FACTORS INFLUENCING DIFFERENTIATION OF PINTO BEAN SEED DISCOLORATION

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Factors Influencing Differentiation of Pinto Bean Seed Testa Discoloration

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Pinto breeding line NE 94-4 (Phaseolus vulgaris L.) showed seed coat yellowing in on-farm-field trials in Nebraska in 1996 and 1997. After reviewing field and weather data of the on-farm-trial sites, hail, high temperature and sprinkler irrigation appeared to be involved with the increase in seed coat yellowing. The goal of this research was to determine the cause(s) of testa yellowing. Moisture was the factor chosen for further exploration.

Two greenhouse experiments (misting and injection) and one lab experiment (moistened filter paper) were conducted. The experimental design was a split-plot in the misting experiment, and a randomized complete block design in the injection and moistened filter paper experiments. In all experiments, breeding line NE 94-4 and Pinto bean variety UI 114 (control) were used. At the end of each experiment, yellowing was recorded visually. Data from all three experiments were analyzed using the mixed model procedure of SAS.

In the misting experiment, only variety/line, and the interaction between variety/line and pod were significant. In the injection and moistened filter paper

experiments, the treatment effects were significant with NE 94-4 showing more yellowing than UI 114.

Thin layer paper chromatography (TLC) was used to identify the pigments in the Pinto bean seed testa. Some flavonoids and carotenes were identified. Sulpheritin (aurones, flavonoids) was found only in the seed coat of yellowed NE 94-4. On the other hand, daidzein and genistein (isoflavonoids) were identified only in the seed coat of UI 114.

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INTRODUCTION

Legumes are grown and consumed in nearly all parts of the world. In many developing countries, 20% of the available protein is provided by legumes. Legumes are also an integral part of dietary protein for approximate 50% of the world's population (Deshpande et al., 1983). Of the twenty leguminous species commonly used for food, dry beans, *Phaseoulus vulgaris* L., are consumed in the largest quantities in the U.S. as well the world (Deshpande et al., 1983).

'Pinto', 'Great Northern', and 'Light Red Kidney' beans are the main bean varieties produced in the central high plains states of Colorado, Nebraska and Wyoming. Among the three 'most-consumed' dry bean varieties (Schwartz et al., 1996), Pinto beans have become the most popular variety. Thus, the combined popularity and global economic worth of Pinto beans makes them an important crop to study.

Color is very important for most fruits and vegetables including Pinto beans. The shining mottled color of Pinto beans attracts consumers' attention. This mottled coloration is primarily determined by genotype (Prakken, 1974). However, environmental factors and diseases can cause discoloration (yellowing) of the testa. Discolored Pinto bean seeds are not purchased by consumers. Thus, growing varieties where even a small percentage of the beans are discolored is to be avoided. In 1996 and 1997, breeding line Pinto NE 94-4 showed seed coat discoloration in on-farm field trials in Nebraska (Figure 1). While other varieties also showed some discoloration (Figure 2), Pinto NE 94-4 was scheduled to be released due to its other excellent characteristics such as high yield. Due to the yellowing, breeding line Pinto NE 94-4 was not released and the question arose as

to how this yellowing occurred as all previous selections in NE 94-4's parentage were made against yellowing and toward quality seed coat color.

After reviewing weather and field data from the on-farm field trials, it was determined that hail, high temperature and sprinkler irrigation could be involved with the increase in seed coat yellowing. Irrigation (moisture) was chosen as the factor for further exploration. Additionally, investigation of the pigments in the Pinto dry bean testa was undertaken to determine what pigments(s) may be involved in the yellowing of the seed coat.

