and 2.1 for the early, medium, late, and all timings application timings (Figure 2.7). The percent of other aberrant progeny observed as a result of the water treated controls were 7.6, 13.9, 16.7, and 16.7 for the early, medium, late, and all timings application timings, respectively. The percent of other aberrant progeny observed as a result of the ethephon treatment were 12.5, 13.2, 16, and 12.5 for the early, medium, late, and all timings application timings (Figure 2.8). A full summary of progeny classification can be found in table 2.6. No significant effects were detected from application timing, treatment or their interactions for the proportions of any classification of progeny in this study.

DISCUSSION AND CONCLUSIONS

The significant differences in height observed between the control and ethephon treated clones at the early, late and all application timings demonstrated ethylene presence in the tissue. Differences in height between control and ethephon treatments in the late and all timings applications demonstrated the dwarfing effect of ethylene. These observations are in agreement with effects documented in *Hordeum vulgar* and *Triticale hexaploid* where late boot stage applications resulted in significant dwarfing (Dahnous et al., 1982). The medium control had a greater than expected reduction of height compared to the other controls. The physical application of the water control may have contributed to a thigmo response, thigmomorphogenesis. Thigmo, or contact, stress has been shown to result in a dwarfing effect in *Lillium* sp. (Hiraki and Ota, 1975), *Pisum sativum* (Mitchell, 1977; Akers and Mitchell, 1984), and *Solanum tuberosum* (Mitchell, 1992). These dwarfing or growth retarding effects can be highly sensitive and have been demonstrated to be a result of auxin-ethylene interactions in the plant (Hiraki and Ota, 1975; Mitchell, 1977). More research is necessary to further understand this observation.

The absence of a detectable significant effect may be due to a limited sample size, as the experiment was designed to detect effect sizes that would be practically applicable to improve the efficacy of Kentucky bluegrass breeding programs. These findings could alternatively be a result of inadequate ethephon concentrations, improper application timing, or a combination thereof and should be investigated further based on the observed trend (Figure 2.7). While lacking significance, the increasing proportion of hybrids detected as a result of the late ethephon application suggests later applications may impose a greater effect. It is also possible the ethephon treatment is also affecting the sexual embryo development, like the imposed stress

experiments on manipulation of apomixis (Gournaris et al., 1991; Carman et al., 2015; Kumar, 2017; Rodrigo et al., 2017). A cyto-embryological evaluation of ethephon treated ovules would be necessary to evaluate this possibility.

Other growth regulators or phytohormone inhibitors may prove to be more effective alone or in combination with ethephon, as phytohormone interactions are common in plants (Iqbal et al, 2017). Giberellin inhibitors or absicic acid inhibitors appear to have potential, based on experimentation in somatic embryogenesis (Jiménez, 2005), and should be considered for future research. There appears to be a continuous spectrum of developmental stages in floral development and could require a treatment that would persist without harm to the sexual embryo development while preventing the initiation of the aposporous initial.

The other aberrant classification may indicate the need for further investigation into how Kentucky bluegrass progeny arise (Table 2.4). While it can be understood that most of this group would comprise self-pollination events, it may include more. Aneuploidy has been well documented in Kentucky bluegrass (Brown,1941; Hartung, 1946; Åkerberg, 1939; Grazi et al., 1961; Eaton et al., 2004) and is also a concern in this study with the differential classification of clones and other aberrants. Whether chromosomes can be lost in the development of the apomictic embryo development process or only as a product of the sexual embryo development is unclear and may suggest further complications with the other aberrant and clonal classifications. Such aneuploidy events could result in near clonal appearing allele phenotypes except for several alleles to zero allelic differences from clonal progeny which may complicate the allele tolerance for clonal classification. Future research should aim to identify the developmental origin of aneuploids in the species.

The occurrence of polyhaploids, or B_I progeny, has also been well documented in Kentucky bluegrass (Mazzucato et al., 1996; Huff et al., 2003) and is likely to populate the other aberrant group. The reproductive origins of these progeny have yet to be experimentally determined. Continuing research should aim to identify the range of possible outcomes of apomictic reproduction in Kentucky bluegrass.

