ABSTRACT

HEINE, AUSTIN JAMES. Comparison of Pollination Bags for Mass Production of Controlled Cross Seeds in Loblolly Pine. (Under the direction of Dr. Steven E. McKeand.)

Over the past 10 years in the southern US, deployment of full-sib families has gained prominence relative to traditional improved loblolly pine (*Pinus taeda* L.) seedling stock such as open-pollinated families or seed orchard mixes. To produce controlled cross seed, a pollination bag must be used to isolate female strobili from outside pollen contamination, and a known pollen is applied at time of maximum female strobilus receptivity. In the spring of 2014, the members and staff of the NC State University Cooperative Tree Improvement Program designed and installed a study to compare four pollination bag prototypes. Bags from PBS International were compared to the industry-standard Lawson pollination bag with and without a support wire. Open-pollinated female strobili were also added for comparisons. Based on preliminary results from the 2014 installation, another round of prototype testing was established in the spring of 2015 and spring of 2017. The main objective of this study was to compare conelet and cone survival of these bags to the industry standard Lawson pollination bag and recommend the most efficient bag for producing controlled cross loblolly pine seed.

To quantify the efficacy of each bag, conelet survival four months after pollination and cone survival following cone harvest were calculated. The 2014 and 2015 replications of this study are now complete. Significant differences were found for conelet and cone survival between the industry-standard Lawson pollination bag and several PBS prototypes and the Lawson pollination bag with a support wire. Results suggest that stiffer/stronger material or the addition of a support wire to Lawson pollination bag increases cone survival at least 10% over the Lawson pollination bag. Conelet survival results from the 2017

installation suggested that the newest bag design with a folded flap had a higher probability of conelet survival than all previously tested bag types. The new design with a folded flap decreases damage to strobili, presumably caused by wind, and increases conelet survival. With over 1.4 million pollination bags installed in the southeastern United States in both 2016 and 2017, the 10% increase in cone survival will substantially increase seed production.

Keywords: *Pinus taeda*, seed orchard, cone analysis, controlled pollination, conelet survival

Comparison of Pollination Bags for Mass Production of Controlled Cross Seeds in Loblolly Pine

by
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BIOGRAPHY

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Chapter 1. Review of Literature on Pollination Bags, Reproductive Biology, and Additional Factors Affecting Seed Yield in Controlled Crosses of Loblolly Pine

Introduction

Loblolly Pine (*Pinus taeda* L.) is among the most important commercial forest tree species in the United States and in the world (Schultz 1997). Because the wood of loblolly pine is highly desired and used to produce many products and because it is a species widely adapted to grow in a variety of landscapes, it is the most commonly planted tree species in the United States. Over the last 60+ years, plantations of loblolly pine have increased substantially (South and Harper 2016) and now comprise about 19% of forests in the southern US (Huggett et al. 2013). To meet this tree planting demand, nurseries around the South grow genetically improved loblolly pine in large quantities each year. Of the one billion seedlings produced by nurseries in the South in the fall of 2013, approximately 97% were conifers, and 67% were bareroot loblolly pine (South et al. 2016). Additionally, since 1977, nearly 100% of all loblolly pine plantations in the southeastern United States have been established using genetically improved seedlings (Aspinwall et al. 2012).

In order to produce these genetically improved trees, more than 4,000 hectares of seed orchards have been established in the southern United States since the early 1960's, primarily for loblolly pine and slash pine (*Pinus elliottii Engelm)* (Bramlett 1991). Seed orchards are established by grafting cuttings from tested and genetically proven superior parents onto rootstock. These clones produce progeny that are superior for traits such as

increased volume growth, stem straightness, and resistance to fusiform rust disease (caused by the fungus *Cronartium quercuum* f.sp. *fusiforme*). Rootstock is planted at a wide spacing (e.g. 10m x 5m up to 15m x 15m) to maximize crown size and number of cones produced. The purpose of a seed orchard is to provide an area where improved genetic material can be easily accessed and large quantities of genetically superior seed can be produced (Zobel et al. 1958).

In 2016, over 27,000 kilograms of improved loblolly pine seed were harvested from seed orchards around the Southeast by members of the NC State University Cooperative Tree Improvement Program (NCSUCTIP 2017). Once extracted and cleaned, this seed is sent to nurseries to be grown into high quality seedlings. In the 2015-2016 planting season, over 782 million loblolly pine seedlings were grown in the South (Enebak 2016). At the end of each growing season, these seedlings are packed, shipped, and then planted.

In loblolly pine seed orchards, seed is produced for seedling production in three primary ways: as seed orchard mixes of open-pollinated seed, as an open-pollinated (OP) family, or as a full-sibling (full-sib) cross. Traditionally, loblolly pine seed orchard managers harvested seed to sell as seed orchard mixes. In this approach, open-pollinated seed is collected from selected clones and then bulked together to be sold as a heterogeneous mix. The advantage of this mix is that the planted area will have a higher level of genetic diversity when compared to planting a single family. The disadvantage of a seed orchard mix is that the landowner does not realize the genetic gains that can be made with other techniques. This technique has been used in southern pine seed orchards since the early 1950's (Bramlett 1991). However, with the further development of a seedling market, many orchard managers have moved away from producing and marketing seedlings as seed orchard mixes.

Planting seedlings of single open-pollinated (OP) families started in the 1970's (Gladstone 1975, Duzan and Williams 1988) and has become the most common method for collecting and marketing loblolly pine (McKeand et al. 2003). In this approach, cones are collected from ramets of a single clone that are pollinated by the wind-borne pollen cloud in that seed orchard. Even though the female may have good genetics, pollen is not guaranteed to come from the other genetically superior trees in the orchard. In theory, for the seeds that are pollinated from pollen outside of the seed orchard, genetic gains are reduced by half (Bridgwater et al. 1998). To produce this open-pollinated seed, clones have been established in seed orchards in various patterns ranging from totally at random to randomized complete block designs and other systematic designs. Two important goals of most of these orchard designs were to minimize inbreeding and maximize gain from the orchard pollen cloud (van Buijtenen 1971).

The most recent major transition occurring in many loblolly pine seed orchards is the mass production of large quantities of full-sib seed to increase the amount of genetic gain. Gains from full-sib families can be as high as 40% over non-improved material for plantations harvested at rotation ages 25-28 years (Li et al. 1999). Based on yearly surveys of members of the NCSU Cooperative Tree Improvement Program, full-sib seedling production has increased almost every year since 2000. In the past 5 years, full-sib seedling production has increased from 30 million seedlings in 2012 to over 116 million seedlings in 2017.

Full-sib seed is produced when female strobili [1]are isolated from outside pollen using a pollination bag, and the desired pollen is applied. With the transition to full-sib seed production, issues that plagued orchard managers seeking open-pollinated seed, are no longer relevant. For example, orchard designs to minimize inbreeding or maximize cross-pollination in orchard pollen clouds are no longer primary concerns, but instead, new problems have emerged.

Several factors impact cone survival and seed yield of controlled pollinated loblolly pine strobili, including pollen quality, timing of pollen application, damage from insects, and damage from naturally occurring events such as wind and freezes. Another factor suspected to cause variation in cone survival and seed yield is the type of pollination bag used to isolate the female strobili from outside pollen. The type of pollination bag used and the relationship it has with seed yield and cone survival is the primary focus of this research and is the reason that the NCSU Cooperative Tree Improvement Program began the Pollination Bag Study that is described in chapter 2. This literature review aims to define what research has been done on pollination bags and what is known about the factors that contribute to the production and loss of controlled cross seed in loblolly pine.

Reproductive Biology of *Pinus taeda*

In order to understand how loblolly pine seed is produced, it is important to understand cone and seed development and morphology. Loblolly pine is a monoecious tree

[1] The correct botanical term for female strobili in loblolly pine is megasporangiate strobili. The terms female strobili and flowers are used frequently instead of megasporangiate strobili. Microsporangiate strobili are the male strobili, commonly referred to as catkins. The microsporangiate strobili/catkins produce pollen.

species, having both male and female reproductive structures on the same tree. The female strobili (commonly referred to as female flowers), typically form in long-shoot terminal buds in the upper quarter of a mature tree's crown. This long-shoot terminal bud is a resting bud network comprised of an apical meristem with an axis of lateral shoots below (Greenwood 1980). Male strobili typically form in the lower quarter of each mature tree's crown and form in clusters on short shoots (Williams 2009).

The entire cycle for flowering and seed production in loblolly pine takes over 24 months, beginning in mid-summer, prior to the spring when pollination occurs, when both male and female strobili are initiated from primordia. In the spring following initiation of primordia, female strobili emerge from the bud scales that protected them over the winter. The female strobilus structure is composed of many ovuliferous scales that spiral around a main axis. This phyllotactic or spiral design of the strobilus resembles a wind turbine that forces air around the axis and down each scale to the scale's base, aiding in the pollination process (Schultz 1997). The central portion of each strobilus is composed of fertile scales. At the base of each fertile scale is a pair of ovules, each of which has the potential to become a developed seed. At the very tip and in the most basal portion of each strobilus are infertile scales that do not produce functional ovules (Bramlett et al. 1977). As each female strobilus emerges, it will transition through different stages of receptivity as the scales flex open and then close in a few days.

Bramlett and O'Gwynn (1980) developed a categorizing system for female strobilus development that is in common use today. Female strobili begin at stage 1 when buds are barely visible on the vegetative shoot and end at stage 6 when the scales on each strobilus have swollen shut, making the ovules no longer receptive to pollen. While the female strobili

are opening, the male strobili begin shedding pollen. Pollen is released when it dehydrates to less than 10% water content, aiding in its ability to travel through the air (Fernando et al. 2005).

Both female strobilus receptivity and male strobilus pollen shed are controlled by accumulated heat sums and precipitation. For this reason, peak pollen shed and maximum female strobilus receptivity are usually synchronous (Boyer 1981). Female strobili are at maximum pollen receptivity at stage 5 (Bramlett and O'Gwynn 1981), when scales are flexed so that they form a right angle to the cone axis. When this occurs, pollen grains pass through the micropyle into the pollen chamber of the ovule. As the scales mature and swell, the opening shrinks and eventually is sealed completely, preventing any additional pollen from entering the ovules. After this, the pollination process is complete, and the female strobilus is then classified as a conelet (Williams 2009).

Before scales swell shut, only a small number of pollen grains can enter each ovule's pollen chamber. To determine the number of pollen grains per ovule Bramlett and Matthews (1983) dissected conelets and found that when female strobili were maximally receptive to pollen (stage 5), they had an average of 3.7 pollen grains per ovule in controlled pollinations. The open-pollinated control had an average of 3.8 pollen grains per ovule. In another study, when 0.25, 0.5, and 1 cc of pollen were applied, there were no significant differences in number of pollen grains per ovule, and when data for these three quantities of pollen were combined, they had an average of 2.2 pollen grains per ovule. When 2 cc's of pollen were applied there was an average of 2.8 pollen grains per ovule. Open-pollinated ovules had an average of 4.0 pollen grains per ovule in this study (Bramlett et al. 1985).

To enter the ovule, pollen grains float through the air and fall on the micropylar arms. The micropylar arms, which aid in the pollination process, are located at the base of each cone scale and are pointed toward the cone's axis. At night, an aqueous and protein rich substance known as the pollen drop, is exuded from each ovule. As it rolls down the micropylar arms it picks up pollen. When pollen is inside of the pollen drop at night, it rehydrates, and by early morning, the pollen drop retracts, bringing hydrated pollen grains into the micropylar chamber (Williams 2009). Once the pollen grains are inside the micropylar chamber, and have fully rehydrated, pollen tubes will germinate and begin elongating. For an ovule to further develop and initiate seed coat formation, it must have at least one pollen grain that germinates inside of the pollen chamber (Matthews and Bramlett 1986). If no pollen grains are present in the pollen chamber, or if they all fail to germinate, the ovule will abort and can later be identified as a first-year abort (Bramlett et al. 1977).

