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Title: Induced Mutation for Improving Cascade and Centennial Hop.

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Hop, *Humulus lupulus* L. is a dioecious perennial species. The female hop plant produces flowers that mature into 'cones' which are used as a raw material in the beer brewing process to impart bitterness, flavor, and aroma. The craft beer industry in the current global beer market continues to experience growth as consumers sustain the demand for high quality, full-flavored beers. The majority of the world's hops are grown in the United States and Germany. Washington, Idaho, and Oregon are the leading hop growing states in the U.S.

The hop industry relies on a limited number of cultivars that are recognized for their brewing profile and consumer familiarity with the product. For these reasons, brewers are very reluctant to change hop cultivars in their recipes, particularly aroma hop cultivars. This limits the use of traditional crossing strategies when the breeding goal is to generate a replacement cultivar for an aroma type that enjoys wide industry acceptance. Hop cultivars are highly heterozygous, and progenies produced from traditional crossing techniques may express a large number of traits which are substantially different from the female parent. In contrast, induced mutations such as those caused by gamma radiation, change only one or a few specific traits of an elite cultivar, and can possibly improve agronomic performance without significantly changing the brewing profile. Available literature is lacking on the use of gamma radiation on hop. Therefore,

the objective of this research was to determine the lethal dose of gamma radiation to kill 50% (LD_{50}) of micropropagated nodal sections of Cascade and Centennial hop cultivars, document the vigor of surviving genotypes and determine the percent DNA change or genetic distance of each cultivar within each treatment.

The experiment was arranged as a three-factor completely randomized design (CRD) the factors being cultivar (Cascade, Centennial), gamma radiation dosage (0,2,3,4 Gy), and replicates (three). A treatment combination consisted of a Petri plate containing 50 nodal sections of a cultivar assigned to a gamma dosage. Following irradiation, plants were evaluated in tissue culture and in the greenhouse.

The LD_{50} of surviving genotypes in tissue culture, growth (cm), presence of roots, and plant division were recorded. The LD_{50} of surviving genotypes after greenhouse establishment and plant growth was also recorded. Genotypes were randomly selected within each treatment combination for DNA extraction and sequencing to obtain high molecular weight DNA for PCR pyrosequencing with an Illumina HiSeq 3000 machine. After SNP calling, pairwise genetic distance estimates for each treatment/cultivar were calculated using Bray-Curtis dissimilarity estimates. Genetic distances between treatments samples versus control samples were used to quantify genetic changes.

Survival ratings of Cascade plantlets after irradiation suggest an increase in gamma dose appears to be associated with a decreased survival. An LD_{50} for Cascade plantlets is estimated to be 4 Gy, this suggested rate are consistent with the previous study of hop (Getty, 2015). The LD_{50} after greenhouse establishment is around 3.5 Gy for Cascade. Gamma dosage significantly affected shoot growth in Cascade. Plants subjected to 2 Gy of gamma radiation had the highest mean shoot growth in contrast to 4 Gy which had the shortest mean shoot growth. Gamma

dosage did not significantly affect root growth although, a numeric trend suggested the treatment irradiated with 2 Gy had the highest presence of roots and 4 Gy had the lowest. A strong correlation between survival of Cascade plantlets was observed with shoot and root growth. This suggests a dose with a higher presence of root emergence, shoot growth, and plantlet production will have a higher probability surviving in tissue culture and greenhouse establishment.

Survival ratings from Centennial plants did not suggest a trend of higher radiation dose resulting in decreased survival. However, a biotic contamination in the stock plants presumably increased the death rate of Centennial plants in all treatments as indicated by the low survival of non-treated plants. For this reason, drawing conclusions from the Centennial data was more difficult. An LD_{50} for Centennial plantlets is estimated to be around 3 Gy or 4 Gy after greenhouse establishment.

Higher radiation dosages resulted in a larger genetic distance from the control in Cascade and Centennial hop. Cascade and Centennial followed the trend of LD_{50} ratings, treatments irradiated with the highest level of radiation reached an LD_{50} and had the highest genetic distance compared to the control. Cascade explants irradiated with the highest level of radiation (4 Gy) had the highest genetic distance from the control, in contrast to the lowest dose (2 Gy) which has the lowest genetic distance from the control.

Mutation-based breeding has the potential to improve well-adapted hop cultivars by modifying relatively few traits to increase their productivity or quality while maintaining the unique brewing characteristics of the original cultivar. The study describes a method that can be used to obtain an effective dose to induce mutations and an LD_{50} for nodal sections of micropropagated Cascade and Centennial hop using gamma radiation.

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Induced Mutation for Improving Cascade and Centennial Hop

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Brooke A. Getty, Author

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CHAPTER 1. LITERATURE REVIEW

1.1 HOP USE

Humulus lupulus L*,* the hop plant, provides an important ingredient for beer brewing. The female hop plant produces flowers that mature into 'cones,' which are used as a raw material in the beer brewing process to provide bitterness, flavor, and aroma attributes.

Historically, hops were used in herbal remedies to treat a wide range of ailments. Records from the Middle Ages describe anti-inflammatory properties of hops and anti-microbial effects (Biendl and Pinzl, 2009). Eventually, the ability of hop to provide bitterness, aroma, taste and antimicrobial properties to beer allowed it to replace gruit, a mix of wild herbs and spices, in the brewing process (Small, 1980; Neve, 1991; Smith et al., 1998; Oliver and Colicchio, 2011).

The first reference to hops being used in beer dates to the 9th century when Abbot Adalhard of the Benedictine Monastery of Corbie in the Picardy in northeastern France, made a record stating that his monks added hop to their ales. By the 11th century, hopped beer was commonplace in France (Small, 1980; Neve, 1991; Oliver and Colicchio, 2011). By 1524, commercial hop production in Europe was initiated and the first cultivated hops were introduced into the United States from Europe by the Massachusetts Company in 1629 (Oliver and Colicchio, 2011) although native American hop lines were found and utilized in abundance by the early American colonists (Schwartz, 1973).

In addition to hop being used in beer, prenylflavonoids found in hop have been proposed as medicinal compounds for human use due to their biological activity. The

primary compounds currently being researched include, Xanthohumol (XN) and its products, iso-xanthohumol (IXN) and the phytoestrogen 8-prenylnarigenin (8PN). Conversion of XN into IXN and 8PN is achieved during beer production and after *in vivo* metabolism by liver microsomes and intestinal bacteria (Nikolic et al., 2006; Possemiers et al., 2008; Possemiers and Verstraete, 2009). Studies with Xanthohumol have shown that it possesses the ability to suppress various cancers (Stevens and Page, 2004; Gerhäuser, 2005; Diederich and Noworyta, 2012; Liu et al., 2015; Gallo et al., 2016) in addition to being therapeutic for other health concerns such as metabolic syndrome, inflammation, and bone disease (Stevens and Page, 2004; Liu et al., 2015). Synthetic production of XN has been explored, however, the process is complicated and the overall yield is relatively low (Khupse and Erhardt, 2007). Thus, extraction, isolation, and purification from female hop inflorescences is still the main method to obtain XN. Iso-xanthohumol has shown to exhibit a wide range of effects such as anti-tumor, anti-inflammatory, antioxidant, and antiangiogenic effects (Negrão et al., 2010; Serwe et al., 2012; Allsopp et al., 2013; Krajnović et al., 2016). The hop compound 8PN has been identified as one of the most potent phytoestrogens based on several *in vitro* investigations (Kitaoka et al., 1998; Milligan et al., 1999, 2002; Schaefer et al., 2003; Zierau et al., 2008). Beer is the main dietary source of these constituents, however, the amount found in most commercial beers is not enough to be therapeutic for human health (Liu et al., 2015).

Other studies have evaluated hop β-acids from dried hop cones as a novel feed additive for anti-microbial purposes. Results from (Cornelison et al., 2006) suggests inclusion of ‘Teamaker’ hops into diets at the rate of 0.5 pounds per ton for broiler

chickens may result in improved growth rate and feed utilization in the absence of antibiotics. Teamaker hop has very little alpha acids and is thought to be more palatable to livestock. Bortoluzzi et al., (2014) demonstrated positive effects of supplementation of hops on productive performance of broilers, and the best results were obtained with addition of 30 mg/kg of hops β-acids. Chilliard, (2009) investigated the effects of beta acid extract of hops on ruminal metabolism of Angus cross steers (*Bos taurus)*. Results from this study did not demonstrate any significant effects of extracts of hops on ruminal fermentation or diet digestibility. Spent hops have also been investigated as a source of eco-friendly repellents for insect pests of stored foods (DeGrandi-Hoffman et al., 2012; Bedini et al., 2015).

These medicinal effects have led to the possibility of using hop not only for beer production but also for other purposes such as medicine or food additives.

1.2 HOP STATISTICS

The craft beer industry in the U.S. has been experiencing rapid growth since 2008 (Brewers Association, 2018). The number of operating craft breweries has grown from eight in 1980 to 537 in 1994 and to over 7,450 in 2018 (Brewers Association, 2018). Even as the number of craft breweries in the United States reaches a saturation point, the global average of hopping rate per liter is increasing as the North American craft beer market continues to be a major influencing factor in the world industry (Piroue, 2018).

The majority of the world's hops are grown in the United States and Germany, with smaller contributions found in various places around the world. In 2018, the United

States produced a total of 49,446 metric tons of hop and Germany supplied 45,000 metric tons (IHGC, 2019).

America's hop growers have responded to the demand for more hops, particularly aroma hops. Production for Idaho, Oregon, and Washington in 2018, the major hop producing states in the United States, totaled 107 million pounds which is a one percent increase from the 2017 crop of 106 million pounds (Hop Growers of America, 2018). Washington, the most important hop-growing state in the US, produced 77.7 million pounds of hops, accounting for roughly 73% of the United States hop crop for 2018. Acreage in Idaho, Oregon, and Washington have seen a consistent, upward trend since 2012 (Hop Growers of America, 2018) (Figure 1). Washington and Idaho showed an increase in acreage but Oregon declined, allowing Idaho to account for 15 percent of the hop crop and Oregon 12 percent. In 2018, the six leading cultivars grown in Washington were Cascade, Citra, Zeus, Centennial, and Simcoe which comprised 49% of the states total crop produced in 2018. Five out of these six cultivars are generally considered "aroma" or "dual purpose" type hops; a similar trend is seen for Oregon and Idaho. According to the Hop Growers of America Statistical Report released on January 2017, the cultivar balance has shifted from 50-50 alpha and aroma/dual purpose hops in 2012 to over 80% aroma/dual purpose cultivars in 2016 (HGA, 2017). The total crop value in 2018 was reported at $583 million dollars, down one percent from 2017 (National Hop Report, 2018; HGA, 2018).

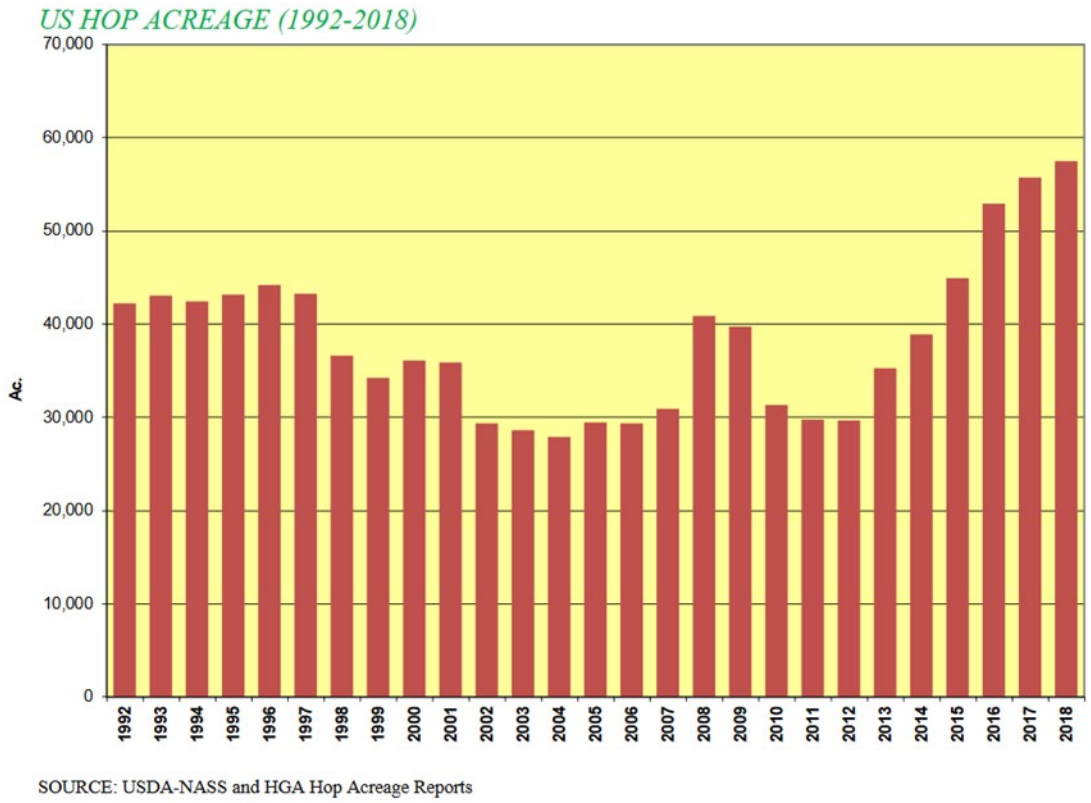

Figure 1. U.S. Hop Acreage (1992-2018)

1.3 BOTANY

Humulus is a small genus of flowering plants in the Cannabaceae family. The genus likely originated in China, where distinct populations developed from isolated plants and dispersed to America and Europe (Small, 1980; Neve, 1991; Murakami et al., 2006). This genus consists of three species, *Humulus japonicas* Siebold & Zuccarini., *Humulus lupulus* L., and *Humulus yunnanensis* Hu (Small, 1980; Neve, 1991). *H. japonicas* is a dioecious, climbing, annual species. It can be found throughout China and Japan, and after importation during 1800's, within the Eastern and Midwestern USA. Little is known about *H. yunnanensis*, although there has been a recent effort to find a living specimen (Boutain, 2014). Of the three *Humulus* species, *H. lupulus* is the

cultivated form used for beer production. *H. lupulus* is composed of five botanical varieties, *H.lupulus* var. *lupuloides*, *H.lupulus* var. *neomexicanus*, *H.lupulus* var. *pubescens*, all from North America; *H.lupulus* var. *lupulus* from Europe; *H.lupulus* var. *cordifolius* from eastern Asia and Japan (Small, 1978). The European and North American varieties have historically been used in brewing. These varieties are indigenous to temperate regions of the Northern Hemisphere but, because of its use in brewing, hop plants are grown in many parts of the world.