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Table 2.1 Least square means for effect of application timing (E: Early, M: Medium, L: Late, A: All Timings) by treatment (C: Water Treated Control, T: Ethephon Treated) on harvest heights measured from the soil level to the tallest seed head to determine bioactivity of the treatments prior to evaluating the effects on rates of apomixis in Kentucky bluegrass.

	AT	**AC**	**LT**	**LC**	**MT**	**MC**	**ET**	**EC**
EC	<.0001	0.0056	<.0001	0.0825	0.0001	0.0007	<.0001	
ET	<.0001	0.0437	0.0013	0.0027	0.7406	0.2556		
MC	<.0001	0.3273	0.0001	0.0314	0.4121			
MT	<.0001	0.0825	0.0007	0.0056				
LC	<.0001	0.1966	<.0001					
LT	<.0001	<.0001						
AC	<.0001							
AT								

Table 2.2 SSR marker identification, accession numbers, primer sequences, and repeat motifs for the molecular markers used to classify progeny differences observed as a result of ethephon treatments to identify the effects of the treatments on rates of apomixis in Kentucky bluegrass. Adapted from Honig et al., 2010.

Marker ID	GenBank Accession No.	Primers (5' - 3')	Repeat Motif
NJPpGA9	HM136689	F: GCCGTAAATAGTGGAGAAGAC R: AAAATCCTGACTGTTGGAGAC	(CT)21
NJPpGA125	HM136695	F: GCAGAACAAAAAGTCATACTGG R: AACCCTATTTAGCCCTCACTC	(GA)8GG(GA)18
NJPpGA446	HM136711	F: TGGCCTACATAATGGTCAAAA R: AAAATTATGGATACCAGCCTACC	(CT)25
NJPpGA749	HM136717	F: CTGCGAGAGTAGCGAACAAT R: AGGGAATCGATCTTGCCTAT	(GA)2TA(GA)17
NJPpGA1071	HM136742	F: CACATTCGGTTTTGGATCAT R: CAGAGAACACGAGCATTGAA	(GA)15GG(GA)23
NJPpGA1119	HM136751	F: TCCCAGCTGGCATTTCTAT R: CGCTGATGCACAGGTTACTA	(GA)19
NJPpGA1153	HM136754	F: TTAAAGACCCTACCAATGCAA R: TTTCATCCTCTCATCGCTTC	(CT)14
NJPpGA9307	HM136755	F: AGCACAAAAGGACACAAAAGA R: CAGGAGCCTTTGATTCTTCA	(GA)20
NJPpGT135	HM136769	F: GCCGCTCTCTTGTGTCATT R: CGGGTAAGGTTTCTGCTTG	(GT)29
NJPpGT9434	HM136773	F: CCCCCTATGCGACACGTA R: GTACCTCGTGCGGCATGT	(GT)15

Table 2.3 SSR markers used in this study with previously observed allele counts per marker (Honig et al., 2018), number of monomorphic, ambiguous, not detected compared to previously observed alleles per marker, number of alleles used from 'Rockstar' for classification and number of alleles used from 'Avalanche' for classification of progeny to evaluate effects of ethephon treatments on rates of apomixis in Kentucky bluegrass.

Marker ID	Previously Observed	Mono-morphic	Ambig-uous	Not Detected	Rockstar Alleles	Avalanche Alleles	Total Alleles
NJPpGA9	8	0	0	0	3	5	8
NJPpGA125	11	3	3	0	2	3	5
NJPpGA446	11	0	2	5	1	3	4
NJPpGA749	6	2	1	0	1	2	3
NJPpGA1071	12	1	1	0	5	5	10
NJPpGA1119	14	1	2	0	4	7	11
NJPpGA1153	8	1	0	0	1	6	7
NJPpGA9307	8	1	1	0	3	3	6
NJPpGT135	13	2	4	0	3	4	7
NJPpGT9434	17	3	4	4	2	4	6
	108	14	18	9	25	42	67

Table 2.4 Table of expected progeny outcomes including gamete origins; allele expectations, as percent of alleles contributed from the respective parent; embryo origin; gamete fusion method; and allele-based classification, used in this study to evaluate the effects of ethephon on rates of apomixis in Kentucky bluegrass. Adapted from Bushman et al., 2018.