Pollen tubes inside of the pollen chamber will elongate very slowly during the first year. Part way through the pollen tubes' growth to the ovule, the tube stops growth and goes into dormancy. During the female strobilus's second spring, one year after pollination, the pollen tube resumes growth and reaches the egg to complete fertilization by early summer of the second year. Once fertilization has been completed, the conelet becomes classified as a cone (Williams 2009). Embryonic and gametophytic tissue will not develop unless the ovule is fertilized. If the ovule is fertilized, it has the potential to develop into a filled seed. Following fertilization, the immature cone begins a period of rapid growth during the summer of the second year until it is a fully developed mature cone in September, approximately eighteen months from time of pollination. Once fully mature, cones are ready to harvest or release seed.

Pollination Bags

In order to produce full-sib seed, the female strobili must be isolated from the orchard pollen cloud using a pollination bag while the desired pollen is applied. The material used for the pollination bag must have small enough pores to preclude entry of pollen but at the same time allow movement of gases and water vapor to prevent the accumulation of moisture, carbon dioxide, and heat in the bags (Sedgley and Griffin 1989). The pollination bag must be large enough to allow for shoot elongation during the time that the female strobili are being isolated. It must also allow pollen that is applied inside of each bag to circulate and not stick to the walls of the bag. Pollen must circulate long enough for pollen grains to deposit onto the scales and micropylar arms of each female strobilus in order to effectively pollinate the female strobili inside the bag. Finally, the isolation bag must allow female strobili to develop properly from stage 1 through stage 6, and develop over a time period where tree breeders have enough time to pollinate. If a bag were to speed up female strobilus development too quickly, breeders would not have enough time to pollinate more than a few bags before female strobili transitioned from stage 5 to stage 6 and were no longer receptive to pollen.

The idea that the type of material used to construct the pollination bag will have an effect on seed yields and cone survival is not new. Over 50 years ago, Snyder and Squillace (1966) listed pollination bags along with pollen-handling procedures and the timing of pollen application as areas where more research was needed. They recognized that there was a major difference in seed yield between open-pollinated and controlled pollinated cones in southern pines.

For many years, breeding programs used sausage casings (clear cellulose bags) to isolate female strobili of loblolly pine. Installing these bags required the installation of an internal support structure that was very time consuming. For mass production of full-sib seed, the industry began using a Kraft paper pollination bag produced by the Lawson Company that is held together by a "shower-proof" (i.e. waterproof) glue. These bags work reasonably well without an internal support structure because they form a box-like shape that makes them "self-supporting". This pollination bag could be installed three to five times faster than using sausage casings (Bridgwater et al. 1998), making the mass production of controlled cross seeds much more feasible. The use of different pollination bag types for breeding in pine was most recently summarized by Bramlett and O'Gwynn (1981) where they described pollination bags made of bleached porous paper, cellulose (sausage casings), and closely woven cotton fabric (used in breeding of western pine species). Since that time, virtually no research on pollination bags for loblolly pine has been published.

The most recent work on pollination bags in loblolly pine was performed by Weyerhaeuser NR Company and can be found in their patent titled *Methods for Modulating Strobili Development in Gymnosperm Trees* (Purnell et al. 2014). Patent number - US 8,677,681 B2 describes several studies on loblolly pine in seed orchards across multiple years. In the original study, seven different bag materials (replicated twice in each of two clones) were tested to see if one could be used to advance and/or delay female strobilus development. Materials tested included a small Kraft paper bag, a medium Kraft paper bag, white non-woven fabric with transparent window, white non-woven fabric painted black, sausage casing, aluminum foil, aluminum foil wrapped around white non-woven fabric, and open-pollinated controls. They found that bag material and color can be used to speed up or

slow down female strobilus development. Aluminum bags maintained female strobilus stages at the stage at which they were bagged for over a month. All other bag types advanced female strobilus development when compared to the open-pollinated control, with sausage casings increasing development the fastest.

In a later study, Purnell et al. (2014) recorded temperature inside some of these bag types and showed that aluminum bags were not significantly different than open-pollinated or control temperatures outside of the bag. At the same time, Kraft bags and white non-woven bags had temperatures significantly higher than temperatures outside of the bags. Kraft bags had cooler temperatures inside of the bags at night. When this is coupled with their ability to advance female strobilus development, these Kraft bags become very unsuitable for orchards that are prone to frost damage. With aluminum bags being the same temperature as the open-pollinated controls but still delaying female strobilus development in comparison with the OP control, Purnell et al. (2014) suggest that light levels are the likely reason for this response. Even though the accumulation of heat sums is strongly correlated with strobilus development in loblolly pine (Boyer 1978), light levels inside of pollination bags also seem to be playing an important factor in female strobilus development. This was tested in a later Weyerhaeuser study where aluminum material with and without perforations was used. It was concluded that even small amounts of light coming through the holes in the bags or diffuse light filtered through the bag material will trigger the female strobili to develop inside of the bags. In order to delay development, female strobili must be bagged using a pollination bag early at pre-stage 1 (buds are noticeable but have not yet separated from the main bud), that totally excludes light.

Pollination bags have been used on many other *Pinus* species and on other plant species. One study very similar to that of the NCSU Cooperative Tree Improvement Program Pollination Bag Study was performed on *Pinus patula* in South Africa where pollination bags constructed of different types of material were monitored for temperature and relative humidity (Nel et al. 2003). Pollen of varying levels of viability was applied to each bag type, and a cone analysis was performed to determine if differences in the number of developed seed existed between pollen viability treatments and between bag types. Materials tested in this study included polythene, micro-fibre (green and white densely-woven cloth with clear window), and cellulose material. There were significant differences among bagging material for cone survival, with the green micro-fibre material having the highest survival in two different study years. They found that internal bag temperatures varied between bag types during the hottest part of the day, with breathable materials (micro-fibre and cellulose) being more similar to the ambient air temperature. Micro-fibre material also had a lower relative humidity than that of the bags made of polythene material, which seemed to trap moisture. Based on cone analysis, the white breathable material (micro-fibre) produced a mean number of developed seed not significantly different from the open-pollinated control. Significant differences in cone survival were found when the pollen application treatments were compared; female strobili that received viable pollen survived much better than those that received 50% viable pollen.

A similar pollination bag study also performed on *Pinus patula*, and a brown paper bag was superior to seven different types of pollination bags for early conelet survival (three months post pollination) (Hagedorn et al. 1997). This study tested a wide range of materials including green and white microfiber (densely-woven cloth), ripstop (parachute material),

paper bags, Jiffy bags (polythene), Sappi tubes (polythene tube with foam rubber stoppers on both ends), and Mondi tubes (made of transparency film with rubber stoppers on both ends). They found that bags with the poorest conelet survival were constructed of material lacking air exchange properties (Hagedorn et al. 1997).

A different study done on *Pinus nigra* Arnold. found that temperatures above 42°C were detrimental to pollen germination and pollen tube growth and were lethal to pollen at 46°C (McWilliam 1957). Temperatures were measured inside of three types of pollination bags (sausage casing, sausage casing with a thin film of aluminum paint covering the upper two thirds of the bag, and a sausage casing enclosed in a brown Kraft bag with ventilation holes) with temperature probes as close to the micropyle as possible. Sausage casings without any type of covering to control temperature reached temperatures that were lethal to pollen when germinated *in vitro*.

Pollination bags are also being used in the production of agricultural crops such as sorghum. In a study done in 2015, pollination bags were assessed not only for their ability to deter birds, but also to see if different bags produced different seed and panicle weights (Schaffert et al. 2016). In this study, Kraft paper pollination bags, Kraft bags covered by a plastic screen, Duraweb® SG2 bags (made from non-woven polyester with smooth paper like surface), and Duraweb® SG1 bags (made of coarse non-woven polypropylene with a point-bonded surface) were assessed across three varieties of sorghum. Non-woven bags provided better protection from bird damage and had higher seed weight, panicle weight, and average seed weight per panicle across all three varieties of sorghum when compared to Kraft bags.

Additional Factors Affecting Seed Yield

When trying to diagnose problems in seed yield or cone survival from controlled crosses in loblolly pine, a variety of other factors must be considered. First and foremost, the timing of when pollen is applied is critical to the success of a controlled pollination. Bramlett and Matthews (1983) found that when controlled pollinations were completed within two days of maximum female strobilus receptivity (stage 5), the average number of pollen grains per ovule was not significantly different from wind-pollinated female strobili. In this same study, they found that multiple applications of pollen did not significantly increase the pollen grain count per ovule when compared with a single pollen application at stages of 4.5, 5, or 5.5. However, if female strobili are at various stages of development inside the bag, multiple pollinations may be beneficial.

Additionally, it is important that pollen of high viability is applied. In a study done by Matthews and Bramlett (1986) to assess the impact of varying pollen quantities and pollen viabilities on filled seed in loblolly pine, they found that adding a higher quantity of pollen with a viability below 50% resulted in minor seed yield increases. Since the pollen chamber can hold up to 7 pollen grains, but typically only holds 2-4 grains per ovule, adding pollen grains of low viability in an attempt to produce more filled seed seems futile. For high numbers of filled seed, high viability pollen should be used. If pollen viability is below 50%, the best way to reach a desired target of filled seed is to increase the number of female strobili pollinated.

Damage from insects is another factor that can have huge negative consequences on cone survival and seed yields. If an orchard crop is not being sprayed and protected, there are a variety of insects that can feed on female strobili from stage of first development,

through the conelet stage, and as cones are developing. Seedbugs, such as *Leptoglossus corculus*, can penetrate through cone scales to extract nutrients from the developing seeds inside of the cone. One documented example of a devastating loss was in 1973 and 1974 in a seed orchard near DeRidder, Louisiana (Goyer and Nachod 1976). Insects destroyed 75% of the crop of loblolly pine cones that was pollinated in the spring of 1973 before they were harvested in 1974. Additionally, due to seedbug feeding, there was a 10% reduction in seed yield for the cones that survived to cone harvest. Second-year aborted ovules (smaller than developed seeds but larger than first-year aborted ovules) and fully-developed seeds that are empty (often called pops[2]) are both classifications of ovules that are caused primarily due to seedbug feeding (Bramlett et al. 1977). Both pops and second year aborts are damaged ovules and will not produce a seedling.

Summary

After reviewing the literature on the use of pollination bags in loblolly pine, it is clear that little research has been performed in recent years. With the exception of the Weyerhaeuser patent (Purnell et al. 2014), the last published paper was Bridgwater et al. (1998) discussing the use of Kraft bags for controlled mass pollination work. Before that, the best summary of different materials used for pollination bags on loblolly pine was given by Bramlett and O'Gwynn in 1981. Sausage casings, Kraft bags, and cloth bags are the only pollination bag material types discussed in the literature that have been employed on loblolly

[2] Pops are developed seeds that are empty, with only a remnant of embryo or gametophyte tissue still present inside of the seed coat (Bramlett et al. 1977).

pine. Fortunately, much work has been done with pollination bags in other pine species and agricultural crops.

Based on this literature review, factors that will likely impact female strobilus survival and seed yields and should be considered when constructing pollination bags for loblolly pine are:

1) Bag stiffness and support. The use of wire supports in pollination bags was necessary to prevent damage to female strobili (Bramlett and O'Gwynn 1981).

2) The material used to construct pollination bags can have a large impact on temperature, humidity, and light levels inside of the bags. This in turn can impact female strobilus development, pollen viability, and consequently cone survival and seed yield. Sausage casings have been proven to speed up female strobilus development and increase temperatures inside the bags (Purnell et al. 2014). Sausage casings have also been found to heat up to temperatures lethal for germinating pollen (McWilliam 1957). Kraft bags have sped up female strobilus development and have had higher temperatures during the day and colder temperatures at night when compared to an open-pollinated control (Purnell et al. 2014). Non-woven white materials have also sped up female strobilus development and had increased temperatures during the day. In sorghum, Duraweb® (non-woven polyester and polypropylene material) led to increases in seed weight and panicle weight when compared to Kraft bags (Schaffert et al. 2016). Woven material, such as micro-fibre, led to higher cone survival when compared to polythene and cellulose material (Nel et al. 2003). Micro-fibre material also had a lower relative humidity than that of the bags made of polythene material, which seemed to trap moisture. Bags with poor air exchange properties led to poor female strobilus survival (Hagedorn et al. 1997).