Hop is an herbaceous, climbing perennial that produces annual shoots each spring from an overwintered crown. The crown consists of multiple shoots, lateral rhizomes, and a long tap root that can extend 1.5 m or more. They are hardy plants that can survive cold winters with temperatures as low as -30ºC (Neve, 1991). As shoots emerge in spring they elongate and begin to climb in a clockwise direction (dextrorse) around most anything within reach. Hop shoots are hexagonal in shape, and downward-pointing trichomes are produced along the stem edges. Hop shoots are considered bines, not vines, since they climb by wrapping around and clinging to a supporting structure using trichomes. Leaves emerge in an opposite arrangement from each node on the stem. Mature leaves vary in shape from cordate to 3-5 lobes depending on ancestry.

Hop is dioecious and one of the few cultivated genera that have true sex chromosomes (Winge, 1929). Hop is wind pollinated and self-pollination is not normally possible, thus plants are genetically highly heterozygous. Plants raised from seed are extremely variable and most are not commercially valuable. Male genotypes are used strictly in plant breeding programs as a paternal parent while select female plants are clonally propagated and used in commercial cultivation (Hill et al., 2016).

Cultivated hop is usually diploid (2n=2x=20) with 9 autosomal bivalents and two sex chromosomes (XX type in female and heteromorphic X and Y type in male plants) (Winge, 1929) although polyploids have been developed (Haunold, 1971). Other studies have found at least six different types of sex chromosomes of *H. Lupulus* (Ainsworth, 1999). Sex expression is determined by X/autosome balance (Parker and Clark, 1991; Shephard et al., 2000; Hill et al., 2016).

Male flowers are produced in loose panicles, and have a perianth of five yellowish-green sepals and five anthers on short filaments. The anthers dehisce large quantities of pollen which are wind born. In commercial production, male hop plants are undesirable because seeded cones may cause an undesirable off-flavor and increase cone weight without benefitting beer flavor. However, Haunold, (1971), demonstrated that triploid male hops increased cone yields without significant seed set as dehisced pollen grains are sterile.

Female hop flowers consist of bracts and bracteoles that enlarge to produce a strobilus, commonly referred to a “cone” when mature, which forms the commercial product. Pollinization and successful fertilization produces an achene housed at the base of bracteoles which are protected by the bracts (Neve, 1991). Pollination, however, is not necessary for the cone to develop.

Glandular trichomes inside the hop cones called ‘lupulin’ glands produce the yellow hard and soft resins containing the chemical components prized in brewing for adding bitterness and aroma to beer along with foam stability (Neve, 1991; Oliver and Colicchio, 2011). Although numerous chemicals are present in these glands, the chemical constituents that are thought to contribute most of the hop bitterness, flavor, and aroma to

beer are found primarily in the resins and essential oils (Vollmer, 2016). The relative proportions of the individual constituents depend on the hop cultivar, the environment, and growing conditions (Sharp, 2013).

The hop bittering acids and polyphenolic material within lupulin are responsible for taste in beer while the oil component is the main source of aroma. The bitter precursors of beer are attributed to the soft resins that contain a class of chemical compounds known as α-acids (humulones) and β-acids (lupulones) (Lewis, 2002). Alpha acids are comprised primarily of five constituent hop acids: humulone, co-humulone, ad-humulone, pre-humulone, and post humulone, with humulone being the most abundant (EBC Technology and Engineering Forum, 1997). Alpha acids have little to no bitterness in their natural form (Hoseney and Hoseney, 1983; Peacock, 1998) and must be isomerized by boiling to convert the alpha acids into bitter-tasting iso-alpha acids (Malowicki and Shellhammer, 2005). Beta acids are a similar mixture to alpha acids and differ in one prenyl side chain (DeKeukeleire, 2000). Beta acids consist of alpha acid analogues including, lupulone, colupulone, adlupulone, prelupulone, and postlupulone. These acids must also go through isomerization to exhibit bitterness but it is believed that they contribute much less in bitterness. Research also suggests beta acids provide antibacterial properties to hops (Larson et al., 1996; Cornelison et al., 2006; Van Cleemput et al., 2009). This bioactivity helps facilitate the death of micro-organisms during wort boiling, which ultimately leads to sterile beer (Van Cleemput et al., 2009; Acworth et al., 2012; Everard et al., 2012).

Essential oils produced in hop contribute a range of aromas in beer and make up a small but important portion of lupulin in hop cones. The composition of essential oil is

extremely complex. There are over 450 identified chemical compounds and it has been suggested that the total number of existing compounds exceeds 1,000 (Roberts et al., 2004). Hoppy aroma in beer is manifested when the myriad of compounds interact (Roberts et al., 2004). These compounds comprise between 0.5% and 3% of a dried hop cone, depending on the cultivar (Table 1) (EBC Technology and Engineering Forum, 1997; Evans, 2006; Eyres and Dufour, 2008). The chemical composition of hop oil is divided into three categories; hydrocarbons, oxygenated compounds, and sulfur-containing compounds. The hydrocarbon fraction contains monoterpenes such as myrcene, sesquiterpenes such as beta-caryophyllene and farnesene, and aliphatic hydrocarbons such pentane and octane. The oxygenated compounds include terpene alcohols such as linalool and geraniol, sesquiterpene alcohols including humulenol, and other compounds such as keytones and esters. The sulfur-containing compounds include thioesters, thiols such as 4-MMP, sulfides such as dimethyl sulfide (DMS), and other sulfur compounds (Sharpe and Laws, 1981; Schönberger and Kostelecky, 2011).

Table 1. Composition of hop material on per mass basis

Principle Components	Concentration (%w/w)
Cellulose + lignin	40.0 – 50.0
Protein	15.0
Alpha acids	2.0 – 17.0
Beta acids	2.0 – 10.0
Water	8.0 – 12.0
Minerals	8.0
Polyphenols and tannins	3.0 – 6.0
Lipids and fatty acids	1.0 – 5.0
Hop oil	0.5 – 3.0
Monosaccharides	2.0
Pectin	2.0

European Brewery Convention *Hops and Hop Products, Manual of Good Practice;* Getranke-Fachverlag Hans Carl: Nurnberg, Germany, 1997.

1.4 HOP PRODUCTION

Hop plants need long summer days of at least 15 hours for maximum production. Therefore, commercial production is typically restricted between latitudes 35^{o} and 55^{o}, either north or south of the equator (Neve, 1991). More than 71.5% of the hop area under cultivation is located in Germany and the USA (IHGC, 2019).

Commercial hops are clonally propagated either by rhizomes or stem cuttings. This produces a uniform crop and maintains cultivar characteristics, such as cone chemistry and foliage color, because traits are fixed in the cloned material. Micropropagation by tissue culture has been established as a multiplication technique and has proven to be viable (Batista et al., 1996, 2000, 2008).

To establish a new crop, herbaceous propagules are planted in spring after the chance of frost has passed, while rhizomes and dormant crowns may be planted in winter. Hop requires a sufficient soil depth to allow the deep-rooted plant to grow, and a well-drained soil with an adequate supply of moisture that minimizes waterlogging. If established correctly and maintained well, the perennial rootstock of hops can have a commercial lifetime of 12-20 years (Benitez et al., 1997).

In the United States, hops historically were planted in hills typically spaced 1.5 meters within rows, and rows were typically spaced 2.8 meters to 3.2 meters apart, however in recent years wider row spacing with shorter plant spacing within-row has become popular. It has become standard practice to provide a permanent trellis structure of poles and wires for climbing support. The height of the trellis varies but the standard height in the United States is typically 5.5 meters. Low trellis systems have been

developed but are not widely used at a commercial level in the United States as there are limited hop varieties available suitable for the shorter height. Each year string or coir is attached to the trellis and anchored into the soil near the plants to provide climbing support for the hop bines.

Each spring, hop shoots emerge from overwintered crowns. This first flush is often removed to synchronize the growth of the crop and decrease the amount of primary inoculum of common pathogens embedded in the crown, principally hop downy mildew (*Pseudoperonospora humuli*) in Western Oregon. As new growth emerges, three to five elongating bines are selected and manually trained onto each string to promote climbing. As plants climb, some farms practice a method of leaf removal called "stripping," where leaves and lateral branches are removed from the lowest part of the bines. This helps minimize pathogens from spreading upwards into the plant canopy.

During the growing season, adequate amounts of fertilizer and irrigation must be applied to provide optimum growth and plant health. The most common soil amendment in the Willamette Valley is 100-150 pounds nitrogen per acre, per year prior to the middle of July (Gingrich et al., 1994). Soils may also require amendment to promote an optimum pH of 5.7 to 6.5. Hops require 2-4 gallons of water per plant per day during the growing season to avoid drought stress and enable the plant to produce high yields, thus irrigation is often used in the dry summer months (Mahaffee et al., 2009).

From July to August, as day lengths shorten, the female inflorescence matures into a cone. During the maturation phase, moisture content of cones will decrease, cone size will increase, and chemistry within the cone change. Studies have shown alpha acid levels will generally reach a maximum as the dry matter content reaches 22 and 24%,

while beta acid levels peaked at a dry matter content of less than 22%. Essential oil content was shown to increase over time as well as compositional changes occurring throughout maturity (Murphy and Probasco, 1996; Sharp, 2013).

Harvest date is highly dependent on the cultivar, growing region, climate, and farming practices but most often occurs between August and September in the Pacific Northwest. In Oregon, rain in mid-September typically encourages the end of harvest and provides early maturing cultivars a competitive advantage.

Hop plants are first removed from the field and the cones harvested via a stationary mechanical picker. After the hops have been picked, they are dried to 8-10% moisture, compressed into 200 pound bales, and typically processed into pellets or extract, or sent to a brewery as whole cones for use. Processing slows down the oxidation of the hop cones by limiting their contact with oxygen.

1.5 BREEDING AND CULTIVAR IMPROVEMENT

Aroma hop improvement is challenging because of the complex chemical profile involved, the perennial nature of the plant, and the unique production infrastructure that is needed. These issues are exacerbated by the long juvenility period in hop establishment (usually three growing seasons in Western Oregon (Neve, 1991)). The α-acids, β-acids, and essential oils produced in hop contribute to a range of flavor and aroma in beer although the relationship between chemical profile and resulting beer attributes is not fully understood (Sharpe and Laws, 1981; Vollmer, 2016). In particular, the composition of hop essential oil is extremely complex, and the inheritance and co-inheritance of essential oil components is not well-studied. Improving aroma hop by

traditional methods is an expensive process that often takes more than 10-15 years of effort before a cultivar can be released for commercial production.

The major techniques for developing new hop cultivars are mutation breeding, pedigree breeding, phenotypic recurrent selection, and mass selection (Haunold, 1981). Mutation breeding can include naturally occurring mutations or the use of chemical and physical mutagens to induce ploidy or random mutations (Haunold, 1981; Trojak-Goluch and Skomra, 2013).

Pedigree breeding is a deliberate attempt to recombine genetic traits from selected parents. In hybridization, progeny often differ substantially from one or both parents. By choosing the parents carefully and evaluating the progeny in detail, the breeder can retain desirable traits and improve less desirable ones (Sleper and Poehlman, 2006). Neither mutation breeding nor pedigree breeding are used to any great extent in hop (Henning, 2006).

Phenotypic recurrent selection refers to the cyclical improvement of a breeding population. This is done by selecting the best male and female individuals for a trait of interest in a population, and randomly intermating the selected plants to produce the next generation. The process is repeated until the desired level of performance is attained (Bernardo, 2002; Sleper and Poehlman, 2006). Because of the additional recombination provided in each generation, new allelic combinations can arise and offer new genotypic and phenotypic possibilities. Phenotypic recurrent selection is used to increase the frequency of favorable alleles in a breeding population and theoretically improve the chance of identifying superior lines. It helps maintain high genetic variability in a

population due to repeated intermating of heterozygous lines, and is useful for improving traits controlled by many genes (Allard, 1999; Sleper and Poehlman, 2006).

Mass selection involves several hundred or thousands of phenotypically superior plants that are selected based on their phenotype. These plants are allowed to intermate, and the seed is harvested and bulked without any progeny test to produce the next generation. This is the simplest and oldest method of crop improvement practiced by breeders. There is no control over pollination, so selected plants are pollinated by both superior and inferior pollen parents potentially increasing the number of cycles required to generate an acceptable population (Allard, 1999; Sleper and Poehlman, 2006).