Progeny Type	Maternal Gamete	Paternal Gamete	Rockstar Alleles Expected	Avalanche Alleles Expected	Embryo Sac Origin	Fusion Method	Allele Based Classification
Apomictic	Unreduced	-	100%	0%	Aposporous	Parth	Clone
B_I Polyhaploid	Reduced	-	~50%	0%	Unconfirmed	Parth	Other Aberrant
B_{II} (Self)	Reduced	Reduced	~75%	0%	Megasporocyte	Fert	Other Aberrant
B_{II} Hybrid	Reduced	Reduced	~50%	~50%	Megasporocyte	Fert	Hybrid
B_{III}^M Hybrid	Unreduced	Reduced	100%	~50%	Megasporocyte	Fert	Hybrid
B_{III}^P Hybrid	Reduced	Unreduced	~50%	100%	Megasporocyte	Fert	Hybrid
B_{IV} Hybrid	Unreduced	Unreduced	100%	100%	Megasporocyte	Fert	Hybrid

Fert. indicates fertilization

Parth. indicates parthenogenesis

Table 2.5 Progeny allele phenotypes represented by counts of alleles contributed from each parent (Allele counts that were not observed in progeny were excluded from the table) across all treatments and application timings in this study to evaluate the effects of ethephon on rates of apomixis in Kentucky bluegrass.

		Avalanche Alleles												
Rockstar Alleles	**Count**	**0**	**1**	**5**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**Total**
	7	1	0	0	0	0	0	0	1	0	0	0	0	0	2
	8	0	0	0	0	0	0	0	0	0	2	0	0	1	3
	9	6	0	0	0	0	0	0	0	0	0	0	0	0	6
	10	3	0	0	0	0	0	1	0	0	0	0	0	0	4
	11	6	0	0	1	0	1	2	0	0	0	0	1	0	11
	12	10	0	0	0	1	0	1	0	1	0	1	0	0	14
	13	6	0	0	1	0	1	0	0	0	1	0	0	0	9
	14	10	0	0	0	1	1	0	2	0	0	0	0	0	14
	15	5	0	0	0	0	0	0	0	1	1	0	0	0	7
	16	10	0	0	0	0	1	0	0	0	0	0	0	0	11
	17	19	0	0	0	0	0	0	0	0	0	0	0	0	19
	18	13	0	0	0	0	0	0	0	0	0	0	0	0	13
	19	26	0	0	0	0	0	0	0	0	0	0	0	0	26
	20	15	0	0	0	0	0	0	0	0	0	0	0	0	15
	21	16	0	0	0	0	0	0	0	0	0	0	0	0	16
	22	11	0	0	0	0	0	0	0	0	0	0	0	0	11
	23	7†	0	0	0	0	0	0	0	0	0	0	0	0	7
	24	46†	0	0	0	0	1	0	1	0	0	1	0	0	49
	25	908	3†	1	0	0	2	0	0	1	0	0	0	0	915
	Total	1118	3	1	2	2	7	4	4	3	4	2	1	1	1152

† Represents progeny classified as clonal in this study

Table 2.6 Progeny classification outcomes reported for the subsamples of progeny from each treated ‘Rockstar’ clone with counts of clonal, hybrid and other aberrant progeny; total aberrant counts, sums of hybrid and other aberrant counts; and proportions of clonal, hybrid and other aberrant progeny. The resulting progeny outcomes from application timing (E: Early, M: Medium, L: Late, A: All Timings) and treatment (C: Water Treated Control, T: Ethephon Treated) to determine the effects of ethephon on rates of apomixis in Kentucky bluegrass are shown.

Treatment Code	Clonal	Hybrid	Other Aberrant	Clonal Proportion	Hybrid Proportion	Other Aberrant Proportion
EC	40	1	7	0.83	0.02	0.15
EC	46	0	2	0.96	0.00	0.04
EC	45	1	2	0.94	0.02	0.04
ET	43	0	5	0.90	0.00	0.10
ET	43	2	3	0.90	0.04	0.06
ET	36	2	10	0.75	0.04	0.21
MC	40	1	7	0.83	0.02	0.15
MC	37	1	10	0.77	0.02	0.21
MC	45	0	3	0.94	0.00	0.06
MT	43	1	4	0.90	0.02	0.08
MT	35	0	13	0.73	0.00	0.27
MT	44	2	2	0.92	0.04	0.04
LC	43	0	5	0.90	0.00	0.10
LC	38	1	9	0.79	0.02	0.19
LC	37	1	10	0.77	0.02	0.21
LT	36	6	6	0.75	0.13	0.13
LT	38	3	7	0.79	0.06	0.15
LT	36	2	10	0.75	0.04	0.21
AC	41	4	3	0.85	0.08	0.06
AC	37	0	11	0.77	0.00	0.23
AC	38	0	10	0.79	0.00	0.21
AT	40	1	7	0.83	0.02	0.15
AT	44	0	4	0.92	0.00	0.08
AT	39	2	7	0.81	0.04	0.15