Additional factors to consider that could impact seed yields and cone survival in loblolly pine include pollen quality, number of pollen applications, timing of pollen applications, and control of insects. It is also important to keep in mind that in several pine species, there are many examples of open-pollinated female strobili producing higher seed yields per cone than controlled pollinated female strobili. All of these factors were considered in the design and implementation of the NCSU Cooperative Tree Improvement Program Pollination Bag Study, and results of this study will be discussed in chapter 2.

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CHAPTER 2. Comparison of Pollination Bags for Mass Production of Controlled Cross Seeds in Loblolly Pine

Abstract

Over the past 10 years in the southern US, deployment of full-sib families has gained prominence relative to traditional improved loblolly pine (*Pinus taeda* L.) seedling stock such as open-pollinated families or seed orchard mixes. To produce controlled cross seed, a pollination bag must be used to isolate female strobili from outside pollen contamination, and a known pollen is applied at time of maximum female strobilus receptivity. In the spring of 2014, the members and staff of the NC State University Cooperative Tree Improvement Program designed and installed a study to compare four pollination bag prototypes. Bags from PBS International were compared to the industry-standard Lawson pollination bag with and without a support wire. Open-pollinated female strobili were also added for comparisons. Based on preliminary results from the 2014 installation, another round of prototype testing was established in the spring of 2015 and spring of 2017. The main objective of this study was to compare conelet and cone survival of these bags to the industry standard Lawson pollination bag and recommend the most efficient bag for producing controlled cross loblolly pine seed.

To quantify the efficacy of each bag, conelet survival four months after pollination and cone survival following cone harvest were calculated. The 2014 and 2015 replications of this study are now complete. Significant differences were found for conelet and cone survival between the industry-standard Lawson pollination bag and several PBS prototypes

and the Lawson pollination bag with a support wire. Results suggest that stiffer/stronger material or the addition of a support wire to Lawson pollination bag increases cone survival at least 10% over the Lawson pollination bag. Conelet survival results from the 2017 installation suggested that the newest bag design with a folded flap had a higher probability of conelet survival than all previously tested bag types. The new design with a folded flap decreases damage to strobili, presumably caused by wind, and increases conelet survival. With over 1.4 million pollination bags installed in the southeastern United States in both 2016 and 2017, the 10% increase in cone survival will substantially increase seed production.

Keywords: *Pinus taeda*, seed orchard, cone analysis, controlled pollination, conelet survival

Introduction

Loblolly Pine (*Pinus taeda* L.) is among the most important commercial forest tree species in the United States and in the world (Schultz 1997). Because the wood of loblolly pine is highly desired and used to produce many products and because it is a species widely adapted to grown in a variety of landscapes, it is the most commonly planted tree species in the United States. Over the last 60+ years, plantations of loblolly pine have increased substantially (South and Harper 2016) and now comprise about 19% of forests in the southern US (Huggett et al. 2013). To meet this tree planting demand, nurseries around the South grow genetically improved loblolly pine in large quantities each year. For example, of the one billion seedlings produced by nurseries in the South in the fall of 2013, approximately 97% were conifers (South et al. 2016). Additionally, since 1977, nearly 100% of all loblolly pine plantations in the Southeast United States have been established using genetically improved seedlings (Aspinwall et al. 2012).

In order to produce these genetically improved trees, more than 4,000 hectares of seed orchards have been established in the southern United States since the early 1960's, primarily for loblolly pine and slash pine (*Pinus elliottii Engelm)* (Bramlett 1991). Seed orchards are currently established by grafting cuttings from tested and genetically proven superior parents onto rootstock. These clones produce progeny that are superior for traits such as volume growth, stem straightness, and resistance to disease. Rootstock is planted at a wide spacing (e.g. 5m x 10m up to 15m x 15m) to maximize crown size and number of cones produced. The purpose of a seed orchard is to provide an area where improved genetic material can be easily accessed and large quantities of genetically superior seed can be produced (Zobel et al. 1958).

In 2016, over 27,000 kilograms of improved loblolly pine seed were harvested from seed orchards around the Southeast by members of the NC State University Cooperative Tree Improvement Program (NCSUCTIP 2017). Once extracted and cleaned, this seed is sent to nurseries to be grown into high quality seedlings. In the 2015-2016 planting season, over 782 million loblolly pine seedlings were grown in the South (Enebak 2016). At the end of each growing season, these seedlings are packed, shipped, and then planted.

In loblolly pine seed orchards, seed is collected for seedling production in three primary ways: as seed orchard mixes of open-pollinated seed, as an open-pollinated (OP) family, or as a full-sib cross. Traditionally, loblolly pine seed orchard managers harvested seed to sell as seed orchard mixes. In this approach, open-pollinated seed is collected from selected clones and then bulked together to be sold as a seed orchard mix. The advantage of this mix is that the planted area will have a higher level of genetic diversity when compared to planting a single family. The disadvantage of a seed orchard mix is that the landowner does not realize the genetic gains that can be made with other techniques. This technique has been used in southern pine seed orchards since the early 1950's (Bramlett 1991). However, with the further development of a seedling market, many orchard managers have moved away from producing and marketing seedlings as seed orchard mixes.

Planting seedlings of single open-pollinated (OP) families started in the 1970's (Gladstone 1975, Duzan and Williams 1988) and is currently the most common method for collecting and marketing loblolly pine (McKeand et al. 2003). In this approach, cones are collected from ramets of a single clone that has been pollinated by the wind-borne pollen cloud that occurs in that seed orchard. Even though the female may have good genetics, pollen is not guaranteed to come from the other genetically superior trees in the orchard. In

theory, for the seeds that are pollinated from pollen outside of the seed orchard, genetic gains are reduced by half (Bridgwater et al. 1998).

The most recent major transition occurring in many loblolly pine seed orchards is the mass production of large quantities of full-sib seed. The purpose of producing full-sib seed is to increase the amount of achieved genetic gain. Gains from full-sib families can be as high as 40% over non-improved material for plantations harvested at rotation ages 25-28 years (Li et al. 1999). Based on yearly surveys of members of the NCSU Cooperative Tree Improvement Program, full-sib seedling production has increased almost every year since 2000. In the past 5 years, full-sib seedling production has increased from 30 million seedlings in 2012 to over 116 million seedlings in 2017 (Figure 2.1).

In order to produce full-sib seed, megasporangiate strobili (commonly referred to as female strobili or flowers) must be isolated from outside pollen contamination using a pollination bag while the desired pollen is applied (pollen originates from the microsporangiate strobili, commonly referred to as catkins). The pollination bag must be constructed of a material with pore sizes small enough to form a barrier to outside pollen but at the same time allow for movement of gases to prevent the accumulation of moisture, carbon dioxide, and heat in the bags (Sedgley and Griffin 1989). To increase success of controlled pollinations, pollen should be applied within two days of maximum female strobilus receptivity, stage 5 (Bramlett and Matthews 1983) when scales are separated and held at right angles to the axis of the cone (Bramlett and O'Gwynn 1980).

For breeding programs, sausage casings (clear cellulose bags) were used to isolate female strobili in loblolly pine in the early years of full-sib production. Installing these bags also required the installation of an internal support structure which was very time consuming.

For mass production of full-sib seed, the industry began using a Kraft paper pollination bag produced by the Lawson Company that is held together by a “shower-proof” (i.e. waterproof) glue. These bags do not require an internal support structure because they form a box-like shape that makes them “self- supporting”. This pollination bag could be installed three to five times faster than sausage casings (Bridgwater et al. 1998), which made the production of controlled cross seeds much more feasible. Another type of material used to produce pollination bags were those made of a closely woven cotton fabric. The use of different pollination bag types for breeding in pine has been studied and was last summarized by Bramlett and O’Gwynn (1981). Over the last 35+ years, virtually no research on pollination bags for loblolly pine has been made publicly available.

Even though the use of Kraft paper bags has increased, the number of bags that a production crew is able to install is limited, and orchard managers have questioned if seed yields from these bags can be improved. For this reason and because the demand for full-sib seedlings increased dramatically, a pollination bag study was initiated during the spring of 2014. Members of the NCSU Cooperative Tree Improvement Program conducted a study that compared prototypes of four PBS International pollination bags to the industry standard Lawson pollination bag. In addition to these five bags, open-pollinated (OP) female strobili and a Lawson bag including a wire for support were added as treatments to this study. The wire support was added because many orchard managers suggested internal support improved the performance of the Lawson bag. The primary goal of this study was to compare the PBS prototypes and Lawson with support wire to the industry standard Lawson pollination bag for mass production of controlled cross loblolly pine seed. Additional objectives were to determine if cone survival could be effectively assessed in June following

pollination in February/March, and to compare open-pollinated strobili to those of pollination bags for cone survival.

Materials and Methods

Study Sites and Experimental Design

The initial study (referred to as the 2014 Study) was installed at nine seed orchard sites across the Southeast in 2014. Three clones per orchard site were used. These were clones that were being actively used for operational production of full-sib seed. Within the crown of each orchard ramet, 10 replications were installed (From this point forward, reps will be referred to as blocks, as that was the experimental unit). Each block consisted of six different treatments (bag types): Lawson (L), Lawson with wire support (Lw), and PBS A, B, C, and D bags (Table A.1). Bags in each block were placed on similar branches and grouped together in the crown as closely as possible (Figure 2.2). During bagging, an effort was made to install the bags in a random order on branches of comparable size and with a comparable number of female strobili. Two open-pollinated (OP) branches were included for comparisons. At time of bagging, each branch was labeled with an aluminum tag with the block number and bag type. For OP branches, a tag was placed just below the female strobili being monitored. When the female strobili inside of each bag were deemed to be receptive, all of the bags in that tree were pollinated at the same time using the same operational pollen lot. Pollen was to be of high-quality, with percent germination from in-vitro pollen germination tests sent to Tree Improvement staff in Raleigh, NC. Each bag was pollinated twice in an attempt to account for any variation inside of bags for female strobilus

development. Following pollination, crews monitored the female strobili and removed the bags once strobili had reached stage 6 (Bramlett and O'Gwynn 1980). At stage 6, the scales are swollen shut and strobili are no longer receptive to pollen.

A portion of this study was installed by the NCSU Cooperative Tree Improvement Program staff at two of the orchard sites at the Georgia Forestry Commission Arrowhead Seed Orchard in Cochran, Georgia and the North Carolina Forest Service Nursery in Goldsboro, North Carolina. At both of these locations, each block included the same six bag types and an open-pollinated branch. These seven treatments were installed in three clones per orchard, with 10 blocks per ramet.

A second pollination bag prototype study was installed in the spring of 2015, referred to here as the 2015 Study. The design of the 2015 Study was similar to that of the 2014 Study, with a few exceptions. As in the 2014 Study, nine orchards participated in the 2015 installation. In each orchard, three clones were used with one ramet per clone. Instead of 10 blocks, only five blocks were installed into each tree. Three of these blocks included seven treatments while two of the blocks included nine treatments (an additional A and redesigned B2 bag from the 2014 Study). The treatment included L, Lw, PBS A, B2, E, F, G, H, and an OP branch. In contrast to the 2014 Study, where OP branches were only installed in two of the blocks during sampling, in the 2015 Study, it was included as a treatment in all blocks. PBS bags used in the 2015 Study were composed of different materials and had different shapes than those in the 2014 Study (Table A.1). Bag types PBS-A, L, and Lw were also used in the 2014 Study and provided connections between the two years (Table A.2). Bags in each block were placed on similar branches and grouped together in the crown as closely as possible.

In the spring of 2017, a final prototype study (referred to as the 2017 Study) was installed to evaluate a new PBS bag design. The bags included in this study were two new prototype PBS bags A2 and I2, a previously tested PBS bag A, and the Lawson bag with a support wire (Lw). An open-pollinated treatment (OP) was also included to make comparisons. The new prototype PBS bags A2 and I2 were of an improved design and material that was expected to reduce wind damage to female strobili and to remain open better than previous designs (Table A.1).