Mutation breeding is the process of exposing tissue to a mutagen in order to accelerate the natural mutation process and generate new genetic traits. The breeder will then select plants that have mutations involving improved agronomic or quality traits. The breeder may release a superior mutant as a new cultivar, or use the mutant as a parent to transfer desirable traits into an otherwise acceptable line. A lot of attention has been given to genetically modified organisms (GMO) or 'transgenic organisms' and so it should be clarified that induced mutagenesis is not considered a GMO in the United States. The USDA groups mutagenesis within classical/traditional plant breeding methods which can be grown on organic systems (USDA, 2013). Mass selection and hybridization are particularly useful for developing novel cultivars for the craft beer industry. Both methods involve sexual recombination, exploiting hop's heterozygosity to produce new flavors and aromas. However, to improve existing aroma cultivars (i.e. develop a replacement cultivar), mutagenesis is potentially more useful because of the

possibility of altering a few agronomic characteristics of an accepted cultivar without significantly changing an industry-accepted brewing profile.

Mutagens fall into two major categories, physical and chemical. In general, chemical and physical mutagens induce changes in DNA sequences and, consequently, change the appearance and characteristics of the treated organism. This is accomplished when a mutagen treatment alters DNA sequences or induces chromosome breakage.

Chemical mutagens typically include alkylating agents (such as the commonly used ethyl methane sulfonate (EMS) and can be used to mutate a wide range of plant material. Seeds can be treated in large quantities and are easily handled, stored and shipped, therefore mutagenic treatment of seeds with chemical mutagens is typically considered the standard method in seed propagated crops (Kodym and Afza, 2003). Hops that have been developed through mutation breeding using chemical mutagens include the first colchicine-induced triploid seedlings, Willamette and Columbia. These two cultivars were developed and released to be higher yielding, seedless replacements for Fuggle (Haunold et al., 1976b; a). In 1976, “Willamette” and “Columbia” were developed and ultimately released as the first triploid (3X=30) cultivars by exposing Fuggle to colchicine and forming a tetraploid form of Fuggle, which was subsequently open pollinated with a diploid male hop of unknown origin. Willamette holds industry importance as a ‘finishing’ hop and is widely grown in Oregon but Columbia is of little importance. The USDA breeding program has also released Mt.Hood, which was a triploid seedling bred from a tetraploid form of Hallertauer (Haunold et al., 1990); ‘Liberty’- another half-sister triploid to Mt. Hood (Haunold et al., 1992); ‘Crystal’, half-sister to Mt.Hood (Haunold et al., 1995); and finally USDA-ARS released ‘Santiam’ a

triploid developed by crossing diploid ‘Tettnanger’ with a tetraploid male (Henning and Haunold, 1999). Compared to other breeding strategies, the USDA breeding program has utilized polyploids to a lesser extent, although not to the extent of the New Zealand breeding program, which expanded on the method and continues to release triploid cultivars.

Physical mutagens are particularly useful in vegetatively propagated crops such as hop, especially when further improvement of outstanding cultivars is desired and it is essential to generally maintain the characteristics of the existing cultivar (Shu et al., 2012b; Suprasanna et al., 2014, 2015). Physical mutagens typically use radiation sources such as gamma rays, X-rays, UV light and particle radiation, including fast and thermal neutrons, beta and alpha particles (Kodym and Afza, 2003).

Induced mutagenesis by ionizing radiation dates back to the beginning of the 20th century. Induced mutations via radiation was used as a tool for plant breeding after the discovery of the mutagenic action of X-rays, once improvements were demonstrated in maize, barley, and wheat in 1928 and 1930 (Stadler, 1928). Initial attempts to induce mutations in plants typically used X-rays, but gamma radiation eventually became more widely used as atomic sources became readily available from newly established nuclear research centers. Gamma rays emit ionizing radiation which affects plant growth and development by inducing cytological, biochemical, physiological and morphological changes in cells and tissues by producing free radicals in cells (Gunckel and Sparrow, 1967; Kim et al., 2004; Wi et al., 2005). Gamma radiation has introduced useful traits in vegetatively propagated crops, including plant size, bloom time, plant architecture, yield, fruit quality and harvest date (Shu et al., 2012a). Three mutant banana cultivars have

been officially released and were all were developed with gamma irradiation. The cultivar ‘Al-Beely’ was released in 2007 in Sudan and showed a 30% higher yield while ‘Klue Hom Thong KU1’ was released in 1985 in Thailand and had improved, large bundles (Datta et al., 2018). ‘Novaria’, a mutant from an irradiated clone of ‘Grande Naine’ was released in 1995 in Malaysia for demonstrating early flowering and improved fruit quality (Novak et al., 1990; Mak et al., 1996). ‘Amasya’ a popular apple cultivar in Turkey had poor agronomic traits such as low yield, biennial bearing, early blooming and poor fruit skin coloring. A proportion of irradiated scions of ‘Amasya’ resulted in superior architectural traits (Atay et al., 2018). The induced mutation process also potentially changed the flowering time in the ‘Amasya’ mutants (Atay et al., 2018). The mutant potato variety ‘Desital’ was officially approved in 1987 in Italy and was developed by irradiation of *in vitro* grown plantlets with gamma rays (30 Gy). The mutant has white skin tubers in contrast to the red skinned parent ‘Desiree’ while the rest of the phenotype was apparently unchanged. ‘Nahita’, another mutant potato cultivar was released in 2016 and has an earlier maturity date than the parent genotype (ARICI et al., 2016).

Gamma radiation’s use as a plant breeding tool requires the initial assessment of a wide range of doses to estimate the sensitivity of target plant materials. Doses leading to 25-50% lethality (LD_{50}) have often been chosen (Shu et al., 2012a). Radio-sensitivity varies between and within species. Data on radio-sensitivity or toxicity are available (Suprasanna et al,; Charbaji, 1999; Kodym et al., 2012). Saif-Ur-Rasheed et al., 2001 observed doses higher than 20 Gy to be lethal to micropropagated potatoes while (Osei-Kofi et al., 1997) determined 45 Gy to be the LD_{50} of irradiated shoot tips of pineapple

(*Ananas comosus*). Previous gamma radiation research in hop suggested that the LD_{50} of nodal explants in tissue culture was less than 5 Gy. In general, literature is lacking on the use of gamma radiation to improve hop.

Induced mutations, using tissue culture is not only potentially useful for aroma hop improvement, but plant tissue culture also aid in the process by increasing the efficiency of mutagenic treatments for variation induction, ease in handling large populations, and rapid cloning of selected variants (Predieri, 2001).

Plant tissue culture is an *in vitro* technique used to maintain or proliferate plant cells, tissues or organs and came to fruition in the beginning of the 20th century (Thorpe, 2007). Tissue culture is carried out within aseptic conditions and uses a nutrient culture medium of known composition. New plants are generated from plant parts such as pollen, leaves, seed, root tip, and embryo (Dagla, 2012).

The environmental conditions plant cultures are maintained in play an important role in their survival and health. The presence or absence of light and its quality along with growth temperatures can be important for culture health, but the media used for tissue culture is also critical for success. Of the various tissue culture media available, they generally have the same basic components but vary in component concentrations. Major components of media include macronutrients, micronutrients, vitamins, amino acids, carbon/energy source, phytohormones/plant growth regulators and agar. The composition of the growth medium, specifically the plant hormones and the nitrogen source play an important role in plant growth and success of the initial plant. Plant growth regulators, or plant hormones, are chemical substances that control normal plant growth such as root and shoot growth (Gaspar et al., 1996). In tissue culture, plant growth

regulators play an important role in growth by inducing and manipulating direct cell programming to obtain a specific type of growth or outcome (Davies, 2010). Among the many known plant hormones, auxins, gibberellins, cytokinins, abscisic acid and ethylene play a major role in coordinating cell growth and development. Auxins and cytokinins are major components of plant tissue culture (Davies, 1995). Auxins are responsible for stimulating cell elongation and influencing a host of other developmental responses, such as root initiation, vascular differentiation, tropic responses, apical dominance and the development of auxiliary buds, flowers and fruit (Addicott et al., 1968; Gaspar et al., 1996; Rai et al., 2011). Cytokinin is responsible for stimulating cell division and inducing shoot bud formation in tissue culture. Cytokinin generally act as antagonist to auxin and often inhibits embryogenesis and root induction (Gaspar et al., 1996).

Somaclonal variation is the occurrence of phenotypic and genotypic variants among regenerated plants in tissue culture (Larkin and Scowcroft, 1981). The phenomenon has been defined as "phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones, namely plants derived from any form of cell culture involving the use of somatic plant cells" (Schaffer, 1990). 'Epigenetic' refers to a transient heritable change in phenotype without a change in the DNA sequence. Somaclonal variation has been used as a method to improve crops (Larkin and Scowcroft, 1981, 1983; Ahloowalia, 1986; Karp, 1995; Brar and Jain, 1998; Jain et al., 1998; Jain, 2001; Krishna et al., 2016; Lestari, 2016). Somaclonal variation can also be a major disadvantage to tissue culture because it jeopardizes genetic uniformity during micro-propagation causing plants to have different phenotypes (Evans, 1989).

Another potential obstacle when irradiating node cuttings is the formation of chimeras. A chimera forms when one of the cell layers in the meristem has a different genetic make-up compared to other layers (Marcotrigiano and Bernatzky, 1995). The architecture of the Shoot Apical Meristem (SAM) enables chimeras to be initiated by having cell layers that remain independent from each other. These distinct cell layers develop into separate tissues within the SAM. The cells of the outermost layer (L1) forms the epidermis. The second apical layer (L2) forms the subepidermal tissue responsible for the outer part of the cortex and gametes. The third layer (L3) forms the deep mesophyll and vascular tissue(Tilney-Bassett, 1991; Trigiano and Gray, 2011). There are three kinds of chimeras based on the spatial arrangement of the genetically distinct cells within these cell layers: (1) periclinal chimeras that have a uniform, genetically distinct layer of cells in the SAM and are considered the most stable form and can be vegetatively propagated, (2) mericlinal chimeras have part of one or more layers that are genetically different, and (3) sectorial chimeras that have a population of cells extending through all the cell layers (Marcotrigiano, 1997).

The hop industry relies on a limited number of clonally propagated cultivars that are recognized for their brewing profile and consumer familiarity with the product. For these reasons, brewers are very reluctant to change hop cultivars in their recipes, particularly aroma hop cultivars. This limits the use of traditional crossing strategies when the breeding goal is to generate a replacement cultivar for an aroma type that enjoys wide industry acceptance (Cascade, for example). Hop cultivars are highly heterozygous, and progenies produced from traditional crossing techniques may express a large number of traits which are substantially different from the female parent. In

contrast, induced mutations such as those with gamma radiation, may change only one or a few specific traits of an elite cultivar, and can possibly improve agronomic performance without significantly changing the brewing profile. Available literature is lacking on the use of gamma radiation on hop. Therefore, the objectives of this work was to evaluate a range of gamma radiation doses that effectively induce hop mutations, and to quantify the genetic change at each dosage.

CHAPTER 2. THE EFFECTS OF GAMMA RADIATION ON CASCADE AND CENTENNIAL HOP

2.1 INTRODUCTION

Conventional breeding in hop improvement faces some hurdles because of the complex brewing profile, the presence of inbreeding in available parents, and low genetic variability due to the relatively small number of available parents. One of the strategies to increase the genetic variability within hop is using induced mutations. Mutation induction can be accomplished by using chemical or physical mutagens. In this context, the major aim in mutation-based breeding is to develop and improve well-adapted plant varieties by modifying relatively few traits to increase their productivity or quality.

For this reason, mutation breeding was chosen to improve existing popular hop cultivars. Literature suggests that induced mutagenesis can be a useful method for plant improvement, and according to the Food and Agriculture Organization and International Atomic Energy Agency (FAO/IAEA) mutant variety database in 2019, it was reported there were more than 3,283 mutant varieties in more than 200 crop species released. Based upon the results of a preliminary study (Getty, 2015), it was hypothesized that 3 Gy of gamma radiation would be sufficient to kill 50% of Cascade and Centennial micropropagated nodal sections.

The goal of this research was to determine the lethal dose of gamma radiation to kill 50% (LD_{50}) of micropropagated nodal sections of Cascade and Centennial hop cultivars, and to document the vigor of surviving genotypes.

2.2 MATERIALS AND METHODS

2.2.1 PLANT MATERIALS

Two hop cultivars were selected for this study based on public availability and their popularity in the craft beer industry. Clean *in vitro* cultures of Cascade, (Brooks et al., 1972) were obtained from the U.S. Department of Agriculture, National Clonal Germplasm Repository (USDA-NCGR) in Corvallis, Oregon. *In vitro* cultures were originally initiated from 0.3-0.5mm meristems of heat-treated shoots from clonally propagated hop plants (Adams, 1975). Clean *in vitro* cultures of 'Centennial'(Kenny and Zimmermann, 1991), were obtained from a commercial propagator (Explant Laboratory, Portland, Oregon).

Plantlets were grown in Magenta GA 7 boxes (Magenta Corp., Chicago, IL) containing NCGR-HUM medium with the addition of 100 mg of Sequestrene Fe (Reed et al., 2003, Aynalem et al., 2006a). Every three weeks, plants were transferred into new media to ensure optimal nutrient levels. Plantlets were maintained at 24±2C^{o} with a 16 hour photoperiod and illumination at 60-80 uM m^2 s^{-1} with a combination of warm and cool white fluorescent bulbs.

To obtain enough plant material for the experiment, each cultivar was micropropagated to fill 18 magenta boxes. Plantlets were initiated in media staggered at one week intervals for three consecutive weeks.