LITERATURE REVIEW

Although the color of the dry bean seed coat is determined by genotype, environmental factors and diseases can affect the coloration of seed coats. Temperature (Hughes and Sandsted, 1975), humidity (Hughes and Sandsted, 1975; Morris and Wood, 1955) and light (Hughes and Sandsted, 1975) appear to be most important external factors for seed coat coloration. Morris and Wood (1955) indicated that moisture content had more influence on the color changes of pinto beans than of other varieties. They also reported that at a lower moisture content (below 10.4%), the color changed less. Hughes and Sandsted (1975) observed that the seed coat of 'California Light Red Kidney' beans had further darkened when exposed to high temperature (24°C) and relative humidity (80%) than lower temperature (12°C and 1°C) and humidity (30%) after 6 months of storage. They also observed that white fluorescent and ultraviolet sources rapidly darkened California Red Kidney beans, with the ultraviolet light darkening the beans more than the fluorescent light after 2 months of storage. The high temperature and humidity caused a dull dark brown color while both light types (cool-white and ultraviolet) produced a bright glassy dark brown color. Hughes and Sandsted (1975) suggested that darkening due to light might involve a pigment change only in the seed coat. While darkening caused by high temperature and relative humidity probably involved changes in constituents throughout the seed.

The term color is based on the human perception of light and refers to a narrow band of the electromagnetic spectrum, specifically the visible spectrum (Hendry, 1993). Acceptability of the pinto bean seed coat color is determined by background color and

patterns (Ghaderi et al., 1984). Average values of different color components can be measured by a Hunter colorimeter, however, selection for the specific type of pinto pattern is based upon visual judgments (Ghaderi et al., 1984). The post-harvest color of dry pinto bean seed coats are due to the carotenoids (Gross, 1991) and the flavonoids (Pietta, 1998), which are the chief classes of plant pigments in nature. Because most beans are not consumed fresh, the color of dry bean seed testa is a very important qualitative characteristic. These pigments, carotenoids and flavonoids, in the bean seed coat are not only crucial for an appealing coloration but important for nutrition as well.

Flavonoids.

The flavonoids are one of the most numerous and widespread groups of natural constituents. They are universally distributed among vascular plants. Except for catechins and proanthocyanidins, they mainly consist of glycosides of flavones, flavonols, flavanons and anthocyanins. The other classes of flavonoids, isoflavonoids, chalkones and aurones, occur less frequently (Pietta, 1998). The most colorful flavonoids are the anthocyanins. Flavonoid color varies from pale yellow, yellow, orange, and blue to red. Of the non-anthocyanin flavonoids, most are colorless or at least pale yellow, largely found in vascular plants and particularly angiosperms (Hendry, 1993). They are present in flowers, fruits, stems, leaves and roots. Frequently, they are located in the epidermal cells.

1. Distribution of flavonoids in common beans.

The seed, seed coat (testa), and hypocotyl of common beans were studied for the presence of flavonoids. The compounds Pg, Cy and Dp 3-glucosides, Pg and Cy 3,5-

diglucosides (anthocyanins) in Bush bean seed coat; (Timberlake, 1975); Dp 3-glucoside in the Adzuki bean seed coat (Timberlake, 1975); Dp, pt and Mv 3-glucosides, Mv 3-5-diglucoside in Adzuki bean (Timberlake, 1975) were identified. And also, 3-xylosylglucoside as the flavonol (Kaempferol) found in *Phaseolus vulgaris* seed (Timberlake, 1975). Kievetone, licoricidin and its analogue phaseolin were identified in virus or fungal-infected French bean seed as isoflavones (Wong, 1975). In Adzuki bean hypocotyl, Mv 3,5-diglucoside (Timberlake, 1975), in Phaseolus vulgaris, flavonols and aurones were extracted (Donnelly, 1975).

2. Functions of Flavonoid in plants.

The role of flavonoids in the plant kingdom mainly corresponds to participation in the light phase of photosynthesis as catalysts of electron transport and/or as regulators of iron channels involved in photophosphorylation (Morel et al., 1998). Since flavonoids are astringent, they can represent a defense system against insects and the other organisms, which are harmful to plant (Morel et al., 1998). Astringency causes the precipitation of proteins (enzymes) through the formation of water-insoluble molecular complexes (Jovanovic et al., 1998). Catechins and other flavonols possess astringent characteristics.