The 2017 study was installed at six orchards throughout the southeastern US. At each orchard, managers were asked to sample three clones (one ramet per clone for a total of three trees). These were clones used for operational production of full-sib seeds. Within each tree, 10 blocks were installed with each block containing all five treatments (PBS-A, PBS-A2, PBS-I2, Lw, and OP). As in the previous studies, bags in each block were placed on similar branches and grouped together in the crown as closely as possible. Bag types PBS-A and Lw were the only bag types used across all three study years (Table A.2), providing connections between the years for analyses.

Measurements and Assessments

In order to determine the success of each bag, several measurements were recorded throughout the 18-month process of cone development. At all orchard sites, the number of female strobili at time of bagging, conelets[1] at time of bag removal, conelets after the June drop, and cones[2] alive at time of harvest were recorded. After pollination and the time of contamination from outside pollen sources was over, all bags were removed to prevent

[1] Once a female strobilus is pollinated, it is then classified as a conelet (Williams 2009).

[2] When fertilization occurs, the conelet is then classified as a cone (Williams 2009).

further bag damage to the conelets. Bag removal typically occurs two weeks after pollination (Bramlett and O'Gwynn 1981). As bags were removed, the number of conelets alive for each bag type was recorded. The next assessment period was in June. At the time of the "June drop", about four months after pollination, the number of surviving conelets for each bag were counted. June drop, also referred to as conelet drop in the literature, has been documented in many other species of *Pinus*. Conelet drop is defined as the premature loss of first year female strobili, which starts at time of receptivity and has concluded approximately four months following pollination (Sweet and Thulin 1969). It is caused by a variety of factors such as inadequate pollen, insect damage, cold injury, or mechanical injury. June drop was recorded in this study to test if bag success for female strobilus/cone survival in loblolly pine could be measured at a point in time much sooner than when cones are ready for harvest, 18 months after pollination.

Approximately 18 months after pollination, the cones were harvested. For each treatment bag, the number of cones surviving at time of harvest were recorded. This measure of success, using the ratio of total harvested cones over the number of female strobili initiated, was described as cone efficiency (Bramlett et al. 1977). The 2017 Study does not have cone survival data, as cones will not be fully developed until October of 2018.

In addition to these measurements, cones were collected and sent to the NCSU Cooperative Tree Improvement Program in Raleigh, NC for processing. Cones were collected for two purposes, to determine seed yield per cone and to be used for cone

analysis[3]. Cone analysis and seed yield for the 2015 Study is still ongoing and will not be discussed in this paper.

Statistical Analysis

After data from all three study years had been combined, the first data step performed was to create a variable called tree. Tree concatenated variables orchard and clone to create a unique id for each tree name. Some clones were unintentionally used in multiple orchards, and the models used in this analysis were trying to estimate clonal effects across orchards. This experiment was not designed to estimate clonal effects, so the variable tree was created so that these models would converge.

Data were checked for data errors and outliers. The variable "Bag Removal Survival" was created by dividing the number of conelets alive at bag removal by the number of female strobili bagged. The variables "June Survival" and "Cone Survival" were also created using the ratio of surviving conelets in June to female strobili bagged and cones alive at time of harvest to female strobili bagged. Means were calculated per orchard, tree, and bag type for each of these variables. One orchard suffered a severe cold event in 2017 where conelet survival at bag removal was negatively impacted for all bag types, and no conelets were recorded in June. Winter 2016-2017 was much warmer than usual, and female strobilus emergence occurred approximately two weeks earlier than the previous study years. On March 16th and 17th, nighttime temperatures reached -6.5°C at this more northern orchard

[3] Cone analysis is a procedure for processing a cone in a way that will provide information about production efficiency of the cone's seed orchard and allow orchard managers to diagnose problems with their orchard (Bramlett et al. 1977).

location. Mean conelet survival at bag removal for this orchard was 64%, the lowest among all orchards in all study years for conelet survival at bag removal. Due to survival results at this orchard being much different than those of other orchards and the differences being a reflection of freeze damage, not a reflection of typical bag performance, this orchard was dropped from all analyses.

Also shown from the variables “June Survival” and “Cone Survival” were instances where survival was greater than 100%. The most likely reason for these observations is that the number of female strobili bagged was undercounted. Counting an incorrect number of female strobili at time of bagging is an easy mistake to make, because female strobili are counted at early stages of development prior to bagging and can be easily missed. For “June Survival” there were 71 observations where survival was greater than 100%. These observations were dropped from the data set and all analyses as they were clear sampling errors. After the 71 observations were dropped there were still 19 observations with “Cone Survival” greater than 100%. These were also removed from the data set and all analyses.

Because the primary objective of this research was to compare bag types to see which bag performed best, and because open-pollinated branches were only installed in two of the blocks in most orchards in the 2014 Study, the open-pollinated treatment was dropped from all models. After conclusions had been reached about bag type effects, the open-pollinated treatment was added back to these analyses in order to make comparisons between bag types and the open-pollinated female strobili.

Female Strobili at Time of Bagging

The following linear mixed model was fit to test if bag types had the same numbers of female strobili bagged ($H0$: $\mu_1 = \mu_2 = \mu_3, \ldots, \mu_n$).

$$Y_{ijklm} = \mu + B_i + R_j + O_k + T_l + S(T)_{m(l)} + BR_{ij} + BO_{ik} + \varepsilon_{ijklm} \qquad \text{Eq. 1}$$

Where:

Y_{ijklm} is the number of female strobili at time of bagging of bag type *i*, in year *j*, in orchard *k*, in tree *l*, in the *m*th block nested within tree *l*,

μ is the overall mean,

B_i is the fixed Bag Type effect,

R_j is the fixed Year effect,

O_k is the random Orchard effect with expectations ~N $(0, \sigma_O^2)$,

T_l is the random Tree effect with expectations ~N $(0, \sigma_T^2)$,

$S(T)_{m(l)}$ is the random Block within Tree effect with expectations ~N $(0, \sigma_R^2)$,

BR_{ij} is the fixed interaction of Bag Type by Year effect,

BO_{ik} is the random interaction of Bag Type by Orchard effect ~N $(0, \sigma_{BO}^2)$,

ε_{ijklm} is the random error effect with expectations ~N $(0, \sigma^2)$.

The model was fit using the MIXED procedure in SAS software (SAS Institute Inc. 2011).

Conelet and Cone Survival

Conelet survival at bag removal (two weeks post pollination), conelet survival in June (four months post pollination), and cone survival at time of harvest (18 months post pollination) were assessed. At each assessment for each bag type, the counts of conelets or

cones alive over the counts of strobili bagged was the variable of interest and was treated as binomial count data with binomial distribution. For example, for cone survival, the number of cones alive at time of harvest over the number of female strobili bagged was the response variable (y = n/N) where *n* is the count of cones at time of harvest (18 months post pollination) and *N* is the count of female strobili bagged. Such a response variable is called binomial counts and is expected to have a binomial distribution. A generalized linear mixed model was fit to each response variable (conelets alive at bag removal, conelets alive in June, and cones alive at time of harvest) to determine the effect of bag type on the probability of conelet or cone success (survival) at each assessment period. The model fit across all years is shown in equation 2 (Isik et al. 2008).

$$Y_{ijkl} = \log(\pi/(1-\pi)) = \mu + B_i + R_j + O_k + T_l + BR_{ij} + \varepsilon_{ijkl} \qquad \text{Eq. 2}$$

Where:

Y_{ijkl} is the response variable, binomial count of conelets or cones alive,

π is the probability of conelet or cone success,

μ is the overall mean,

B_i is the fixed Bag Type effect,

R_j is the fixed Year effect,

O_k is the random Orchard effect with expectations ~N $(0, \sigma_O^2)$,

T_l is the random Tree effect with expectations ~N $(0, \sigma_T^2)$,

BR_{ij} is the fixed interaction of Bag Type by Year effect,

ε_{ijkl} is the random error effect with expectations ~N $(0, \sigma^2)$.

The random effect "block" was dropped from the final model because it did not explain any variance.

The same model can be presented in matrix form for ease of interpretation and assumptions of generalized linear models (Isik et al. 2012).

$$E\{\mathbf{y}|\mathbf{u}\} = g^{-1}(\mathbf{X\beta} + \mathbf{Zu}) \qquad \text{Eq. 3}$$

Where:

y = (*n x 1*) response vector,

u = vector of random effects,

g^{-1} = inverse link function,

X = (*n x p*) design matrix of rank *k* for the (*p x 1*) fixed effects **β**,

β = vector of fixed effects,

Z = (*n x q*) design matrix for the (*q x 1*) random effects **u**,

Random effects, **u**, are assumed to be normally distributed with a mean equal to 0 and variance matrix **G**,

The inverse link function was used to calculate solutions of the fixed bag type effect on the probability scale:

$$g^{-1} = e^{n} = 1/(1 + e^{n}) \qquad \text{Eq. 4}$$

The model was fit using the GLIMMIX procedure of SAS software (SAS Institute Inc. 2011). To determine if bag types were greater than the industry standard Lawson (L) pollination bag, the LSMEANS statement was run in the GLIMMIX procedure using the Dunnett's test where bag type L was set as the reference. To determine if bag types were

significantly different from each other, Tukey adjusted post-hoc test was used at an alpha of 0.05. Additionally, odds ratios were estimated to show the relative differences between bag types for survival.

Plots were constructed to compare early assessments of female strobilus survival to final survival rates at time of cone harvest. Both conelets at bag removal and conelets in June were compared to cones at time of harvest. To construct these plots, the mean strobilus survival per bag type at each tree was plotted, and a regression line was fit with a 95% confidence interval. Observations where mean conelet survival at bag removal was greater than 100% were dropped from the analysis. Observations where mean cone survival at time of harvest was higher than mean conelet survival in June were also dropped. The open-pollinated branches were also not included in these plots.

Results

Number of Female Strobili Bagged

Number of female strobili bagged per bag type was inspected to assure there was no initial bias from orchard managers when bags were first installed. The bag type main effect was not significant (Pr = 0.3938) for female strobili bagged. The year effect and interaction effect between year and bag type were both not significant (Pr = 0.2832 and Pr = 0.2951 respectively). The orchard effect was the only random effect not significant as indicated by the small ratio of its covariance parameter estimate over its standard error (1.17). The mean number of female strobili bagged across all bag types was 8.2 or approximately eight female strobili per bag.

Conelet Survival at Bag Removal

The number of conelets alive at bag removal (two weeks post pollination) provided the earliest assessment of bag performance. At time of bag removal, the bag type effect was highly significant (Pr<.0001). The fixed effect year and the interaction effect of year by bag type were not significant (Table 2.1) and were dropped from the model. Random effects orchard and tree were significant as suggested by the large ratios of their covariance parameter estimate over their standard error (2.35 and 3.56 respectively).

Dunnett's one-tailed t-test indicated bag types PBS-C, PBS-D, and PBS-B2 were the only bag types not performing greater than bag type L for conelets alive at bag removal. The plot of odds ratios (Figure 2.3), which shows all bags compared to bag type L for survival at bag removal, visually displays the same results as the Dunnett's test. Bag types PBS-C and PBS-D performed worse than bag type L with 26% and 37% lower odds of survival, respectively. Bag type PBS-H performed the best for conelet survival at bag removal with an odds ratio of 2.84. This can be interpreted that the odds of a female strobilus bagged using PBS-H surviving to a conelet at bag removal is 2.84 times more likely to succeed than one bagged using bag type L.

Conelet Survival at Time of June Drop

Number of surviving conelets in June (four months post pollination) differed significantly (Pr<.0001) among bag types. The fixed interaction term year by bag type was also found to be highly significant for conelet survival in June (Table 2.2). The fixed effect year was not significant. The random effects of orchard and tree were significant, as

suggested by their high ratios (2.08 and 4.46 respectively) from their estimate over their standard error.

Odds ratio estimates were calculated for each bag type compared to bag type L (Figure 2.4). The best bag for conelet survival in June was PBS-A2; female strobili bagged using PBS-A2 were 2.93 times more likely to survive to June than female strobili bagged using bag type L. Results of Dunnett's test showed all bag types except for PBS-C and PBS-D performed significantly better than bag type L for conelet survival in June. Using Tukey comparisons among least squares means at an alpha of 0.05, PBS-A2 was significantly different than all other bag types for conelet survival in June, and PBS-I2 was significantly different than all bag types except for PBS-G and PBS-H.