To prepare for irradiation, 100x25 mm sterile, polystyrene, Petri dishes were filled with approximately 20 ml of NCGR-HUM medium, in which the Sequestrene Fe was omitted (Aynalem et al., 2006a).

2.2.2 EXPERIMENTAL TREATMENTS

The experiment was arranged as a three-factor completely randomized design (CRD) the factors being cultivar (Cascade, Centennial), gamma radiation dosage (0,2,3,4 Gy), and replicates (three). A treatment combination consisted of a Petri plate containing 50 nodal sections of a cultivar assigned to a gamma dosage. Nodal sections were excised into 1 to 1.5 cm sections, leaves were removed and each section was planted upright in Petri plates previously prepared with NCGR-HUM medium (Figures 2-3) (Reed et al., 2003; Aynalem et al., 2006a). Nodal sections were exposed to the appropriate radiation dose within two hours of being dissected and placed into a Petri dish.

Figure 2. Cascade plantlets being dissected into 1-1.5 cm nodal explants

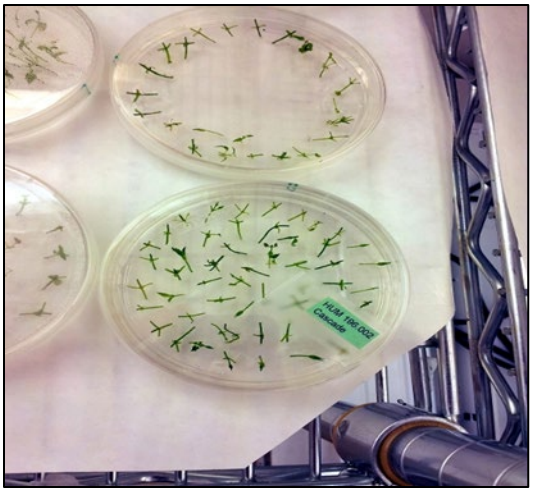

Figure 3. Cascade hop, excised nodal explants with leaves removed planted upright on media

2.2.3 IRRADIATION

Irradiation was conducted at the OSU Radiation Center using cesium-137 (Cs^{137}) as a radiation source. Three gamma radiation dosages (2,3,4 Gy) were administered, and a non-irradiated control was included for each cultivar. The irradiation method used here is considered 'acute' as a result of the plants being subjected to a brief burst of radiation as opposed to long term exposure over the plantlets lifetime.

For each radiation session, three Petri dishes were stacked in the radiation chamber and were removed at timed intervals in order to be subjected to 2, 3, or 4 Gy. Since the Cs^{137} radiation source was located in the bottom of the chamber, it was assumed that the top Petri dish might receive a slightly different dose than the bottom Petri dish. For this reason, each Petri dish was randomly assigned to a chamber location (lower, middle, upper) for each of the six irradiation sessions. As each Petri dish reached its radiation dose, it was removed from the chamber causing the positions to be rotated. The average dose for all three Petri dish while they were in the chamber was 0.09 Gy hr^{-1}. Nodal sections were cut within two hours prior to irradiation.

2.2.4 PLANT GROWTH IN TISSUE CULTURE

Following irradiation, plants were left in the Petri dish for seven days, maintained at 24 ± 2ºC with a 16h photoperiod and illumination at 60-80 uM m^2 s^{-1} with a combination of warm and cool white fluorescent bulbs. After seven days, the irradiated explants were transferred into individual 16x125mm glass tubes with modified NCGR-HUM medium with the addition of 100 mg Sequestrene Fe (Aynalem et al., 2006a). Surviving explants were transferred to new tubes containing fresh media every three weeks for 90 days. During each transfer, any callus that was present was excised and discarded. In most cases, one axillary shoot would elongate and grow while the other shoot would become stunted. Since it was assumed that each nodal section contained two axillary buds that, following irradiation, would generate two separate genotypes that were unique from the mother plant, the healthiest axillary shoot was excised from each explant and transferred onto NCGR-HUM medium. The other axillary shoot and original root stock were discarded. This was done in order to obtain and separate mutated cell lineages and chimeras on the meristems from the original root stock. In some cases, neither axillary shoot elongate and were stunted. In these cases, the axillary shoots were not removed and the entire explant was moved into new media to promote growth in subsequent days.

2.2.3 LD_{50} IN TISSUE CULTURE

Ninety days after irradiation, surviving genotypes in each treatment combination were recorded to determine the LD_{50}. A genotype was counted as a survivor if the plantlet had elongated, roots had emerged, and was a healthy green color. Root measurements

were scored on the presence of roots. Shoot growth was measured in centimeters with a ruler outside of the tube from the media surface to the plant apex. Plantlet source was recorded as either separated from the original root stock or original root stock. Viable axillary shoots that had been separated from rootstock were transplanted into a greenhouse 90 days after irradiation.

2.2.4 GREENHOUSE ESTABLISHMENT

Micropropagated hops are typically grown aseptically on tissue culture media with near 100% humidity, ideal temperature, and artificial lighting. Plants grown under these conditions are sensitive to sudden changes in environment and require acclimation to greenhouse conditions to minimize stress that might damage or kill the plant. Sterile 10.16 cm pots were filled with RESiLIENCE® Sunshine soilless potting mix and thoroughly watered. Each plantlet was carefully removed from their tube with tweezers and residual MS media was gently washed off the roots with tap water. Plantlets were carefully potted, thoroughly watered with tap water, and labeled accordingly. Pots were placed in trays and covered with a 17.78 cm vented humidity dome to increase local humidity for acclimation. A 50% shade cover was added over the humidity domes to reduce overheating the microenvironment around the plants. Greenhouse temperatures were maintained between 21-23ºC. To acclimate plants to lower humidity, vents on the dome were opened directly after transplanting. Four days after transplanting, the shade cover was removed and after one week the domes were removed. If the plants began to wilt, the dome was replaced for 1-2 more days. Pots were fertilized with a full-strength

Miracle-Gro® Water Soluble All Purpose Plant Food (24-8-16) two times per week after planting.

2.2.5 LD_{50} AFTER GREENHOUSE ESTABLISHMENT

Thirty days after greenhouse establishment, surviving genotypes in each treatment combination were recorded in order to determine the final LD_{50}. A genotype was counted as a survivor if the plant had elongated and was a healthy green color.

Each plant was scored for vigor by was measuring the height of the plants in centimeters. Disease incidence was scored sixty days after establishment using a nine-step ordinal scale (0-9) where 0= no disease symptoms and 9 = nine or more lesions present.

2.2.6 STATISTICAL ANALYSIS

The Centennial plant stock used in this experiment was apparently infected with an unknown pathogen which resulted in the Centennial data being non-normal (Appendices E-H). Thus, all traits were analyzed by cultivar. Plant survival, shoot growth, rooting percentage, and division percentages were analyzed using an analysis of variance where gamma dosage and rep were considered fixed effects. Significant treatment effects were separated using Fisher's LSD. Correlation coefficients were calculated using Pearson's method. All statistical analyses were performed in RStudio version 1.2.1335 (Rstudio Team, 2015).

2.3 RESULTS

2.3.1 SURVIVAL RESPONSE OF GAMMA IRRADIATED PLANTS

Plantlet survival in tissue culture and plant survival after greenhouse establishment was not affected by gamma dosage as it was not statistically significant for survival in either cultivar although there appeared to be a numerical decrease in survival for Cascade with increasing gamma dosage. Centennial did not show a similar trend as plantlets exposed to 3 Gy of gamma radiation had a numerically higher survival rate compared to other treatments. An unknown contamination affected the Centennial stock plants and this probably increased the death rate of Centennial plants in all treatments as indicated by the low survival of non-treated plants (Figures 4,5) (Appendix I).

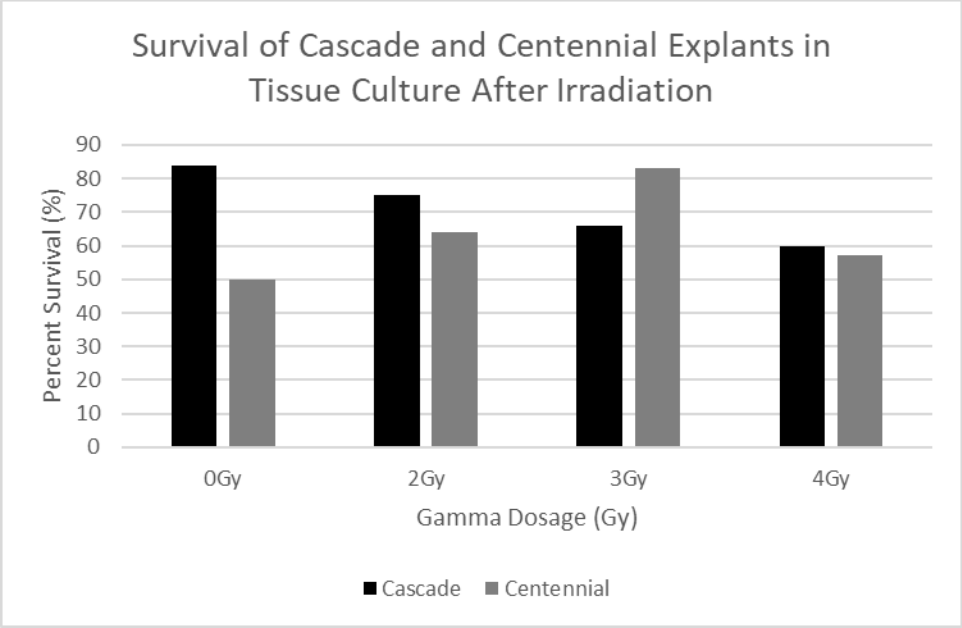

Figure 4. Percent survival of Cascade and Centennial explants that survived 90 days after irradiation while in tissue culture.

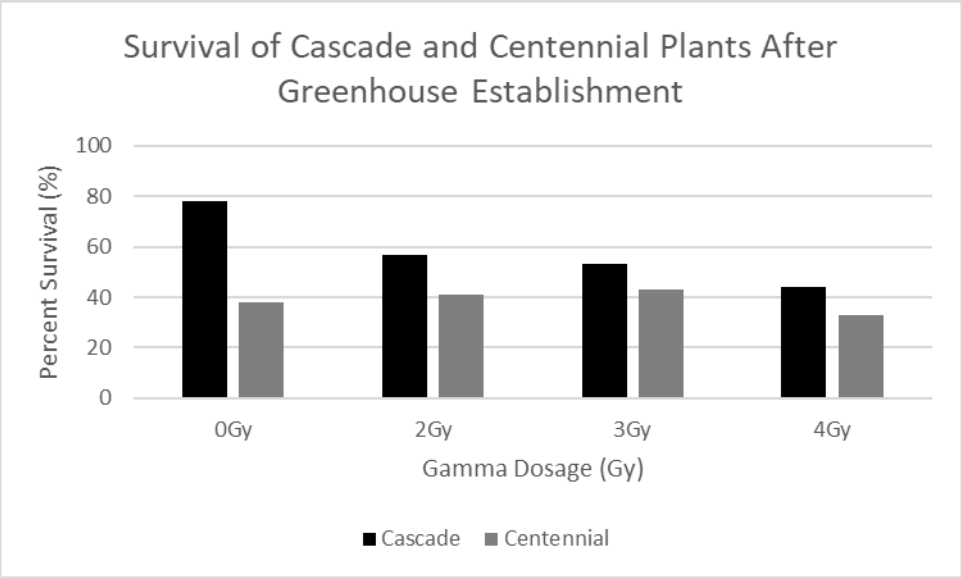

Figure 5. Percent survival of Cascade and Centennial plants that survived 90 days after greenhouse establishment.

2.3.2 SHOOT DIVISION OF NODAL SECTIONS

Division of shoots were significantly impacted by gamma radiation for Cascade and followed a trend of increasing gamma radiation decreasing the number of divided shoots from the main plantlet. The treatment with the highest percentage of plantlets divided from the rootstock was the control and 2 Gy while the lowest percentage was 4 Gy (Figure 6).

Centennial did not show a similar trend as plantlets exposed to 3 Gy of gamma radiation had the highest number of plants divided from the rootstock. Fifty-eight percent of the plantlets in the control group were divided from the rootstock, in contrast to the Cascade control group where 100% were divided (Figure 6). This low percentage of plantlets divided from their rootstock is probably due to the contamination in Centennial stock plants.

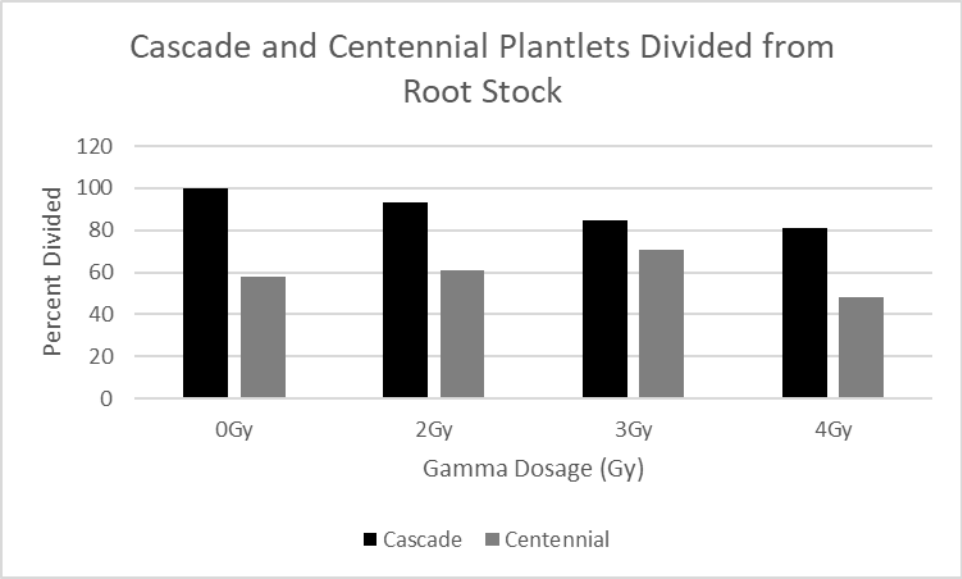

Figure 6. Mean of plantlet divided and plantlet division and planted into media following irradiation

2.3.3 SHOOT GROWTH RESPONSE OF IRRADIATED AND CONTROL PLANTS

Shoot growth in culture was significantly impacted by gamma dosage for Cascade. The highest mean shoot growth was observed for those plants in the control treatment and plants subjected to 2 Gy of gamma radiation, while the shortest plants were those treated with 4 Gy of gamma radiation (Figure 7) (Appendix I). Plant growth in the greenhouse was not affected by gamma dosage for Cascade. The highest mean shoot growth were plants in the control group and those treated with 3 Gy of gamma radiation (Figure 8).