Isoflavones are important plant-protective phytoalexins (Jovanovic et al., 1998). Phytoalexins are antimicrobial metabolites that are absent or present in low amounts in healthy plants and that accumulate in high concentrations in or around damaged cells (McClure, 1975). Because of their antioxidant characteristics, several flavonoids serve as antioxidants for ascorbic acid by chelating metals from the reaction mixture (McClure, 1975). Flavonoids are also responsible for plant part coloration as described by their composition products, such as anthocyanins, which would give red colors. However, the

coloration varies according to co-pigmentation and to chelation with metals (Morel et al., 1998). Another function of flavonoids in plants is the attraction of birds, bees and insects that carry pollen from one plant to another, thus aiding pollination (Salisbury and Ross, 1991). The attraction of fruits is also another function of flavonoids. Plants insure wide dissemination of seeds by having attractive colored fruits which will be eaten by animals and birds. McClure (1976) cited that flavonoids can also screen plants from damage due to ultraviolet radiation.

3. Pharmacological and clinical application of the flavonoids.

It is likely that many flavonoids, either from medicinal plants or from diet, play an antioxidant role mainly in the digestive system by inhibiting the enzyme-catalyzed formation of reactive oxygen species and/or by scavenging them (Briviba et al., 1998).

Briviba et al (1998) reported that individuals that consumed soybean have a lower cancer rate. Researchers confirmed that genistein and daidzein (isoflavonoids) found in soybeans may be responsible for the reduced cancer risk. Additionally, cancer occurrence and severity was decreased when newborn animals were treated with only three single doses of genistein (Briviba et al., 1998).

Quercetin (flavonol) also reduces the number of tumors in certain animal models, but not in human models (Hertog et al., 1998). However, Hertog et al (1998) reported that quercetin found in onion could lower the risk of stomach cancer as individuals consumed onion consumers have lower rate.

Some studies have also shown that the flavonoids, especially quercetin, could prevent cardiovascular diseases (Hertog et al, 1998). Moreover, flavonoids may protect

against some coronary heart diseases, such as arteriolosclerosis and thrombotic tendencies (Samman et al., 1998).

4. Factors affecting flavonoid biosynthesis.

Light. Many workers have considered flavanoids to have a function in screening plants from ultraviolet radiation since they all have absorblity in the 200 to 380 nm region of the visible spectrum (McClure, 1975). It is also clear that in many cases these same wavelengths are very effective for inducing higher levels of flavonoids (McClure, 1975). Most studies were done on the anthocyanins, and indicated that blue and indigo (deep reddish blue) lights were effective. Red light was slightly effective. Green light was not effective in promoting anthocyanin accumulation (McClure, 1975).

Temperature. Most observations showed that foliar anthocyanins increase with the onset of cold weather (McClure, 1975). In fruits, on the other hand, anthocyanin levels increased as the levels of other flavonoids, especially leucoanthocyanin decreased. In most cases, lower and sustained lower temperature causes an increase in PAL (phenylalanine ammonia lyase), which is an enzyme involved in flavonoid synthesis (McClure, 1975).

Mineral nutrients. As mineral nutrition controls the metabolism of the total plant, it is not surprising that an imbalance of minerals would change the balance of flavonoids. Several researchers have determined flavonoid levels in response to fertilization using the macronutrients nitrogen, phosphorus and potassium (McClure, 19575). The effect of N, P, and K^+ on isoflavones in *Trifolium pratense* was studied and found that phosphorus deficiency markedly increased isoflavones while the other elements had no specific effect (McClure, 1975). Another study conducted on the same plant indicated that nitrogen,

phosphorus and sulfur deficiency almost doubled the levels of flavonoids (McClure, 1975).

Mechanical damage and pathogen attack. Pathogen attack may also alter the flavonoid composition either by wounding or by processes that depend upon the presence of a pathogenic metabolite to change the flavonoid accumulation (McClure, 1975). This was validated by the following experiment. This experiment showed that the PAC (phenylalanine ammonia lyase, an enzyme responsible for flavonoid synthesis) level of immature pods from Phaseoulus vulgaris and Pisum sativum increased within 8 hours of inoculation with several pathogenic and nonpathogenic microorganisms (Gross, 1991).