Cone Survival at Time of Harvest

Cone survival at time of harvest was assessed, but as a reminder, cone survival has not yet been collected from the 2017 Study. When assessing cone survival for the first two study years, the main effect bag type was highly significant (Pr<.0001) for cone survival at time of harvest. The fixed effects of year and the interaction between year and bag type were not significant (Table 2.3) and were dropped from the model. The random effect tree was significant as suggested by the high ratio (3.68) of its estimate over its standard error. The low ratio (1.18) of the estimate over the standard error for orchard suggests it was not significant.

Bag type Lw performed the best with an odds ratio estimate of 2.02 (Figure 2.5). This can be interpreted that a female strobilus bagged using bag type Lw is over twice as likely to survive to cone harvest than a strobilus bagged using bag type L. The odds ratio

estimate of PBS-D, which performed worse than bag type L, was 0.76, which can be interpreted that a female strobilus bagged using bag type PBS-D is 24% less likely to survive to cone harvest than one bagged using bag type L. Dunnett's test was also used to compare each bag type to the reference bag type L, and all bags except for PBS-C and PBS-D performed significantly better than bag type L (Pr<.0015).

Least squares mean strobilus survival for all bags at each assessment period are presented in Table 2.4. Using these means, a plot was created to show the survival of each bag type at each assessment period through time (Figure 2.6). Major differences in survival are not noticed until conelet survival was assessed in June, approximately four months after pollination. The data clearly show two different groups for conelet survival in June with bag types L, PBS-C, and PBS-D having lower success compared to the rest of the bags.

Scatter plots were created to show the relationship between conelet survival at bag removal and cone survival at time of harvest, conelet survival in June and cone survival at time of harvest, and conelet survival at bag removal and conelet survival in June. All scatterplots were highly significant (Pr<.0001). A weak positive relationship was found between conelet survival at bag removal and cone survival at time of harvest (r=0.32) (Figure 2.7), but conelet survival in June had a positive and high correlation with cone survival at time of harvest (r=0.79) (Figure 2.8). A moderate positive relationship was found between conelet survival at bag removal and conelet survival in June (r=0.42) (Figure 2.9)

After comparisons were made among bag types, the open-pollinated treatment was added back to the data to make comparisons between open-pollinated strobili and controlled pollinated strobili. As expected, open-pollinated strobili performed very well with an odds ratio estimate for conelet survival in June of 3.59 (Figure 2.10) and cone survival at time of

harvest of 3.36 (Figure 2.11). This can be interpreted that open-pollinated strobili were 3.36 times more likely to succeed to cone harvest than female strobili bagged in bag type L. The closest bag type to the OP treatment for cone survival was Lw with an odds ratio estimate of 2.02. The least squares mean survival for OP cones at cone harvest was 0.81, 10% better than the best bag type Lw.

Discussions

For a pollination bag to be an effective alternative to the Lawson bag, it must be superior for one or more of the following attributes: 1) Increase female strobilus survival per bag, ultimately resulting in more cones at cone harvest. This would likely happen by reducing risk from wind damage and temperature extremes. 2) Increase seed yield of each cone in the pollination bag. 3) Decrease labor demands, which is more difficult to quantify.

The design and implementation of this study was successful in that it allowed for detection of differences in female strobilus survival among different pollination bags. The first step to this successful study was orchard managers installing bags without bias. The bag type effect was not significant (Pr = 0.3938) for number of female strobili bagged, suggesting bags were installed on a comparable number of female strobili for each bag type.

Even though the bag type effect was highly significant (Pr <.0001) for conelets alive at bag removal, conelets alive at bag removal was not a good indicator for early bag performance. There was a moderate relationship (r = 0.42) between survival at bag removal and survival in June (Figure 2.9) and a weak relationship (r=0.32) between survival at bag removal and survival at time of cone harvest (Figure 2.7). This was likely due to survival being very high at time of bag removal (94% survival was the lowest of any bag type), and

damage to female strobili caused by bags was not fully apparent. One possible explanation is that female strobili damaged by the bags had not abscised at time of bag removal, and therefore had an exaggerated survival. Also, if any female strobili did not receive adequate pollen due to differences in pollination bag material, conelet mortality would not yet be apparent after only two weeks since pollination. In open-pollinated cones of young orchards, it is common to see female strobili develop but then die within two months of development because there is an inadequate pollen supply in the young orchard (Bramlett et al. 1977).

Differences among bags for female strobilus survival became much more clear when they were assessed in June, approximately four months after pollination. The bag type effect was highly significant in June for conelet survival ($Pr<0.0001$), and this was due to conelet abortion, presumably from bag damage to female strobili. These results help verify the belief that bag stiffness and design is important for female strobilus survival. Bramlett (1993) described one of the major causes for losses of conelets as damage from the pollination bag. Developing female strobili/conelets are very delicate and any rubbing against the bag can cause damage, and if severe, the conelet usually dies (Bramlett 1993). At this assessment period, it was clear which bag types were going to perform poorly for strobilus survival. PBS-C, PBS-D, and bag type L had similar conelet survival in June (Figure 2.6). Also, the newest prototypes, PBS-A2 and PBS-I2 had a conelet survival in June higher than other pollination bags. Overlapping confidence intervals in Figure 2.10 suggests that prototype PBS-A2 is not significantly different than the open-pollinated treatment for conelet survival in June.

The scatter plot showing the relationship between mean conelet survival in June and mean cone survival at time of harvest (Figure 2.8) shows a strong positive relationship

(r=0.79). Knowing that conelet survival at four months has a strong relationship with cone survival 15 to 16 months later is useful for orchard managers wanting an early indication of what they might expect from their future cone crop. Even though there is a strong relationship, there are still some bags with conelets alive in June that totally failed at time of cone harvest, probably due to damage from insects. With this strong relationship between conelet survival in June and cone survival at time of harvest, there is reason to believe that prototypes PBS-A2 and PBS-I2 will have a higher survival at time of cone harvest than previously tested pollination bags.

Differences in bag performance for cone survival at time of harvest was the most significant finding of this research. The installation of PBS bag prototypes and Lawson pollination bags compared different materials and bag designs. With the exception of bags PBS-C and PBS-D, all PBS prototypes tested in the 2014 Study and 2015 Study and bag type Lw were clearly superior to bag type L for cone survival at time of harvest. PBS bags C and D tended to collapse onto the female strobili inside of the bags, which was the likely cause of their lower survival. Although bag type L was not as prone to collapse on to the female strobili, apparently, it was not able to hold its shape as well as PBS prototypes and bag type Lw, especially in high winds. When a pollination bag collapses onto a female strobilus or rubs against it in high winds, damage is done to the strobilus (Bramlett 1997), and the strobilus will frequently abort.

In regards to the different bag shapes/designs, PBS-A and PBS-C bags were the same design and PBS-B and PBS-D bags were the same design. Differences in survival between these bags would indicate that the bag design did not affect strobilus survival, but instead, differences can be attributed to the type of material used. The primary reason for the sharp

differences in female strobilus survival between these two groups of pollination bags could be due to the differences in the rigidity of the material used in producing these bags. The same argument can be made when comparing the survival of Lawson with and without a support wire. Both treatments L and Lw were constructed of the same material, so the 16% difference in cone survival at time of harvest (Table 2.4) can be attributed to the addition of the aluminum wire for support and a likely reduction in damage to female strobili.

When comparing conelet survival in June, the newest prototypes PBS-A2 and I2 had the highest survival among bag types (Table 2.4), and Tukey comparison tests suggest that PBS-A2 is superior to all other bag types for conelet survival in June. The one thing in common for the new prototype bags that separates them from previous bags is that these bags have a new design where the top of each bag has a flap of material that is folded down. It appears that these flaps are reducing the amount of wind that the bags are catching, making the bags more streamlined in the wind. Also, the flaps appear to reinforce the upper shape of the bag, giving it more rigidity and preventing the bag from collapsing inward. Keeping the top of the bag from collapsing inward could be important in reducing damage to female strobili as terminal shoots begin elongating, and female strobili move upwards inside of the bag. Again, if the relationship seen across the previous two studies continues to hold between conelet survival in June and cone survival at time of harvest, PBS-A2 and PBS-I2 should perform very well for cone survival, indicating this bag design to be superior to all other prototypes tested.

As expected, open-pollinated branches were superior to all pollination bags for conelet survival in June and cone survival at time of harvest. With a cone survival of 0.81, open-pollinated strobili had a 10% higher probability of survival to cone harvest than the best

pollination bag and over 25% higher probability of survival to cone harvest than strobili bagged in bag type L. While the higher survival for open-pollinated strobili compared to pollination bags has been generally accepted by tree breeders, there is little published documentation of the reduction in survival for loblolly pine. In a study with *Pinus patula*, open-pollinated branches had cone survival of 59% (1997) and 78% (1998) while the best pollination bag had survival of 43% (1997) and 46% (1998) (Nel et al. 2003).

Conclusions

Results from this study verified common knowledge of loblolly pine orchard managers that open-pollinated cones have a higher survival than cones produced using pollination bags. For the OP treatment, conelet survival in June and cone survival at time of harvest was higher than all bag types. These results suggest that cone production using pollination bags will require additional research in order to produce yields equivalent to those of open-pollinated cones in loblolly pine. To determine if these survival differences are due to damage caused by pollination bags, variation in pollination bag temperature, humidity, or light levels or due to pollen issues such as pollen viability, pollen vigor, pollen age, or simply the timing or frequency of pollen application, additional research is needed.

The strong positive relationship between conelet survival in June and cone survival at time of harvest is important. Knowing that conelet survival at four months had a strong relationship with cone survival 15 to 16 months later is useful for orchard managers wanting an early indication of what they might expect from their future cone crop. The weak relationship between conelet survival at bag removal and cone survival at time of harvest

indicates that two weeks after pollination is too early to draw conclusions about future cone crops.

An important finding from this study is that rigidity/stiffness and design of pollination bags appear to have a major impact on female strobilus survival. Pollination bags should have either a support wire or be constructed of a material that is rigid enough to allow the bag to hold its shape and minimize contact with female strobili during high winds. With the exception of PBS-C and PBS-D, which were made of a material that was not rigid, the PBS bags and bag type Lw had over a 10% increase in mean cone survival at time of cone harvest compared to bag type L. Survival differences shown in June for conelet retention indicate that the newest prototypes, PBS-A2 and PBS-I2 are superior than previously tested prototypes due to their new design. With preliminary findings for seed yields from bags showing that pollination bag type had no significant effect on seed yield per cone (data are still being collected), the best way to increase seed yields per bag is to increase strobilus / conelet survival. Using a stiffer material to construct pollination bags, using a support wire, and the use of a design with a flap that folds down to prevent bags catching in the wind and to stay open appear to be ways to increase strobilus survival and reduce damage to developing strobili.

In the spring of 2017, members of the NC State University Cooperative Tree Improvement Program installed 1,444,707 pollination bags in their operational orchards (NCSUCTIP 2017). Using the average number of surviving cones at time of cone harvest across all study years at approximately 5 cones per bag and the preliminary results for average seed yield per cone in the 2014 Study being approximately 79 seed per cone, a 10% increase in cone survival, would have produced 57,065,927 additional seeds.

Whether or not it is time and cost-effective to install support wires with Lawson bags versus installing a PBS bag that does not require an additional support wire will be up to orchard managers to decide. One thing that is certain is that with the demand for full-sib seedling production rapidly increasing every year, the need for improving the efficiency of full-sib seed production will inevitably continue to increase.

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Table 2.1 F tests for conelet survival at bag removal (a) and variance components for random tree and orchard effects (b).

a) Bag type was highly significant for conelet survival at bag removal. Year and the interaction of year by bag type were not significant.

Source	Num DF	Den DF	F	Pr > F
Year	2	2628	0.46	0.6316
Bag type	12	2628	10.89	<.0001
Year*Bag Type	3	2628	0.78	0.5032

b) Random tree and orchard effects were significant as suggested by a large ratio of the covariance parameter estimate over its standard error.