Shoot growth in culture was not affected by gamma dosage in Centennial (Figure 7) (Appendix J). The highest mean shoot growth in Centennial was among plants treated with 3 Gy and the shortest shoot growth was in the control group (Figure 7). Plant growth in the greenhouse was not affected by gamma dosage for Centennial. The highest mean shoot growth was observed for those plants in the control group and plants subjected to 4

Gy of gamma radiation. This can most likely be explained by the unknown pathogen contamination.

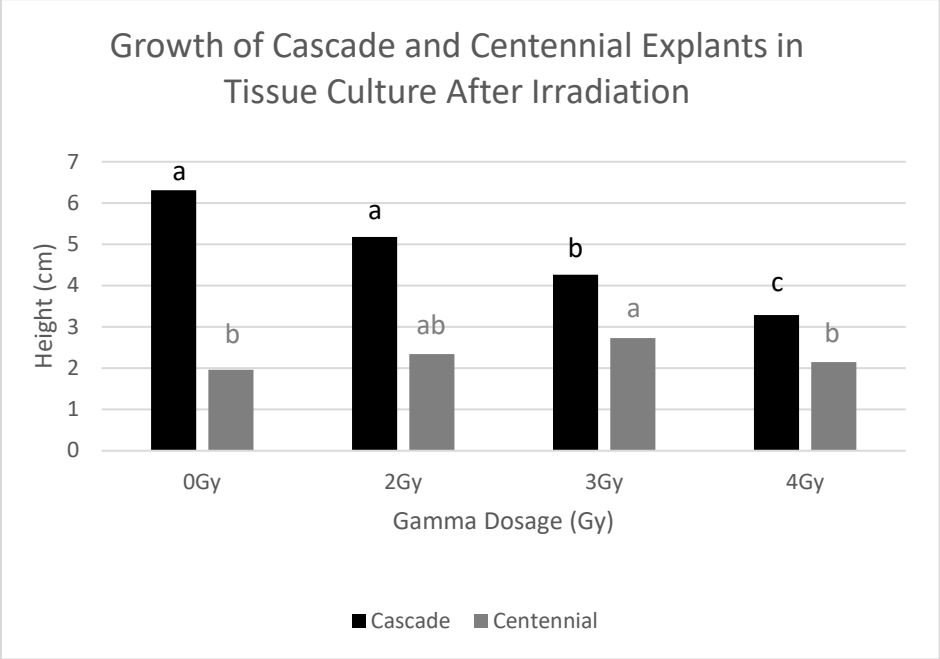

Figure 7. Measurements of growth (cm) of plants in tissue culture 90 days after irradiation

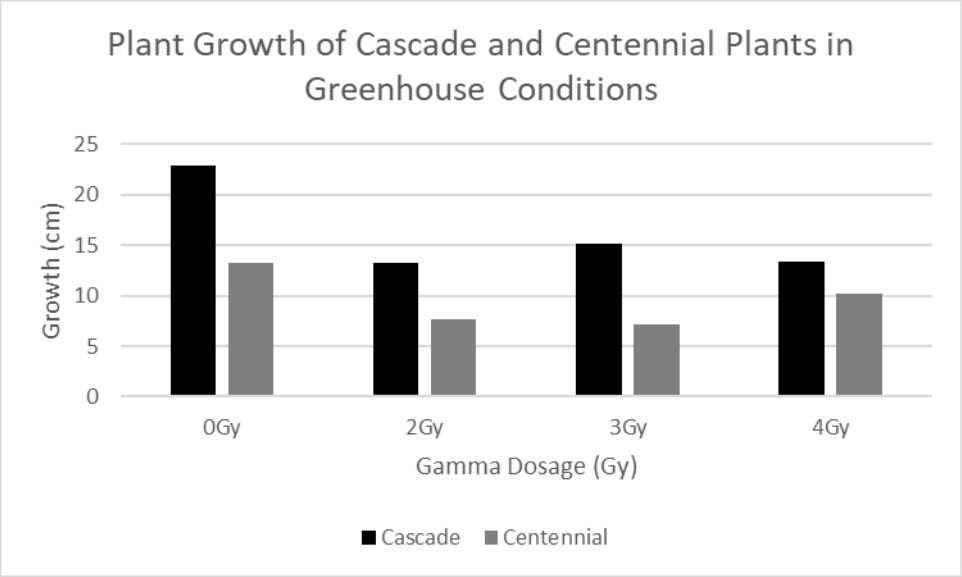

Figure 8. Measurements of growth (cm) of plants in the greenhouse 30 days after greenhouse establishment

2.3.4 ROOT EMERGENCE RESPONSE OF IRRADIATED AND CONTROL PLANTS

Gamma dosage did not significantly affect root growth for either Cascade or Centennial (Figure 9) (Appendix K, L), presumably due to contamination in the Centennial stock plants. Numerically, both cultivars showed a decrease in root emergence as gamma dosage increased (Figure 8). The treatment showing the highest trend of root growth in Cascade plants were irradiated with 2 Gy while the lowest trend of root growth were plants irradiated at 4 Gy (Figure 9). The trend for Centennial was a little different with the most root growth numerically in the 2 Gy treatment and the lowest plants irradiated at 4 Gy (Figure 9).

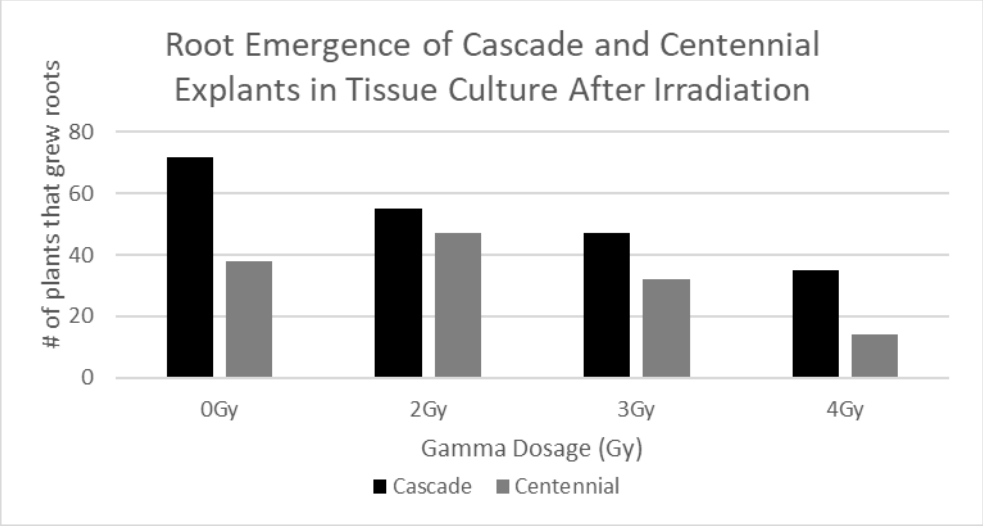

Figure 9. Percentage of means measuring the number of Cascade and Centennial plantlets that grew roots after irradiation.

2.3.5 CORRELATIONS BETWEEN VIGOR RATINGS IN TISSUE CULTURE AND GREENHOUSE CONDITIONS

A significant correlation of survival within Cascade plantlets was observed between root growth, division, shoot growth, and greenhouse survival (Table 2). No

significant correlation was observed between plantlet survival and plant growth after greenhouse establishment. A strong correlation between root growth and shoot growth was statistically significant. Plant survival in the greenhouse and plant growth after greenhouse establishment was significantly associated with root growth in tissue culture. Plant division was significantly correlated to plantlet growth and plant survival in the greenhouse. Plantlet growth was significantly correlated to plant survival in the greenhouse (Table 2).

For Centennial, plantlet survival was significantly correlated with plantlet division, shoot growth, and plant survival in the greenhouse. Plantlet survival was not significantly correlated with root growth or greenhouse survival. Root growth was not significantly correlated with any other measurement including plantlet survival, shoot growth, division, plant survival or growth in the greenhouse. Plant division was correlated with shoot growth and survival in the greenhouse. Shoot growth was correlated with greenhouse survival but not significantly correlated with greenhouse growth although, very close (Table 3).

Table 2. Correlations of Cascade hops: plantlet survival in tissue culture, root growth in tissue culture, shoot growth in tissue culture, division in tissue culture, survival of plants after greenhouse establishment, growth after greenhouse establishment.

Cascade	Survival (TC)	Root (TC)	Divide (TC)	Growth (TC)	Survival (GH)	Growth (GH)
Survival (TC)						
Root	0.810**					
Divide	0.765**	0.486				
Growth (TC)	0.917***	0.8945***	0.721**			
Survival (GH)	0.836**	0.719**	0.827**	0.776**		
Growth (GH)	0.327	0.691*	0.142	0.563	.388	

a. *p<0.05, **p<0.01, ***p<0.001 b. TC (Tissue Culture), GH (Greenhouse)

Table 3. Correlations of Centennial hops: plantlet survival in tissue culture, root growth in tissue culture, shoot growth in tissue culture, division in tissue culture, survival of plants after greenhouse establishment, growth after greenhouse establishment.

Centennial	Survival (TC)	Root (TC)	Divide (TC)	Growth (TC)	Survival (GH)	Growth (GH)
Survival (TC)						
Root	-0.033					
Divide	0.844**	0.002				
Growth (TC)	0.904***	-0.129	**0.824			
Survival (GH)	0.929***	0.033	***0.917	***0.888		
Growth (GH)	-0.288	-0.157	-0.085	-0.015	-0.156	

a. *p<0.05, **p<0.01, ***p<0.001 b. TC (Tissue Culture), GH (Greenhouse)

2.4 DISCUSSION

A hop breeder must balance plant improvement for both agronomic and brewing-specific traits, a task that is made more difficult due to the dioecious nature of the hop plant and the significant cost associated with hop production. Compounding these issues in aroma hop development includes the complex chemical profile found in the mature hop cone which is comprised of hundreds of chemical compounds (Roberts et al., 2004a). Additionally, how these compounds interact with one another and with the other components in the brewing process is not well understood. Thus, when an industry-standard aroma hop cultivar like Cascade or Centennial encounter production problems (loss of disease resistance, for example), developing a replacement cultivar with a similar brewing profile is exceedingly difficult to do. While direct insertion or editing of DNA is likely possible in hop (Škof and Luthar, 2005; Batista et al., 2008; Matoušek, 2012), social attitudes regarding these techniques appears to be unfavorable at this time. Mutation breeding, however, may offer a possible alternative for hop improvement when the goal is to develop a replacement aroma hop cultivar.

The main advantage of induced mutagenesis in aroma hop breeding is the ability to change one or a few traits of an otherwise outstanding cultivar without significantly altering the remaining, and often unique, aspects of the plant. However, literature is lacking on mutagenesis techniques for aroma hop improvement, and given that hop is a clonally propagated species, gamma-induced mutagenesis might be a reasonable approach. Some preliminary work in this area suggested that gamma radiation doses reported to be sufficient to establish an LD_{50} in other plant species such as sweet potato (Wang et al., 2007, Shin et al., 2011), potato (Yaycili and Alikamanoğlu, 2012), and

ornamentals (Kutty et al., 2019) were too high for Cascade hop (Getty, 2015) so gamma radiation doses less than 5 Gy were used in this work.

Survival ratings of Cascade plantlets after irradiation were not significant in either tissue culture or greenhouse conditions, however there was a numerical trend suggesting an increase in gamma dose appeared to decrease survival in both cases. The process of tissue culturing hop plants itself is not perfect and an 84% survival rate of the Cascade control plants in tissue culture was observed. An irradiation study on strawberry showed similar results, increasing doses of gamma rays decreased survival rate. Lethal effect began at 30 Gy and the survival rate decreased markedly when explants were exposed to gamma rays more than 180 Gy (Rudi et al., 2013). The effect of gamma radiation on minitubers of *Solanum tuberosum* L. suggest doses of radiation (10, 20 and 30 Gy) promoted the emergence percentage of minitubers but the emergence percentage, plant height and root length of minituber plants were significantly inhibited at 40 and 50 Gy (Cheng et al., 2010). Lapade et al., (1995) reported an increase in morphological changes in pineapple as the radiation dose increased. However, this study saw an increase in anthocyanin pigmentation with doses ranging from 5 to 40 Gy of gamma radiation.