Plant growth substances. The effects of plant growth regulators (PGR) on flavonoids are as diverse as their effects on growth. Increase or decrease in flavonoid levels in response to growth regulators depends on the concentration and manner of PGR application as well as the developmental stage of tissue and the genetic composition of the plants (McClure, 1975). In Phaseolus aureus, IAA, NAA and kinetin induced an increase in the flavonoid concentration (McClure, 1975).

Carotenoids.

Carotenoids are brightly-colored pigments occurring in practically all families of plants and animals (Zollinger, 1985). These are the yellow, orange or red pigments that exist in various kinds of colored plastids in roots, stems, leaves, flowers and fruits of different plants. Carotenoids derive their name from the main representative of their group, β-carotene (Gross, 1991). Carotenoids can be divided into two groups: (1)

carotenes that are hydrocarbons and their oxygenated derivatives (2) xanthopylls (Gross, 1991).

1. Distribution of carotenoids in common beans.

Gross (1991) indicated that the pods of the green beans (French or snap bean) have β-carotene. In the raw bean seed, the following carotenoids and xanthophylls are found: neoxanthin, violoxanthin, lutein 3-5 epoxide, lutein cis+trans, α-carotene and β-carotene. The carotenoids of etiolated French bean seedlings were analyzed, carotenoids and xanthophylls (chrysanthemaxanthin and auroxanthin) were found (Gross, 1991). The true leaves of the French bean had mostly β-carotene, lutein and neoxanthin (Gross, 1991). However, violaxanthin was absent in leaves (Gross, 1991; Kantha and Erdman, 1987).

2. Function of carotenoids in plants.

Two functions of carotenoids in photosynthesis are well established. The first, both carotene and xanthophyll have the ability to transfer absorbed energy to chlorophyll (Gross, 1991). Secondly, they prevent photoxidation of chlorophyll (Gross, 1991; Salisbury and Ross, 1991). Another important function of carotenoids involves the fact that most yellow flowers contain carotenoids that serve as attractors to pollinators (Salisbury and Ross, 1991).

3. Role of carotenoids in human nutrition and medicinal application.

The sources of the human nutrient vitamin A are provided by diet. Animal sources provide half of the total vitamin A as preformed vitamin A, and plant sources provide the other half as provitamin A carotenoids (Gross, 1991). Carotenoids universal function appear to be in protecting the living system from the combined lethal effect of light and oxygen (Davies, 1975). Carotenoids protect cells and tissues against the potentially

harmful effects of visible light, because they are very efficient deactivators of singlet oxygen (Zollinger, 1987). Gross (1991) reported that because carotenoid pigments protect green plants against photosensitization, these pigments could be used in the treatment of photosensitive diseases, such as erytropoietic protophorphyria. Because of this, carotenoids are being considered as a potential cancer preventation agent. In animal models, β-carotene and canthaxanthin can prevent or slow down the growth of skin tumors induced by UV–B (290nm-320nm) radiation (Gross, 1991). Human studies showed β-carotene protects against cancer of the lung and other organs (Gross, 1991).

4. Factors affecting carotenoid biosynthesis.

Plant growth substances. Plant carotenoids are influenced reversibly by various endogenous and exogenous plant growth regulators. Studies showed that the application of GA_3 on the shoots of carrot increased the carotene content in the shoot, whereas it decreased in the root (Gross, 1991). Application of abscisic acid to detached 'Rutgers' tomatoes decreased the carotene content by about 50% (Grosss, 1991). Ethephon, endogenous ethylene, and ethylene also increased the carotene content in some tomatoes cultivars (Gross, 1991). Young bean leaves responded to benzyladenine by decreasing the carotenoid content temporarily (Gross, 1991).