Figure 2.1 Image representing the stage of development when the early application timing treatments were administered to determine the potential effects of ethephon applications on rates of apomixis in Kentucky bluegrass. The flowering culm is in the boot and just the tip of the seed head is beginning to become visible.

Figure 2.2 Image representing the stage of development when the medium application timing treatments were administered to determine the potential effects of ethephon applications on rates of apomixis in Kentucky bluegrass. The seed heads have emerged about halfway from the boot.

Figure 2.3 Image representing the stage of development when the late application timing treatments were administered to determine the potential effects of ethephon applications on rates of apomixis in Kentucky bluegrass. The seed heads have emerged and are beginning to elongate.

Figure 2.4 Harvest height plotted against application timing between water control and ethephon treatment. Data demonstrate the main effects and interactions observed as a result of the applications to ensure bioactivity in Kentucky bluegrass prior to evaluating the effects of ethephon on rates of apomixis, error bars represent standard deviation.

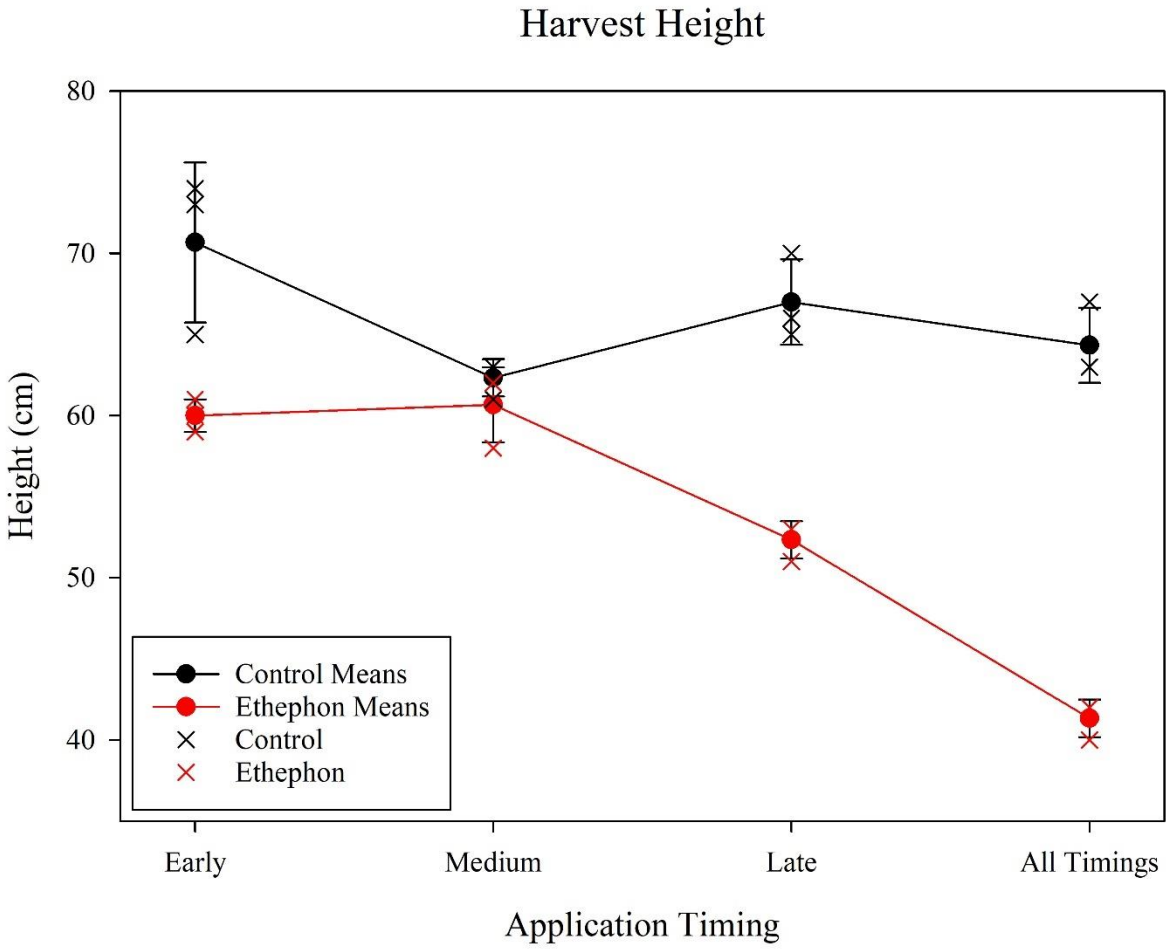

Figure 2.5 The frequencies of parental alleles observed in progeny across all applications to evaluate the effects of ethephon on rates of apomixis in Kentucky bluegrass are summarized with bubble sizes representing root transformed counts and exclude 908 genotypes with identical allele phenotypes to the ‘Rockstar’ Kentucky bluegrass mother.