Gamma dosage significantly affected shoot growth in Cascade in both tissue culture and in the greenhouse. In addition, gamma dosage significantly affected the number of plantlets that were divided from their rootstock. Cascade plants subjected to 2 Gy of gamma radiation were similar in height to control plants in tissue culture and similar to plants that received 3 Gy of gamma radiation once they were transferred to greenhouse conditions. This may suggest that the irradiated plants were more susceptible to environmental stresses once they were removed from the ideal growing conditions of

tissue culture. Cascade explants in the control group and 2 Gy treatment had the highest percentage of plantlets divided from their rootstock. Plantlets that were not divided were stunted and not vigorous enough to be excised and planted into new media. It was clear that plants receiving 4 Gy of gamma radiation were less vigorous in shoot growth, consequently reducing the number of shoots that were divided. Gamma dosage did not significantly affect root growth although, a numeric trend shows the treatment irradiated with 2 Gy had the highest presence of roots and 4 Gy had the lowest.

A strong correlation between survival of Cascade plantlets was observed with both shoot and root growth. Further, division and survival of plants after greenhouse establishment were significantly positively correlated with survival. This suggest, a gamma dose that results in greater root emergence, shoot growth, and shoot yield will have a higher probability of surviving both in culture and greenhouse establishment. If hop improvement is the intended goal, perhaps a 2 Gy gamma dosage would be an appropriate irradiation rate in Cascade to maximize shoot and root growth, and increase the number of plants divided from their rootstock which would provide more plant shoots for selection.

In tissue culture, an LD_{50} for Cascade plantlets was estimated to be around 4 Gy as this was the dose that killed approximately 50% of the population (Figure 4) while the greenhouse data suggested a dosage between 3 and 4 Gy. Contamination in the Centennial stock plants decreased the survival of all Centennial treatments. For this reason, drawing conclusions from the data was more difficult. Survival ratings from Centennial plants did not suggest a trend of higher radiation dose resulting in decreased survival. An LD_{50} for Centennial plantlets is estimated to be around 3 Gy and 4 Gy after

greenhouse establishment if the contamination is not taken into account (Figure 7). Treatments given 3 Gy of radiation had the highest survival in tissue culture and after greenhouse establishment while 0,2,4 Gy had the lowest. Gamma dosage did not significantly affect shoot or root growth for Centennial. Regardless of contamination, Centennial showed a numeric decrease in root emergence as gamma dosage increased. Plantlet survival was strongly correlated with shoot growth and moderately correlated with plant division and survival after greenhouse establishment. Plant survival in the greenhouse was also strongly correlated with plant division and shoot growth in tissue culture. This follows the same trend as Cascade as plantlets with higher root emergence, shoot growth, and division have a higher probability of survival after greenhouse establishment. These gamma radiation rates are consistent with the previous study of hop in which the suggested LD_{50} for gamma-irradiated Cascade hop would be less than 5 Gy (Getty, 2015). These results suggest Cascade hop may be more sensitive to gamma radiation compared to other species such as potato and sugarcane which have an LD_{50} of 20 Gy or higher (Saif-Ur-Rasheed et al., 2001).

The appropriate gamma dosage to utilize will depend on the researcher's goals. If the goal is to develop a replacement for an established aroma hop cultivar, 2 Gy of gamma radiation may be an appropriate dose as the likelihood of generating numerous vigorous plants for selection is higher than irradiation nodal explants at a 4 Gy gamma rate. However, if the goal is to maximize genetic mutations for a research application such as gene discovery, a 3 or 4 Gy gamma irradiation rate may be more appropriate as there would likely be a greater number of genetic changes to study. Also, an adequate number of plants with reasonable vigor could be generated at the 3 or 4 Gy gamma

irradiation rate, at least in Cascade hop. Ultimately, craft beer drinkers, and the brewers that create craft beer, will have the final say in whether mutation breeding will be an acceptable hop improvement technique.

CHAPTER 3. ESTIMATES OF GENETIC DISTANCE

3.1 INTRODUCTION

The cultivated hop, *Humulus lupulus* L., is an important crop used in beer brewing. The female hop plant produces flowers commonly known as “cones” which are used as a raw material for beer brewing and they provide bitterness, flavor, and aroma attributes. Bittering acids and essential oils accumulate in lupulin glands that exist in large numbers within the cone and impact most of the bitter taste, flavor, and aroma in beer. Improvements of these components is one of the most important goals in hop breeding.

Mutagenized populations are important resources to generate and identify desirable genetic variation of useful traits for crop improvement. When the lack of genetic variability hampers the breeding progress, mutagenesis can introduce genetic variation, reveal gene function, and aid in the characterization of candidate genes involved in biological functions. Mutagenized populations are useful to screen for altered phenotypes and physiological responses, and also as a genomics research tool. The overarching goal is to generate new phenotypes via random mutation, screen the mutated individuals for a desired phenotype, and possibly release the individual as a new cultivar. Mutagenized individuals may also be useful in gene discovery studies.

3.1.1 HISTORY OF HOP BREEDING AND GENETICS

The first reference to hops being used in beer dates back to the 9th century when Abbot Adalhard of the Benedictine Monastery of Corbie in the Picardy, in northeastern France, made a record stating that his monks added hops to their ales (Delyser and

Kasper, 1994). As hops in beer became more popular, cultivation spread to other countries eventually leading to the deliberate breeding efforts beginning in the 20th century.

The earliest breeding program was initiated at Wye College in 1904. Wye College has since produced many of the early bittering English cultivars such as Brewer's Gold, Bullion, and Northern Brewer (Haunold, 1981). As hop breeding began to take off in Europe, World War I forced a reduction in hop acreage and much of the agriculture in Europe was destroyed. This allowed the hop growing industry to expand in the United States during the late 19th century and pre-prohibition (Michael A. Tomlan, 1992).

After prohibition ended in 1933, the USDA-ARS established a hop research program in 1933 in Corvallis, Oregon and it is still in operation today. The first hop cultivar to be released from the program was Cascade in 1974 (Brooks et al., 1972). Cascade was released as an aroma hop and has become the signature aroma and flavor for the craft beer industry, and it is still one of the most cultivated hop cultivars today (USDA, 2017).

The analysis of genetic variation plays an integral role in genomic studies to better understand plants and animals at the DNA level. Genomic variation is presented as DNA polymorphisms e.g. small-scale insertions/deletions (indels), polymorphic repetitive elements, microsatellite variation, and single nucleotide polymorphisms (SNPs). Single Nucleotide Polymorphisms are useful in genomic studies because they can be directly related to phenotype differences, be associated with a disease, or indicate relationships between individuals within a population (Takagi et al., 2015; Xing et al., 2015; Byrne et al., 2017).

Within the last decade, genetic studies have shifted away from the application of automated Sanger sequencing (Sanger et al., 1977) for genome analysis which had dominated the industry. The demand for technologies that deliver fast, inexpensive and accurate genome information, propelled the development of next-generation sequencing (NGS) methods such as Genotype by Sequencing (GBS). The GBS technique is used in the field of genetic sequencing to discover SNPs, and to enable advances in whole genome assembling. Genotyping by Sequencing uses restriction endonucleases to reduce DNA complexity by targeting a small portion of a genome (Poland and Rife, 2012). This enzyme-based method is coupled with DNA barcoded adapters to produce multiplex libraries of samples analyzed on NGS sequencing. Genotype by Sequencing was developed largely, from major investment in technology development to complete the human genome, an endeavor set out to complete the mapping and understanding of all human genes. Plant scientists have adapted this technology, created by the broader genetic research community to plant genetics.

As genotyping and sequencing methods have advanced, genetic control over important traits in hop became easier to study. Applications of GBS in hop genetics and breeding help realize this goal by implementing Marker-Assisted Selection (MAS) population studies, germplasm characterization, linkage map development, association mapping, and GWAS studies to determine differences between cultivars and genetic distance (Henning et al., 2016). Henning et al., (2016) reported a high density genetic map using GBS to find markers associated with downy mildew resistance. Their result includes a set of four SNP markers identified to be statistically associated with downy mildew resistance in hop. This information will someday be useful for hop MAS.

Genotype by Sequencing has been used in Quantitative Trait Loci (QTL) studies researching disease resistance, genetic linkage map creation, sex determination, alpha acid content, and yield (Koie et al., 2005; Seigner et al., 2005; Cerenak et al., 2006, 2009; Henning et al., 2011). More recently, Henning et al., (2017b; a) utilized a recently published genome (Hill et al., 2017) to identify markers tightly linked with powdery mildew (*Podosphaera macularis* (Wallr.) U. Braun & Takam) resistance and short-stature growth in hop. Seigner et al., (2005) screened wild hops from various regions around the world for effective resistance genes and closely associated molecular markers are being identified to select for resistance to hop powdery mildew. Henning et al., (2017b; a), utilized mapping populations designed for the traits of interest to identify QTLs.

The application of mutation techniques has played a significant role in plant breeding and genetics and advanced genetic studies (Shu et al., 2012b; Suprasanna et al., 2014, 2015). To date, studies involving hop improvement via induced mutagenesis are limited to polyploid induction (Haunold, 1971), thus, research is needed to address the feasibility of techniques driving smaller-scale genetic changes for hop improvement. The goal of this research was to estimate the level of DNA change in Cascade and Centennial hop cultivars subjected to various gamma radiation doses.

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIALS

Clean *in vitro* cultures of Cascade hop were obtained from the U.S. Department of Agriculture, National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon. *In*

vitro cultures were originally initiated from 0.3-0.5mm meristems of heat-treated shoots from clonally propagated hop plants (Adams, 1975). Clean *in vitro* cultures of Centennial hop were obtained from Explant Laboratory in Portland, Oregon.

Plantlets were grown in Magenta GA 7 boxes (Magenta Corp., Chicago, IL) containing NCGR-HUM medium with the addition of 100 mg of Sequestrene Fe (Reed et al., 2003; Aynalem et al., 2006b) (Aynalem et al., 2006a). Every three weeks, plants were transferred into new media to ensure optimal nutrient levels. Magenta boxes were maintained at 24±2ºC with a 16 hour photoperiod and illumination at 60-80 uM m^2 s^{-1} with a combination of warm and cool white fluorescent bulbs.

The gamma radiation factor has three levels 2,3,4 Gy of radiation and a non-irradiated control was included for both cultivars. Plants were acclimated to greenhouse conditions ninety days after irradiation in an Oregon State University greenhouse.

3.2.2 PLANT DNA EXTRACTION

Eight genotypes were randomly selected from each rep within each treatment combination for DNA extraction. Plant leaf tissue consisting of juvenile leaves approximately 3-4 cm in diameter were collected and immediately placed on ice. The fresh tissue was subsequently weighed to obtain 90-110 mg of leaf tissue per sample. After weighing, leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. Grinding was repeated three times to ensure breakdown of cell wall material and facilitate the release of nuclear contents. The frozen plant material was scraped into collection vials for DNA extraction according to the procedure modified from Qiagen Plant DNeasy Kit (Qiagen Inc, USA) procedure (Henning et al., 2016). Disruption using

the TissueRuptor or TissueLyser was not used as the process tended to create excessive amounts of small fragment DNA. Extraction Buffer AP1 650 ql and 8 ql RNase A was added to ground plant material in a collection tube. Subsequently, the lysate was shaken vigorously and inserted into a water bath. Incubation period of the lysate was increased to a minimum duration of 30 minutes but no longer than 60 minutes. Increased quantities of Buffer AP1, RNase A, and incubation period—over that recommended by Qiagen Plant DNeasy Kit—ensured complete cell lysis, destruction, and removal of RNA. Neutralization Buffer P3 190ql was added to the lysate, mixed, and incubated on ice for five minutes. Increased quantities of Buffer P3 ensured a more purified DNA sample. Lysate was then centrifuged for five minutes at 20,000 x g (14,000 rpm). The QIAshredder step was eliminated as this process created excessive amounts of small fragment DNA. The flow-through fraction was transferred into new tubes without disturbing the cell-debris pellet. Wash Buffer AW1 (700ql) was added to the liquid fraction and mixed by inverting the tube 15-20 times. 650 ql of the mixture was then pipetted into the DNeasy Mini spin column and placed in a 2ml collection tube. The tube was centrifuged for one minute, and the flow-through was discarded. The previous step was repeated with the remaining sample. After the flow-through and collection tube were discarded, the DNeasy Mini spin column was placed into a new 2ml collection tube. Approximately 500 ql Buffer AW2 was added into the DNeasy Mini spin column, and centrifuged for 2 minutes at 20,000 x *g* (14,000 rmp) to dry the membrane. The DNeasy Mini spin column was transferred to a 1.5ml or 2ml microcentrifuge tube and 50 ql Buffer AE was pipetted directly onto the DNeasy membrane. The quantity of Buffer AE was decreased from 100ql to increase the final DNA concentration. The Buffer AE was

allowed to incubate on the DNeasy membrane for five minutes at room temperature (15-25ºC), and then centrifuged for 1 minute at 2,000 x *g* (6,000 rmp).

A Qubit® Fluorometer (Life Technologies Inc., USA) was used to quantify the concentration of DNA in each sample. The DNA samples were subsequently prepared and analyzed on an agarose gel to determine the level of shearing in addition to potential RNA contamination. In addition, 50 random DNA samples were cut with EcoR1-HR to determine if DNA samples would sufficiently cut for Illumina® sequencing (Illumina Inc., USA) library preparation.

3.2.3 DNA SEQUENCING

Seven samples per replication were randomly selected resulting in a total of one-hundred and forty four genotypes sequenced utilizing three plates with forty-eight genotypes multiplexed per lane. The Center for Genome Research and Biocomputing at Oregon State University performed all library preparation and sequencing. Library preparation was achieved by the restriction enzyme ApeK1 to cut the high molecular weight DNA, ligating adapters and bar-codes, and bridge PCR amplification of short fragments for pyrosequencing on an Illumina HiSeq 3000 (Elshire et al., 2011).