Source	Estimate	Standard error	Estimate/SE
Tree	0.7019	0.1974	3.56
Orchard	1.6751	0.7128	2.35

Table 2.2 F tests for conelet survival in June (a) and variance components for random tree and orchard effects (b).

a) Bag type and the interaction of year by bag type were highly significant for conelet survival in June. Year was not significant.

Source	Num DF	Den DF	F	Pr> F
Year	2	2841	0.12	0.8909
Bag type	12	2841	38.19	<.0001
Year*Bag Type	3	2841	7.95	<.0001

b) Random tree and orchard effects were significant as suggested by a large ratio of the covariance parameter estimate over its standard error.

Source	Estimate	Standard error	Estimate/SE
Tree	0.5644	0.1266	4.46
Orchard	0.4359	0.2091	2.08

Table 2.3 F tests for cone survival at time of harvest (a) and variance components for random tree and orchard effects (b).

a) Bag type was highly significant for cone survival at time of harvest. Year and the interaction of year by bag type were not significant.

Source	Num DF	Den DF	F	Pr > F
Year	1	2045	0.00	0.9679
Bag type	10	2045	36.52	<.0001
Year*Bag Type	2	2045	2.29	0.1014

b) Random tree effect was significant as suggested by a large ratio of the covariance parameter estimate over its standard error. Random orchard effect explained a smaller variance in cone survival at time of harvest.

Source	Estimate	Standard error	Estimate/SE
Tree	0.6719	0.1826	3.68
Orchard	0.2318	0.1966	1.18

Table 2.4 Estimated mean probability of conelet/cone survival (with standard errors) at each conelet/cone assessment period per bag type. The estimates for cone survival at time of harvest were based on the first two years (2014 and 2015). Thus, data for cone survival at time of harvest were not available for bag types PBS-A2 and PBS-I2 that were included in the 2017 Study.

Bag Type	Conelet Survival at Bag Removal	Conelet Survival at June Drop	Cone Survival at Time of Harvest
Lw	0.98 (0.01)	0.79 (0.03)	0.72 (0.03)
PBS-A	0.97 (0.01)	0.80 (0.03)	0.70 (0.04)
PBS-A2	0.99 (0.01)	0.85 (0.02)	.
PBS-B	0.97 (0.01)	0.79 (0.03)	0.71 (0.04)
PBS-B2	0.96 (0.02)	0.74 (0.04)	0.66 (0.05)
PBS-C	0.95 (0.02)	0.64 (0.04)	0.54 (0.04)
PBS-D	0.94 (0.02)	0.59 (0.04)	0.49 (0.04)
PBS-E	0.98 (0.01)	0.75 (0.03)	0.69 (0.04)
PBS-F	0.98 (0.01)	0.78 (0.03)	0.69 (0.04)
PBS-G	0.98 (0.01)	0.79 (0.03)	0.66 (0.04)
PBS-H	0.99 (0.01)	0.80 (0.03)	0.70 (0.04)
PBS-I2	0.98 (0.01)	0.83 (0.03)	.
L	0.96 (0.01)	0.65 (0.04)	0.56 (0.04)

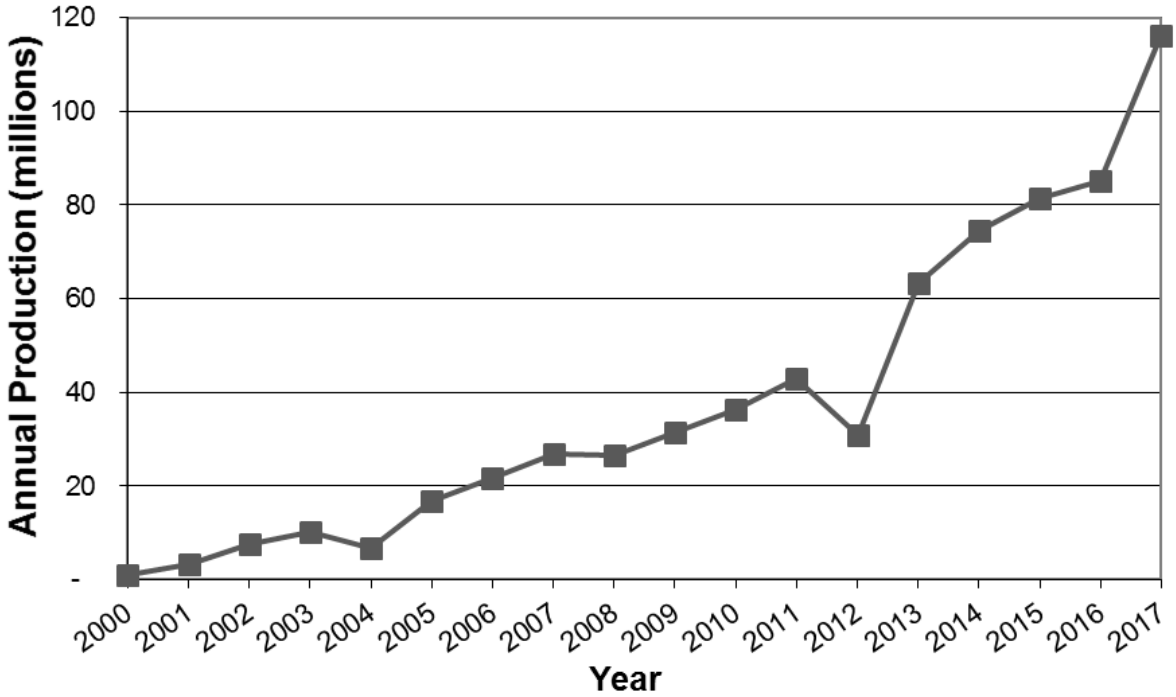

Figure 2.1 Annual mass production of controlled crosses of loblolly pine seedlings. These data have been collected annually from members of the North Carolina State University Cooperative Tree Improvement Program.

Figure 2.2 PBS bags being installed at Arrowhead Seed Orchard in the Spring of 2014. Bags in the red oval are an example of one block in the 2014 Study with six bag types and an open-pollinated branch. Bags in the same block were placed closely together on similar branches on each tree.

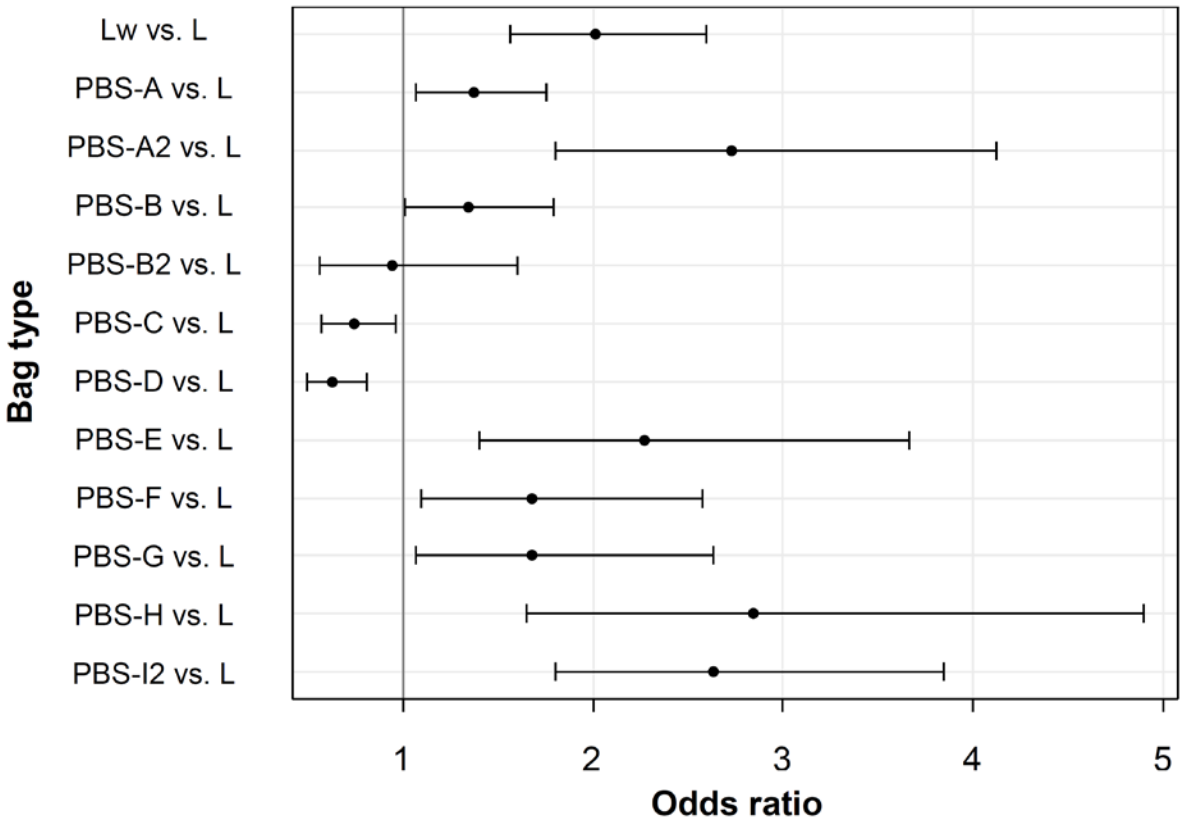

Figure 2.3 Odds ratios for conelet survival at time of bag removal (two weeks post pollination) with 95% confidence limits. Bag types PBS-B2, PBS-C, and PBS-D were the only bags not performing better than bag type L, as indicated by the confidence limits crossing or having values less than 1. PBS-C and PBS-D performed worse than bag type L for probability of conelet survival at bag removal as indicated by confidence limits below 1.

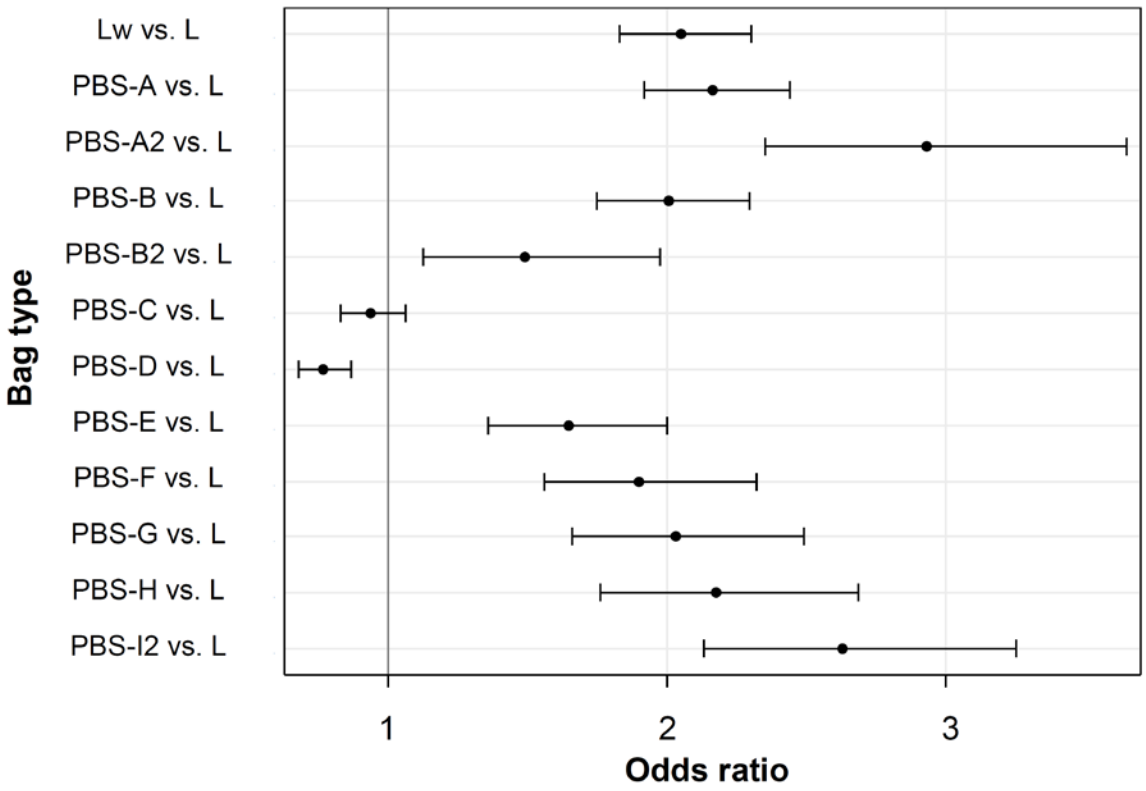

Figure 2.4 Odds ratios for conelet survival in June (four months post pollination) with 95% confidence limits. PBS-A2 had almost 3 times odds of success compared to bag type L. PBS-C did not perform differently than bag type L while PBS-D performed worse than bag type L.