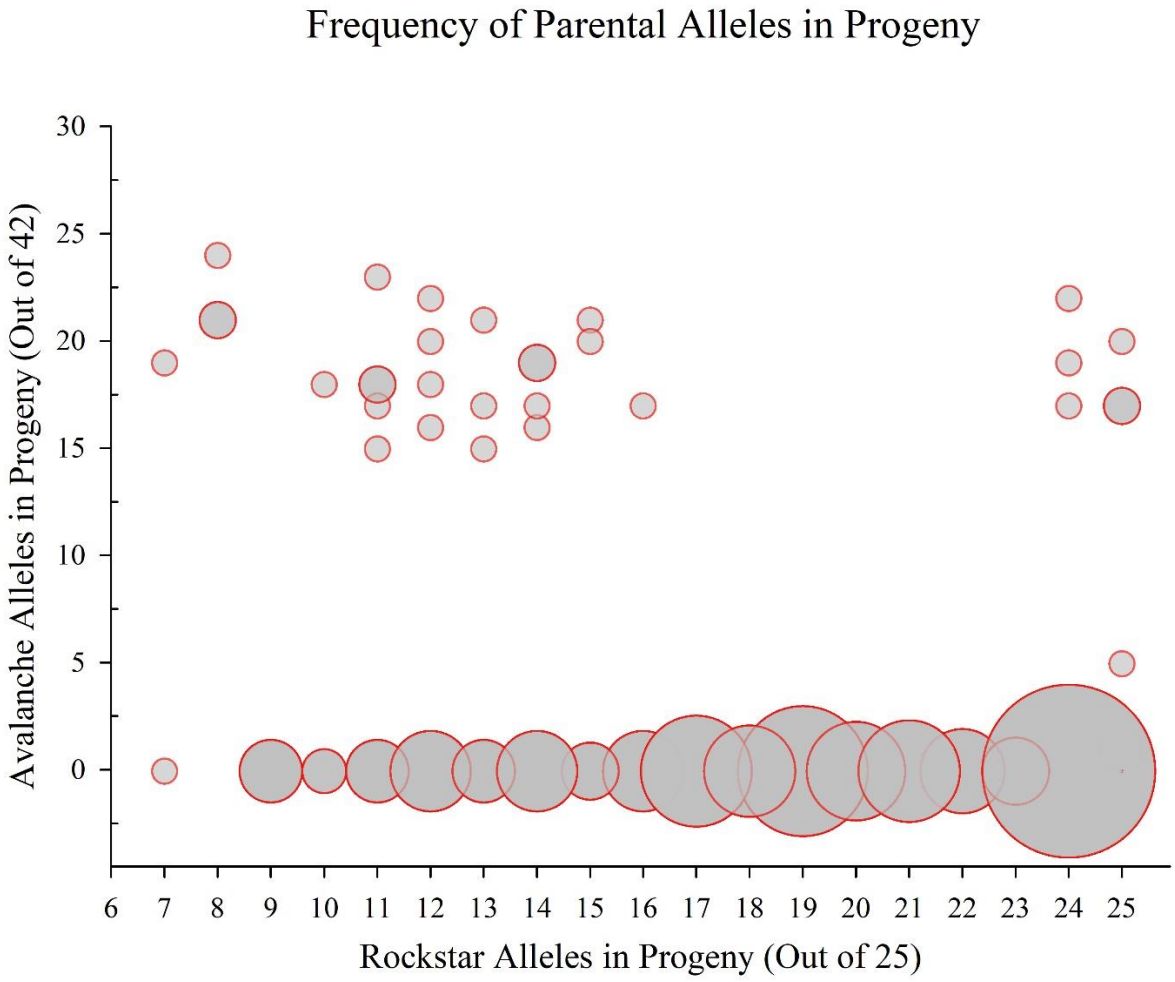

Figure 2.6 Summary of count data representing the clonal progeny observed, out of the 48 progeny subsamples, by application timing comparing the effects of the water control and ethephon treatments to evaluate effects of ethephon on rates of apomixis in Kentucky bluegrass. Error bars represent standard deviation.