3.2.4 SNP CALLING

Tassel 3.0 Pipeline (Ellshire et al, 2011) was used for SNP calling with default settings. The reference genome used for alignment was the Cascade PacBio genome (http;//www.Hopbase.org). The resulting hapmap format file was subsequently loaded into TASSEL 5.2.40 (Bradbury et al., 2007) for marker filtration. The overall data set

was split into two datasets: one for Cascade and one for Centennial. Marker sets were subsequently filtered out using the presence of each marker in 95% of all genotypes as criteria in order to retain markers. All monomorphic and/or polymorphic markers across all individuals were retained in both data sets as data for these contribute towards calculations of genetic distances created by mutation treatments.

3.2.5 DATA ANALYSIS

Pairwise genetic distance estimates for each treatment/cultivar were calculated using Bray-Curtis dissimilarity estimates (Bray and Curtis, 1957) in SYSTAT v. 13.2 (Systat Software, Inc,). Genetic distances between treatments samples versus control samples were used to quantify genetic changes. As there were multiple treatments as well as control treatments (with no mutation treatments applied), genetic distances were averaged within each treatment group and the average genetic distance for the control group was subtracted to provide a measure of how much genetic change had occurred for each treatment. This number presumably gave the level of mutation obtained from each treatment measured as genetic distance.

3.3 RESULTS

Using the reference genome of Cascade for SNP calling resulted in identifying approximately 850K SNP markers. After filtering the markers and dividing data sets for Cascade and Centennial, 50,756 SNPs were identified for the Cascade data set and 65,277 SNPs were identified for the Centennial data set.

The genetic distances varied among each radiation dose for Cascade and Centennial hop. A trend was observed in Cascade and Centennial hop where a higher dose of radiation resulted in a larger genetic distance from the control. Compared to the control, the average genetic distance for Cascade after 2 Gy was 0.0085, 3 Gy was 0.0092, and 4Gy was 0.0096 (Figure 10). Compared to the control, the average genetic distance of Centennial after 2 Gy was 0.0145, 3 Gy was 0.0147, and 4 Gy was 0.0148 (Figure 11).

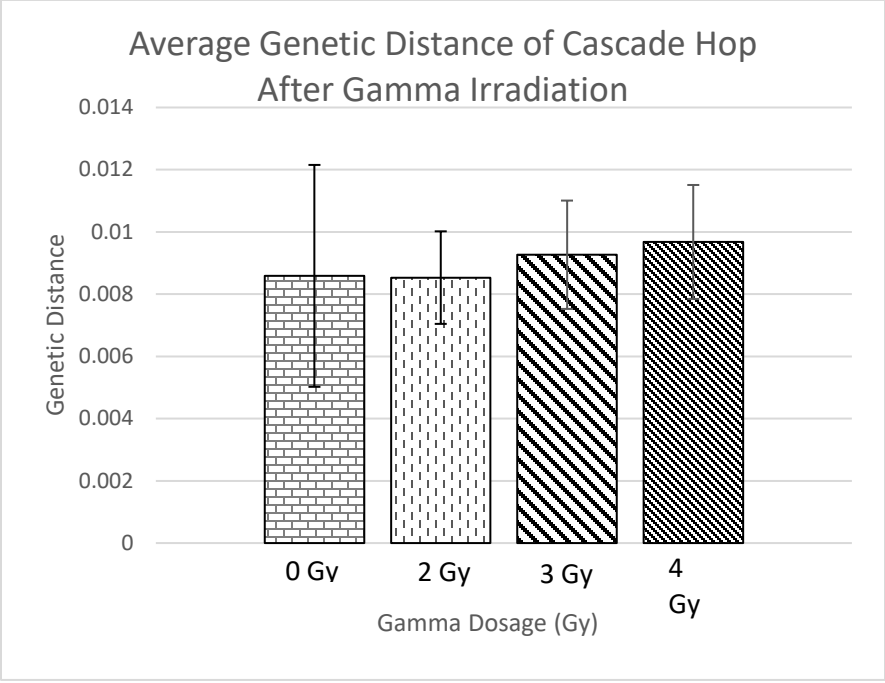

Figure 10. Mean and standard deviation of the genetic distance values of Cascade for each dose based on SNPs.

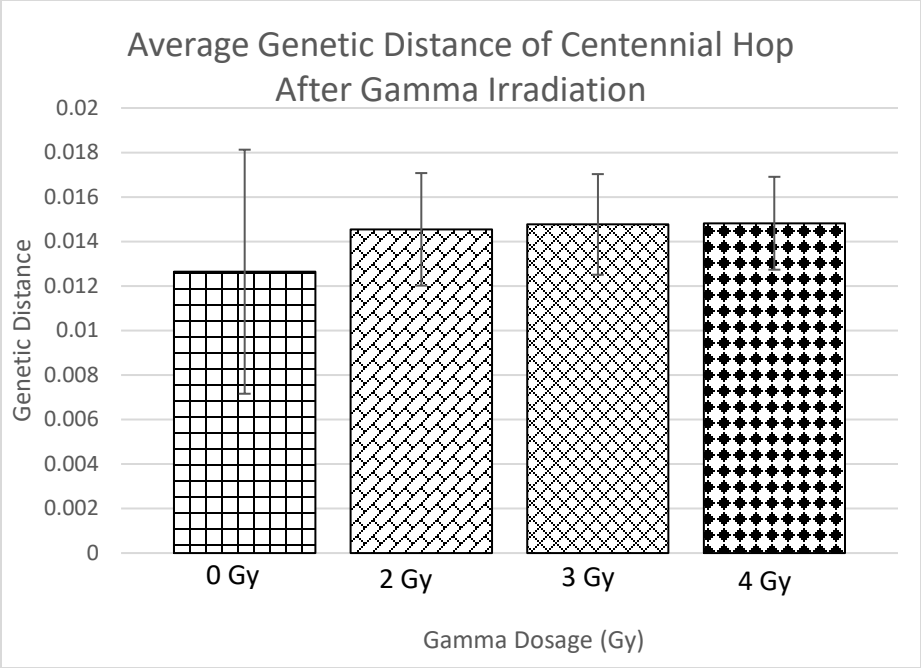

Figure 11. Mean and standard deviation of the genetic distance values of Centennial for each dose based on SNPs

3.4 DISCUSSION AND CONCLUSIONS

Cultivated hop is a domesticated, economically important herbaceous perennial used in the brewing industry as a source of flavor and aroma in beer. Brewers desire new hop lines possessing genetic improvements in yield or disease resistance in order replace popular cultivars without significantly changing their brewing profile. Cross-breeding popular cultivars with heterozygous male lines significantly changes the offspring from the parent cultivar. However, mutation breeding potentially allows for small to large DNA changes (depending upon radiation dosage) while maintaining similar characteristics of the parent cultivar. The goal here was to determine an optimum level of mutation to introduce variation into two popular cultivars without causing significant damage to the lines themselves. The quantification of mutations levels can be determined

by calculating genetic distances of the mutated lines from control lines of Centennial and Cascade.

Recently, USDA-ARS and OSU scientists completed a phased-diploid, draft-quality, fully-annotated genome for the cultivar Cascade (HopBase.org) that can be used to identify genes of interest as well as obtaining a relatively accurate measure of genetic changes that have occurred due to gamma radiation. The following text describes methods for determining genetic distance of Centennial and Cascade hop cultivars after being irradiated with a range of gamma radiation under *in vitro* conditions as well as phenotypic changes observed from mutation treatments.

The LD_{50} of nodal sections of micropropagated plants for Cascade hop is estimated to be 4 Gy or less of gamma radiation (Getty, 2015). In the current study, Cascade plants subjected to 2 Gy had the highest survival, shoot growth and root growth in tissue culture, in addition to the highest survival after greenhouse establishment. Quantifying levels of mutation for each dose can help inform researchers on the appropriate doses to for research or breeding goals.

A higher dose of gamma radiation resulted in a larger genetic distance from the control in Cascade and Centennial hop. This trend was similar to the previous chapter where an increase in radiation showed a decrease in survival, shoot growth, root growth, and plant division in tissue culture, plant survival and growth after greenhouse establishment. Cascade explants irradiated with 4 Gy of radiation had a mortality rate of nearly 40% in tissue culture, the closest dose to an observed LD_{50}, and a genetic distance from the control of 0.0096. Results indicate that this level of mutation resulted in reduced shoot growth, root growth, and plant division (Figure 4). Centennial followed a similar

trend as Cascade, a higher radiation dose resulted in a greater genetic distance from the control. The LD_{50} of Centennial while in tissue culture was 4 Gy of gamma radiation and had a mortality rate of 43%. At this dose, the genetic distance when compared to the control was 0.0148. Cascade explants irradiated with 3 Gy had a mortality rate of 34% in tissue culture and a genetic distance from the control of 0.0092. Decreases in shoot growth, root growth and plant division were similarly reduced to the 4 Gy treatment. Centennial explants irradiated with 3 Gy had a genetic distance of 0.0147 and a mortality rate of 17%. The LD_{50} for Centennial after greenhouse establishment was 3 Gy of gamma radiation. Centennial treatments given 3 Gy of radiation had the highest survival in tissue culture while treatments 0, 2, 4 Gy had the lowest survival. These results do not appear to support the hypothesis that higher radiation doses increase genetic distance by causing mutations and negatively affect growth. However, contamination in the Centennial stock increased mortality and skewed the root and shoot growth data, making interpretation difficult. Cascade explants irradiated with 2 Gy had a mortality rate of 16% and genetic distance from the control of 0.0085. This level of gamma radiation had the highest survival, shoot growth, root growth, and plant division.

Genetic distances for Centennial were higher at each dosage than for Cascade. Possible reasons for this include the reference genome used for identifying SNPs in the study being Cascade. This could have potentially increased the amount of SNPs due to the genetic difference between the two cultivars. If the reference genome had been made from Centennial, the amount of SNPs would be lower in Centennial and higher in Cascade. Another possibility could be higher quality DNA from Centennial samples may have increased the number of SNP calls that were identified.

The controls for both cultivars have standard deviation bars overlapping each treatment. This could indicate the treatments are not significantly different from controls, however, fewer control plants were analyzed reducing the statistical power. A numerical trend supports the hypothesis that an increase in radiation dose decreases survival.

These observations could have commercial significance as these results suggest that 2, 3, and 4 Gy are all effective gamma radiation doses to induce genetic mutations in hop. If the goal is to obtain a relatively high mutation rate for gene discovery or other genetic work, 4 Gy could be an appropriate gamma radiation dose since the mutation rate was the highest and survival, while significantly lower after tissue culture, still produced healthy plant. If the goal is to initially screen for useful characteristics such as abiotic and biotic stresses (tolerance to salt, toxic elements, or fungal toxins for example) for plant breeding purposes, 2 Gy of gamma radiation may be more appropriate since greenhouse survival would be higher. Furthermore, if the breeding goal is to retain brewing similarities to the parental source material but induce minor agronomic changes, it would be reasonable to utilize the lowest gamma radiation dose (2 Gy) and screen larger numbers of mutated individuals to find desired genotypes.

CHAPTER 4. FUTURE WORK

Based upon the traits studied here, these results suggest 2, 3, and 4 Gy of gamma radiation to be acceptable dosages to induce mutations in Cascade and Centennial hop. The dosage chosen can be dictated by the researcher's goals. However, a major drawback in this study was an unknown biotic contamination in the Centennial stock plants. Unfortunately, the measurements were greatly affected by the apparent decrease in survival and overall vigor due to the pathogen. As such, the results of the LD_{50} and vigor for Centennial were inconsistent and likely unrepresentative of how Centennial hop responds to gamma radiation. In addition, only two hop cultivars were surveyed, and analyzing additional hop cultivars from a wider genetic background would be helpful in understanding gamma radiation's feasibility for further hop research and development.

To obtain a more comprehensive idea of how gamma radiation affects hop, new studies could evaluate chemical profile changes, unique mutants (changes in sex, etc.) and response under field conditions. Evaluating the chemical profile of mutants would be advantageous and would allow the potential opportunity to correlate DNA change with the change in chemical profile. This would help breeders refine their radiation doses to a level of radiation that minimally affects the chemical profile, and perhaps aid in gene discovery for the chemical traits. In addition, evaluating mutants over locations and for consecutive years on differences in growth, disease resistance, and morphological changes might be another way to fine-tune appropriate gamma radiation doses for useful mutations. Integrating mutants into a traditional breeding program as parents in cross

breeding could help discover recessive mutations and help assess the mutation's stability over multiple generations.

Expanding the research to evaluate other mutagen types such as EMS may also be useful in determining the best method of mutation breeding in hop. Methods such as EMS and other chemical treatments could prove to be less labor intensive, more efficient in inducing mutation, and possibly produce more useful mutations than gamma radiation.