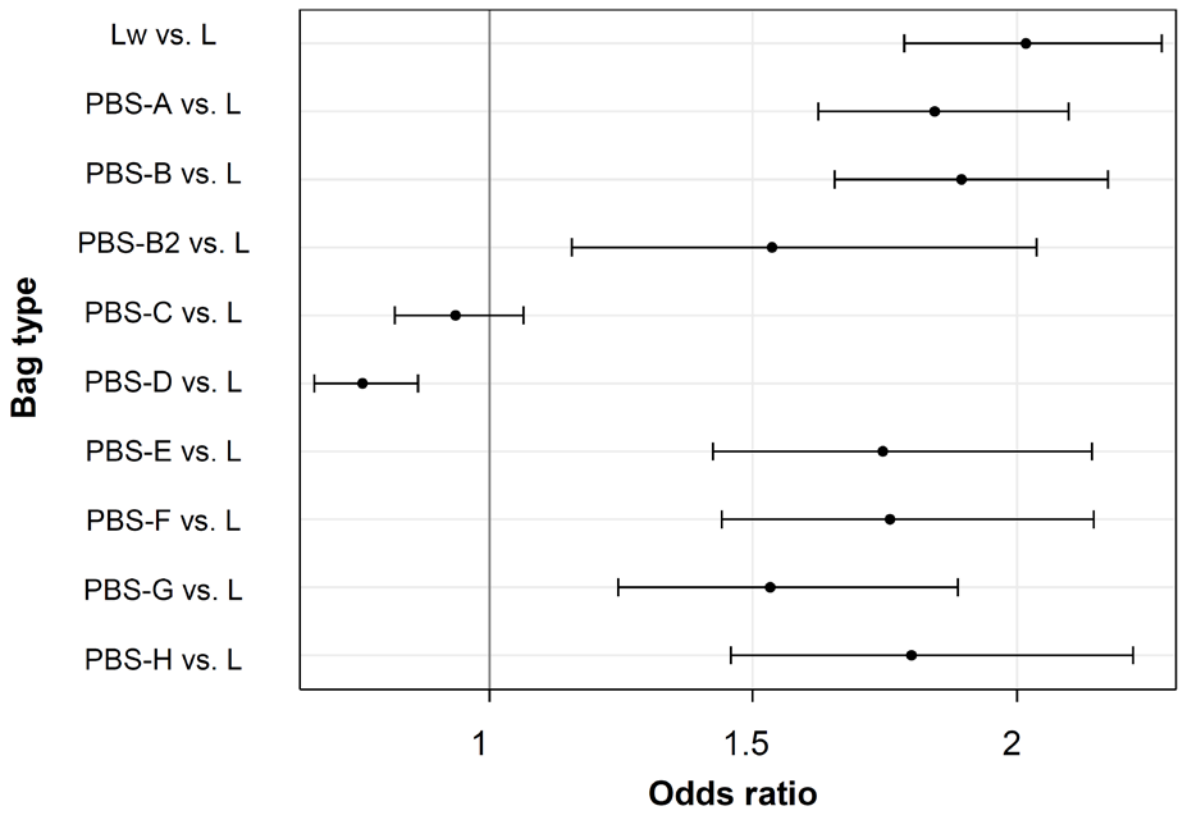

Figure 2.5 Odds ratios for cone survival at time of harvest with 95% confidence limits. PBS-C did not perform differently than bag type L, while PBS-D performed worse than bag type L. Bag types A2 and I2 are not included in this figure, because the cone survival data from the 2017 Study are not yet available.

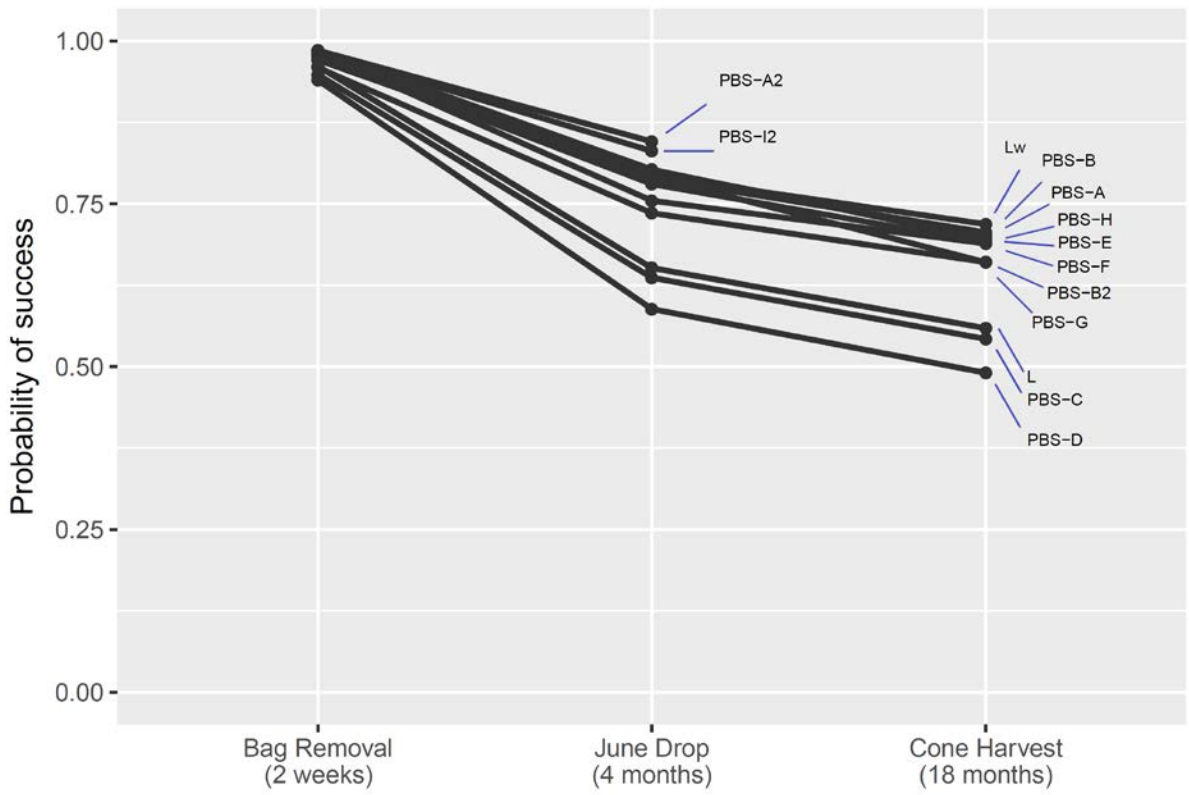

Figure 2.6 Probability of female strobilus success per bag type from bag removal (two weeks post pollination) to June drop (four months post pollination) to cone harvest (18 months post pollination). The critical assessment for survival due to bag type effects appears to be at June drop, four months post pollination. The data clearly show two different groups for June drop. Bag types L, PBS-C, and PBS-D showed lower success compared to the rest of the bags.

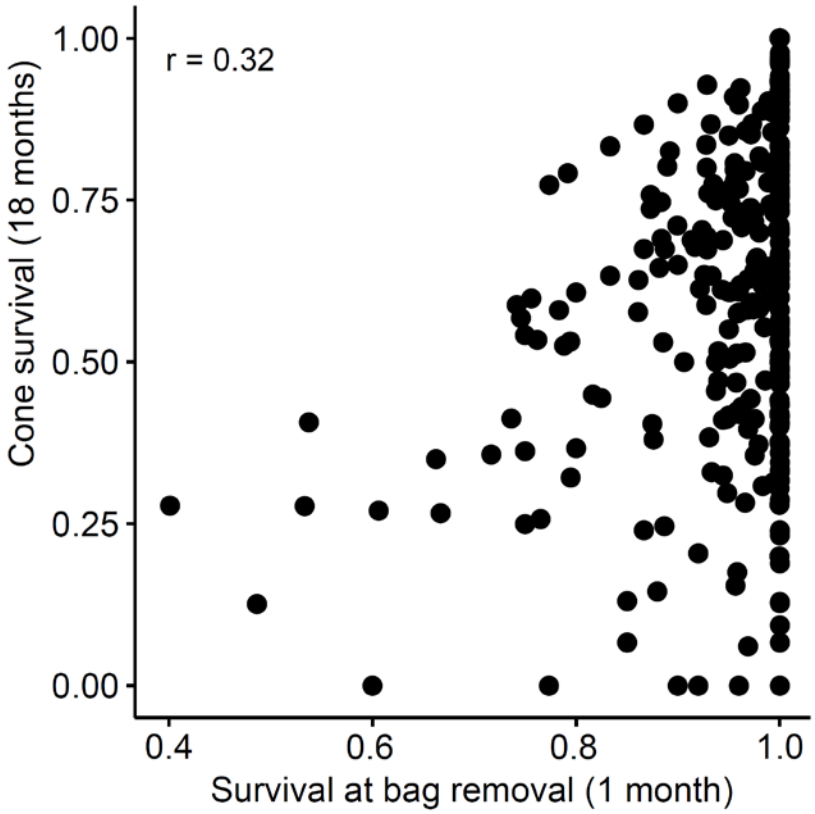

Figure 2.7 Relationship between mean conelet survival at bag removal and the mean cone survival at time of harvest. Means were calculated for each bag type in each orchard tree.

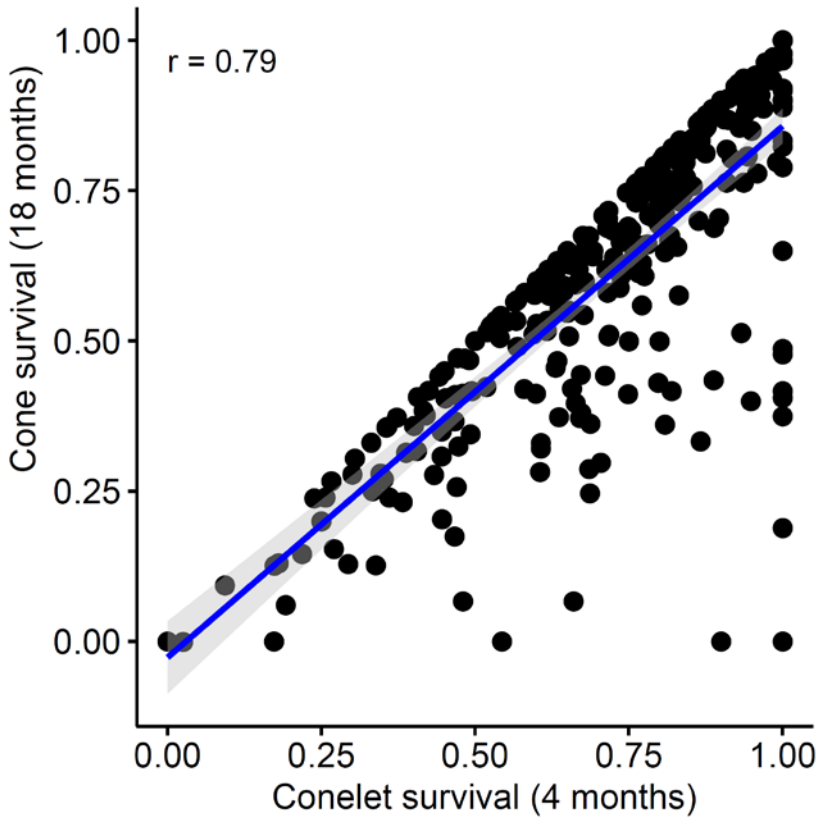

Figure 2.8 Relationship between mean conelet survival in June and the mean cone survival at time of harvest. Means were calculated for each bag type in each orchard tree. Data suggested a linear relationship. However, some conelets did not develop into mature cones, likely due to other factors than bag type, such as insect damage.