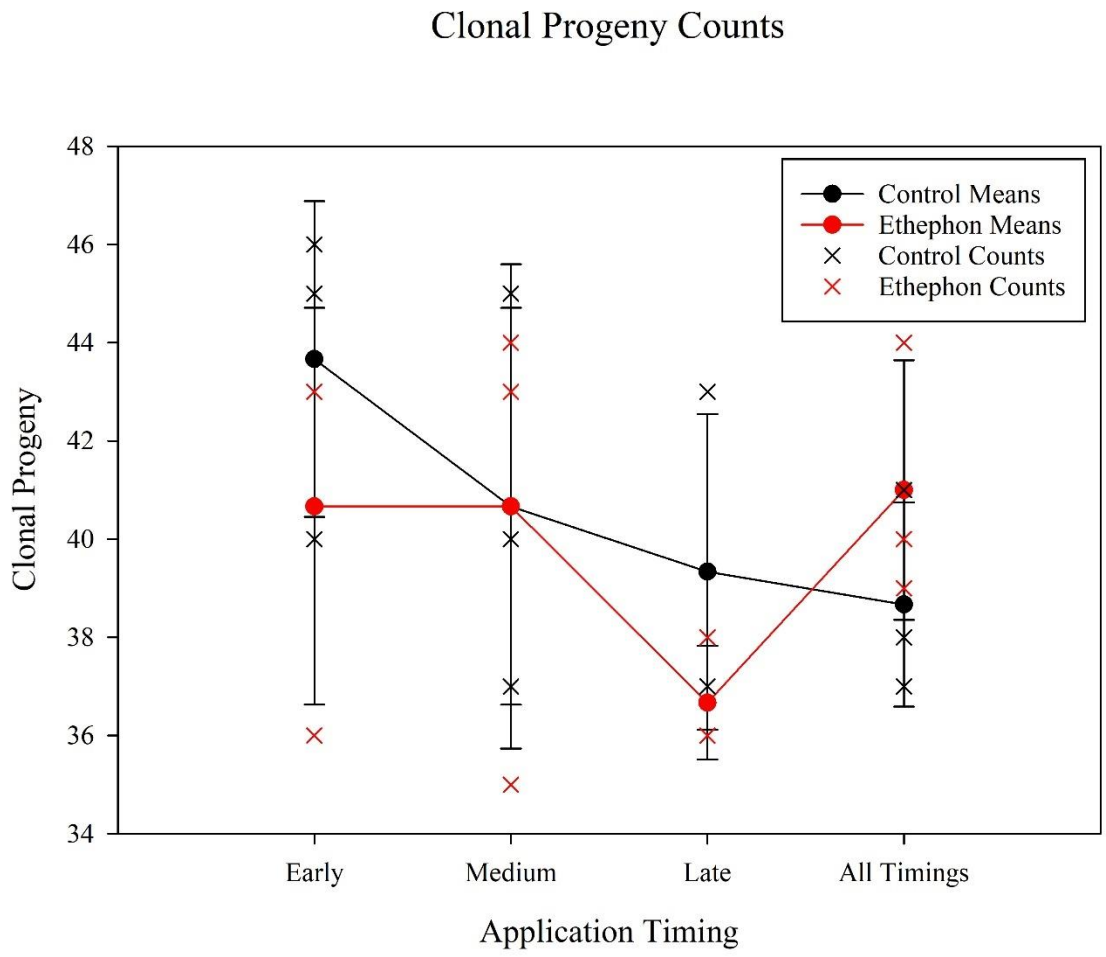

Figure 2.7 Summary of count data representing the hybrid progeny observed, out of the 48 progeny subsamples, by application timing comparing the effects of the water control and ethephon treatments to evaluate effects of ethephon on rates of apomixis in Kentucky bluegrass. Error bars represent standard deviation.