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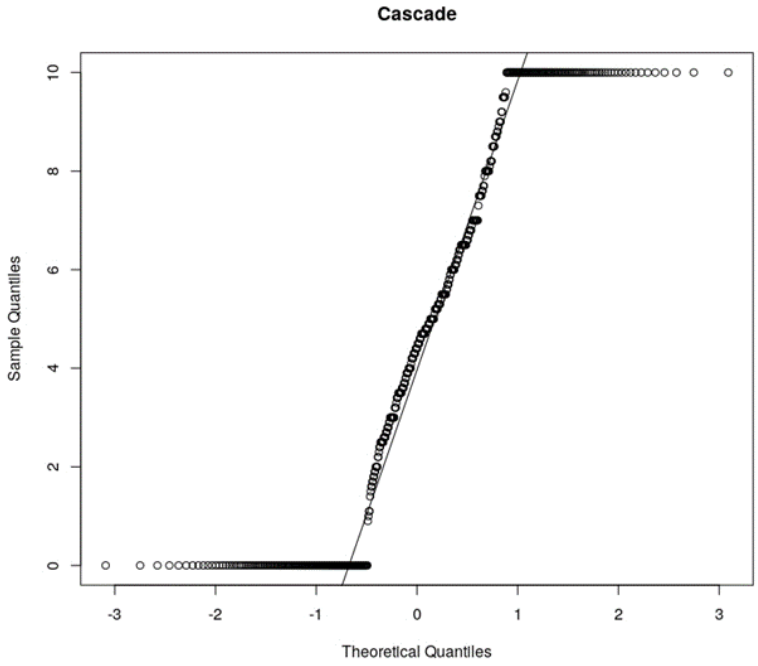
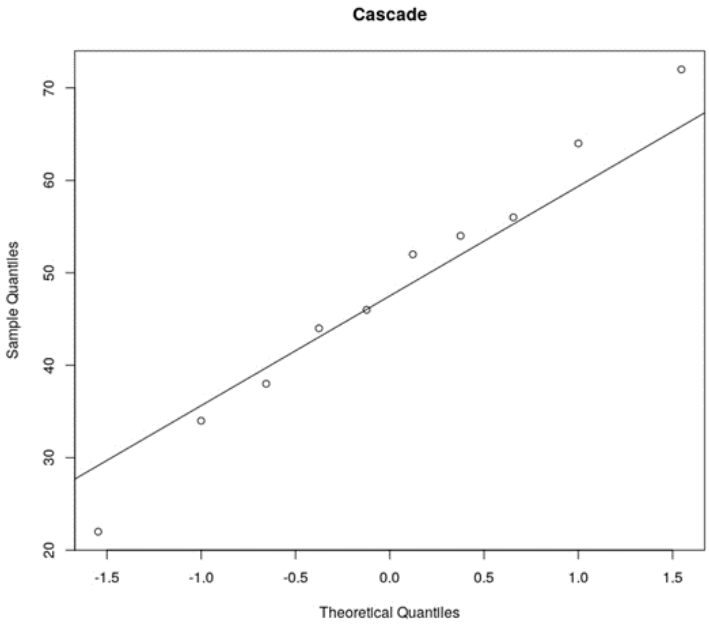
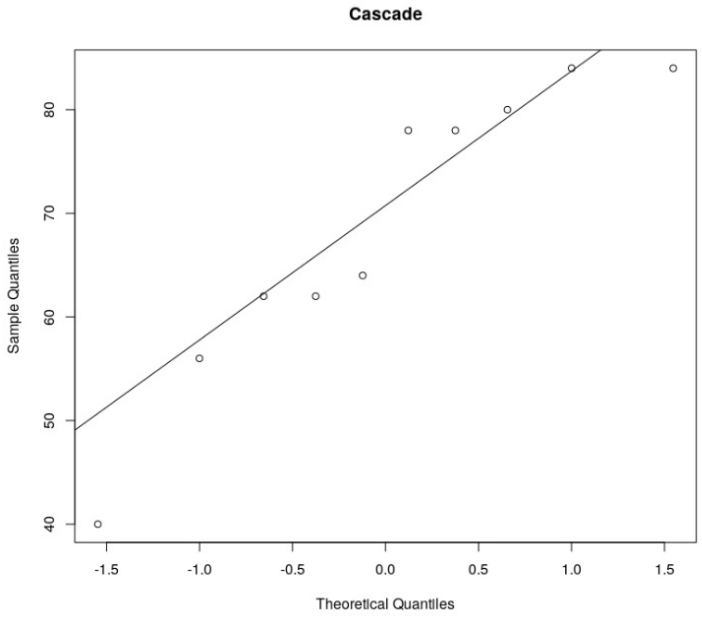
LIST OF APPENDICES



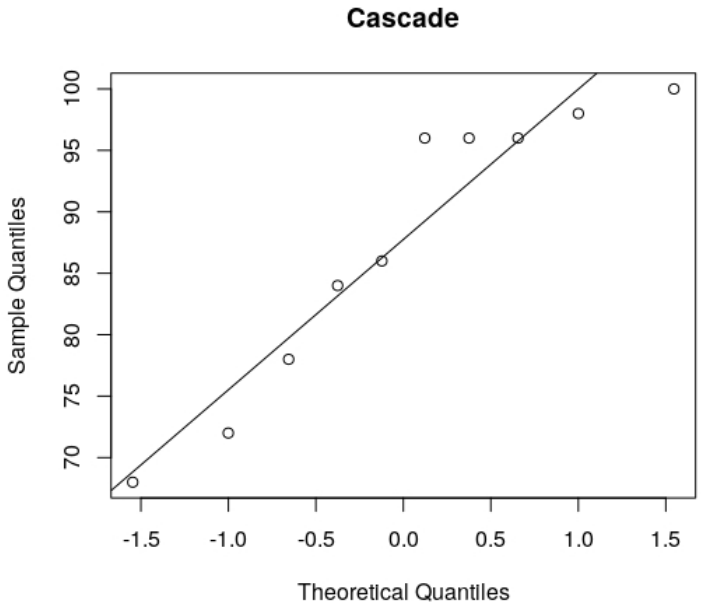
A. Normality test for Cascade on shoot growth (cm)



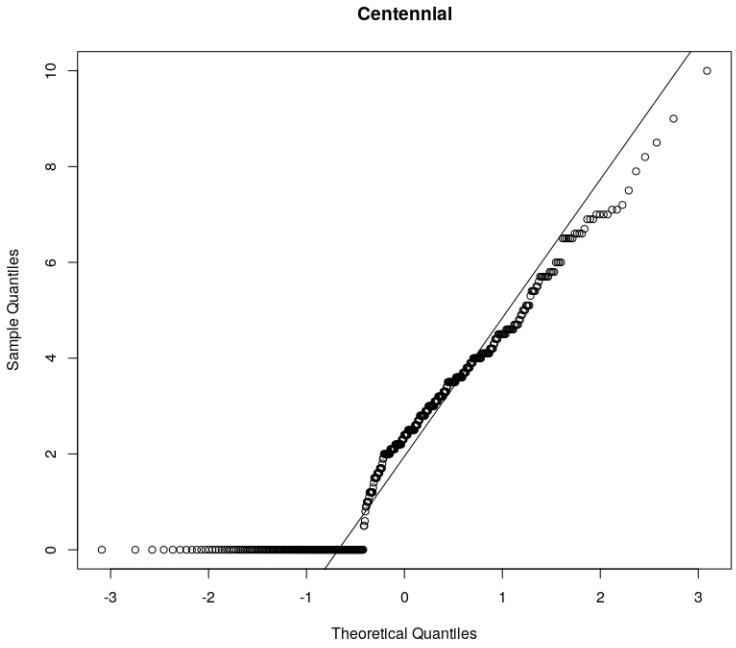
B. Normality test for Cascade on the presence of roots (yes or no) after irradiation



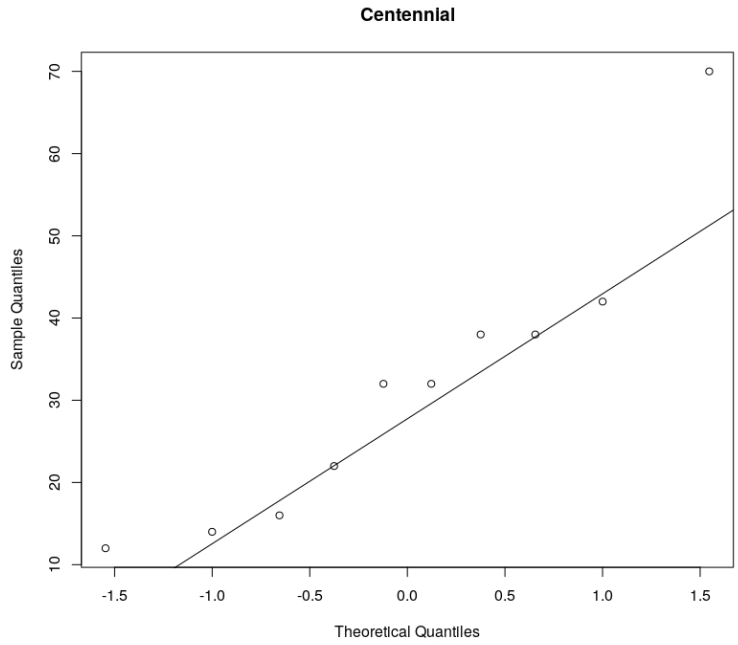
C. Normality test for Cascade on survivability after irradiation



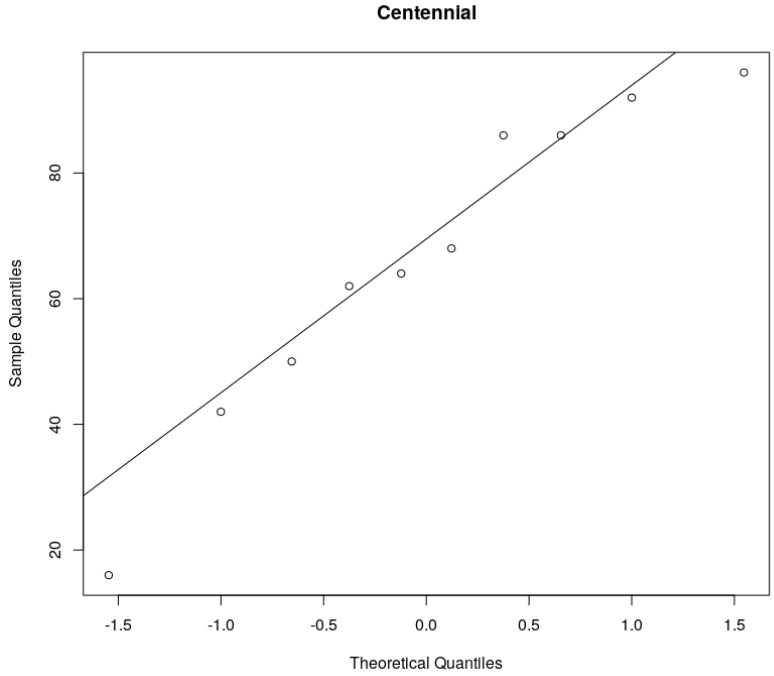
D. Normality test for Cascade on plant division



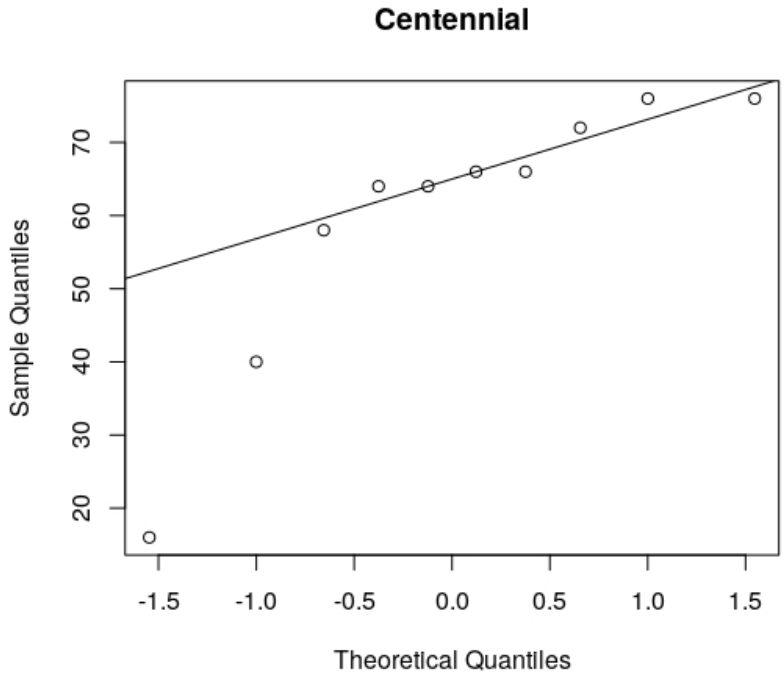
E. Normality test for Centennial on shoot growth (cm)



F. Normality test for Centennial plants on the presence of roots (yes or no) after irradiation



G. Normality test for Centennial on survivability after irradiation



H. Normality test for Centennial on plant division

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gamma	3	460	153.47	11.411	3.03e-07 ***
Plant	1	54	53.75	3.997	0.0461*
Gamma:Plant	3	40	13.37	0.994	0.3952
Residuals	492	6617	13.45		

I. Analysis of variance for Cascade testing the significance of gamma dosage and plant from shoot growth

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gamma	3	35.3	11.750	2.571	0.0536
Plant	1	1.6	1.564	0.342	0.5588
Gamma:Plant	3	9.2	3.057	0.669	0.5715
Residuals	492	2248.7	4.570		

J. Analysis of variance for Centennial testing the significance of gamma dosage and plant from shoot growth

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gamma	3	1195.6	398.5	1.874	0.309
Rep	1	80.7	80.7	0.379	0.582
Gamma:Rep	2	25.3	12.7	0.060	0.943
Residuals	3	638.0	212.7		

K. Analysis of variance of Cascade plants for difference between gamma dosage and replications for root growth

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gamma	3	1651.7	550.6	3.494	0.166
Rep	1	240.7	240.7	1.528	0.304
Gamma:Rep	2	329.3	164.7	1.045	0.452
Residuals	3	472.7	157.6		

L. Analysis of variance of Centennial plants for differences between gamma dosage and replications for root growth