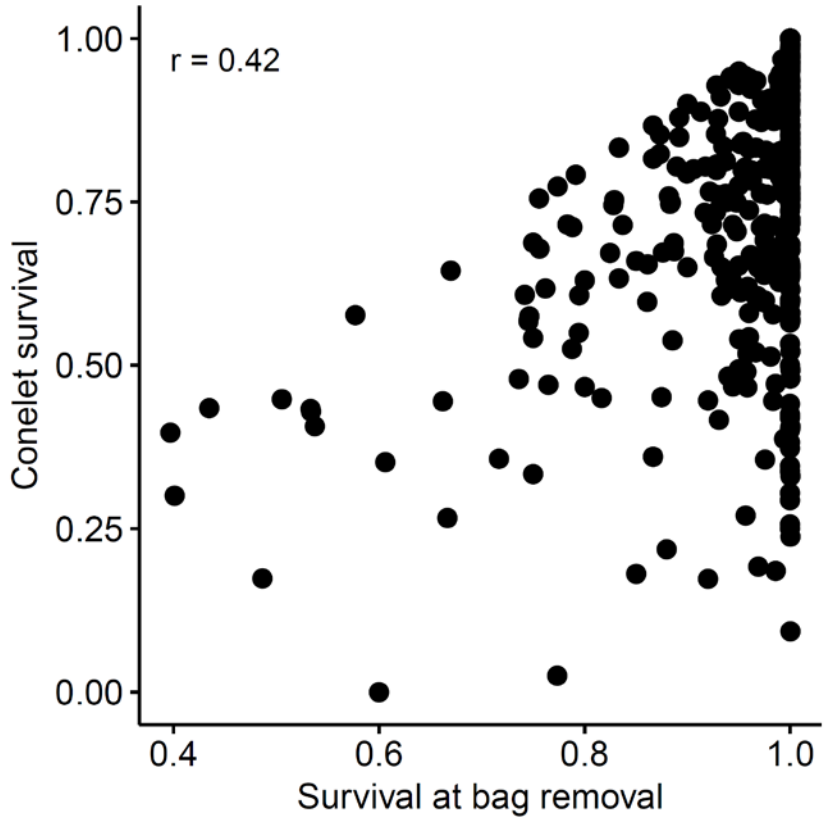

Figure 2.9 Relationship between mean conelet survival at bag removal and mean conelet survival in June. Means were calculated for each bag type in each orchard tree.

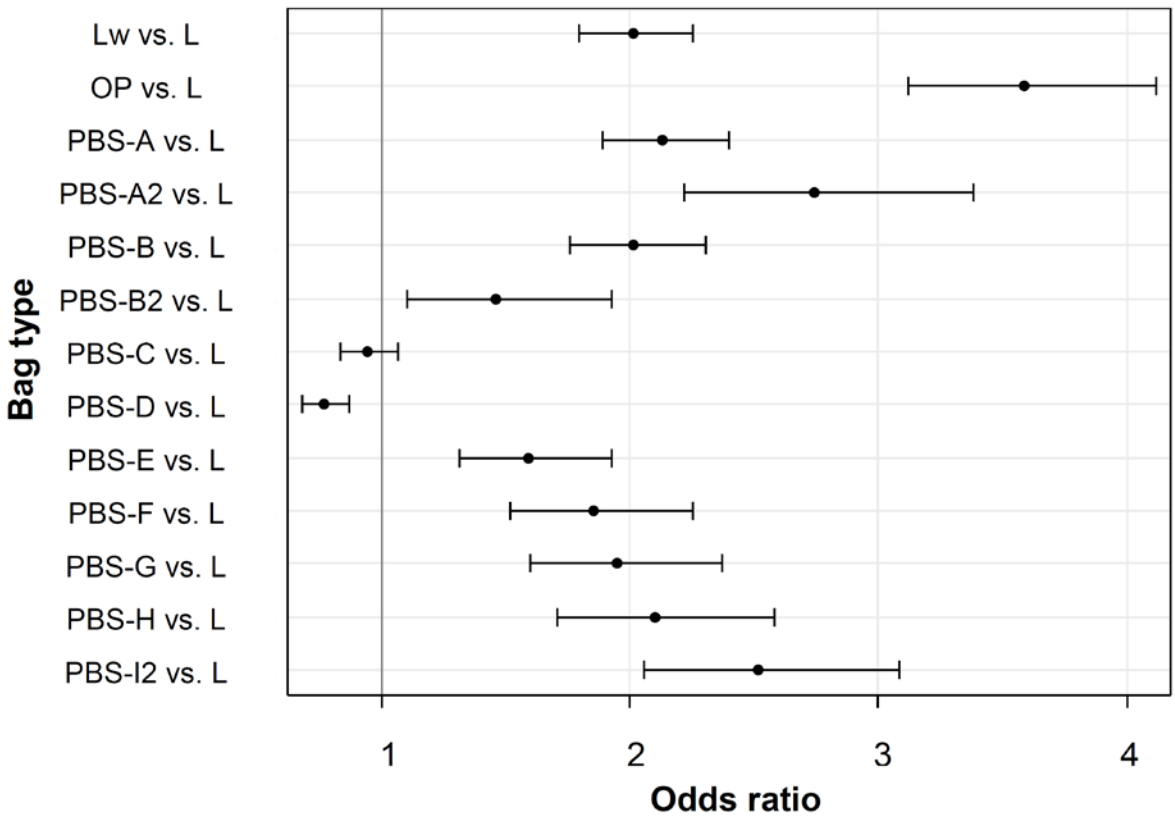

Figure 2.10 Odds ratios for conelet survival in June (including the open-pollinated treatment) with 95% confidence limits. The open-pollinated (OP) treatment performed over 3.5 times better than bag type L for conelet survival in June. Also shown is that one of the newest prototypes, PBS-A2, was not significantly different than the OP treatment for conelet survival in June (shown by overlapping confidence intervals).

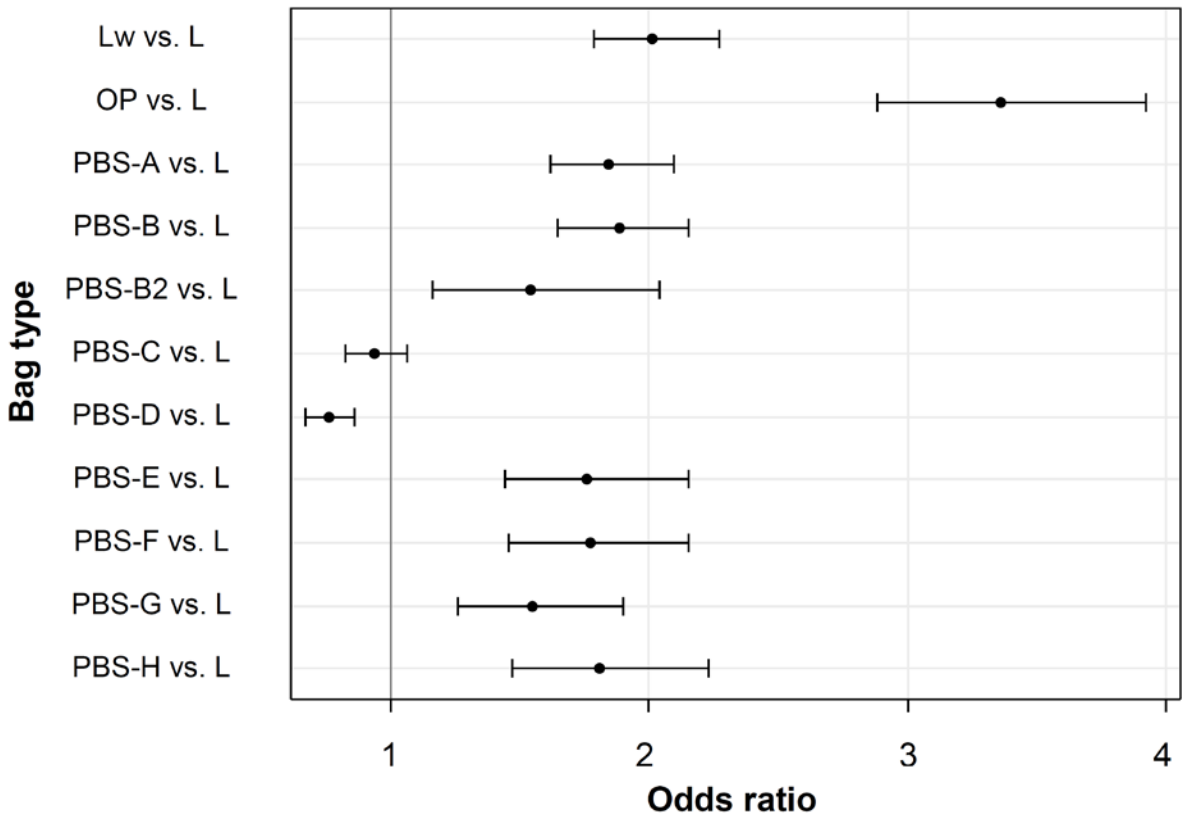

Figure 2.11 Odds ratios for cone survival at time of harvest (including the open-pollinated treatment) with 95% confidence limits. The open-pollinated treatment (OP) performed over 3 times better than bag type L for cone survival at time of harvest. Bag types A2 and I2 are not included in this figure because the cone survival data from the 2017 Study are not yet available.

APPENDIX

Table A.1 Description of pollination bag prototypes used in the study.

Bag Type	Width & Depth (mm)	Length (mm)	Material	Design
PBS-A	158 x 158	550	Smooth surface, “paper-like” material similar to duraweb®	Classic gusseted bag design. Flat.
PBS-B	158 x 158	550	Smooth surface, “paper-like” material similar to duraweb®	Modified design to facilitate staying open. Extra weld along top.
PBS-C	158 x 158	550	Textured surface, softer polypropylene material	Classic gusseted bag design. Flat.
PBS-D	158 x 158	550	Textured surface, softer polypropylene material	Modified design to facilitate staying open. Extra weld along top.
PBS-B2	Cylindrical 160 diameter	460 (open)	Smooth surface, “paper-like” material similar to duraweb®	Stay-open cylindrical design.
PBS-E	158 x 158	550 (flat)	Stiff “paper-like” polyester with no pattern	Classic gusseted bag design. Flat.
PBS-F	Cylindrical 160 diameter	460 (open)	Stiff “paper-like” polyester with no pattern	Stay-open cylindrical design.
PBS-G	158 x 158	550 (flat)	Stiff “fabric-like” polypropylene with pattern	Classic gusseted bag design. Flat.
PBS-H	Cylindrical 160 diameter	460 (open)	Stiff “fabric-like” polypropylene with pattern	Stay-open cylindrical design.

Table A.1 Description of pollination bag prototypes used in the study (continued).

PBS-A2	600 (flat – opens to circle)	550 (flat – opens to 460mm)	Smooth surface, “paper-like” material similar to Duraweb®	Stay open design, with flaps welded down to create a box shape.
PBS-I2	600 (flat – opens to circle)	550 (flat – opens to 460mm)	Lighter weight version of G/H with patterned appearance	Stay open design, with flaps welded down to create a box shape.
Lawson bag 405	170 x 129	520	Kraft paper	Has the same “boxed off” length as PBS design (460mm)

Table A.2 Frequency of bag type occurrence per year. Bag types Lw and PBS-A were the only bag types that occurred in all three study years.

	YEAR			
FREQUENCY	**2014**	**2015**	**2017**	**Total**
L	266	132	0	398
LW	261	127	128	516
PBS-A	263	52	128	443
PBS-A2	0	0	127	127
PBS-B	261	0	0	261
PBS-B2	0	54	0	54
PBS-C	268	0	0	268
PBS-D	261	0	0	261
PBS-E	0	133	0	133
PBS-F	0	134	0	134
PBS-G	0	130	0	130
PBS-H	0	133	0	133
PBS-I2	0	0	125	125