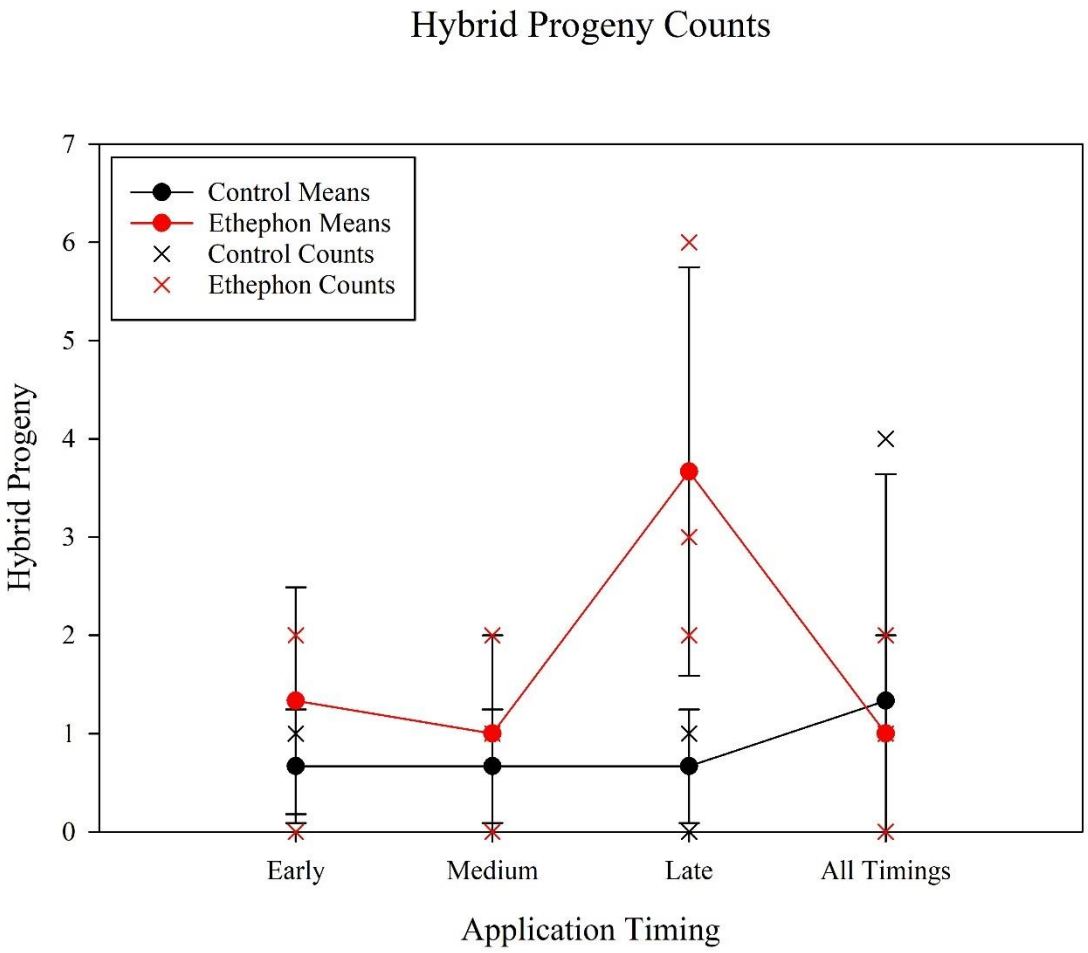

Figure 2.8 Summary of count data representing the other aberrant progeny observed, out of the 48 progeny subsamples, by application timing comparing the effects of the water control and ethephon treatments to evaluate effects of ethephon on rates of apomixis in Kentucky bluegrass. Error bars represent standard deviation.