Light. Light is not essential for carotenoid synthesis. However, studies have shown that light increased carotenoid synthesis (Gross, 1991). Gross (1991) further proposed that far-red light may the decrease the carotene synthesis.

Temperature. Generally, the optimal temperature for carotenoid synthesis is relatively low, and the optimal temperature sensitivity varies from plant to plant. The optimal temperature for lycopene formation in tomatoes is 16°C to 21°C. Above 30°C, it

is not formed (Gross, 1991). Gross also reported that β-carotene is not affected by temperature. For carrots, the optimal temperature for carotenoid formation was found from 15°C to 21°C (Gross, 1991).

Fertilizers. An experiment showed that high levels of nitrogen caused an increase in the carotene content in sweet potatoes and carrots (Gross, 1991). Potassium deficiency in spinach leaves increased the carotene content while an increase of K raised the total carotenoid and lycopene content in tomatoes. However, this also lowered the β-carotene content (Gross, 1991).

Pesticides. In most cases, the pesticides such as chlorcholine chloride containing nitrogen increased the carotene content in carrots (Gross, 1991). One experiment indicated that in summer carrots grown where the soil pretreated with a series of insecticides and herbicides, the carotene content was higher (up to 21%) than in the control. An exception was recorded with the herbicide Dosanex™, which decreased the total carotene content (Gross, 1991). In lettuce grown in soil, the total carotene content was higher in plants treated with herbicides, but was unaffected in plants treated with fungicide treatment (Gross, 1991).

Soil. The influence of the type of soil on the carotene content of carrots was studied and found that carotene levels were higher in carrots grown on sandy soil, moor, muck and brown soil (Gross, 1991). For carrots in general, the more acidic soil types increased the carotene content (Gross, 1991).

Cultural methods. Different cultural methods, such as growing in a plastic greenhouse or field situation as well as the position of the fruit on the plant, affected the carotenoid content of tomatoes (Gross, 1991). The highest carotenoid content was found

in vine-grown tomatoes (Gross, 1991). Direct plastic covering, using perforated polyethylene sheeting applied for a certain time, produced a threefold increase in the carotene content, while in uncovered uncultured conditions, the increase was only twofold (Gross, 1991). In lettuce, the effect was opposite (Gross, 1991).

Inheritance of seed coat color of pinto beans.

The seed coat color of Phaseoulus vulgaris L. depends on the integration of the so-called "yellow-black' colors and the 'red-black' colors (Prakken, 1974). Nine main loci groups categorized I, II, III-a, III-b appeared to be responsible for seed coat color (Prakken, 1974). Prakken (1974) classified nine loci groups as the following:

I. Basic gene or groundfactors.

1. *P*, the basic gene or 'groundfactor' for seed coat color.

2. *T*, the gene necessary for a completely colored seed coat.

II. Three dominant color genes or chromogenous factors.

3. *C*, a 'locus' having numerous 'alleles', indicated as the 'complex locus for ever-segregating and constant mottling'.

4 *D*, the hilumring factor.

5. *J* , the shine factor.

II-a. Three dominant modifying genes or intensifying factors.

6. *G*, the yellow-brown factor.

7. *B*, the gray- greenish brown factor.

8. *V*, the violet factor.

III-b. One recessive modifying or intensifying factor.

9. ***Rk***, the locus for recessive genes (Prakken, 1974).

A number of seed coat color variants were found in beans. Most of the variations can be explained by assuming interactions of three genes, *M, Rk* and *Br*, which are all present in the dominant form in normal Pinto beans (Prakken, 1974). *M,* for mottling, has two alleles: *M*st for striped distribution of mottling and *m* for self-colored beans (Prakken, 1974). *Br* is a color modifier of the mottling. In the presence of *m* , the *Br* gene has no effect, in beans of the constitution *M Br* the mottling is brown, while in *M br* the mottling is green or pink in color. *Rk* is a color gene, affecting the ground color of mottled beans or the seed coat color of self-colored beans. *Rk* produces buff color, while the recessive *rk* produces pink color. Beans of the constitution *M Rk Br* are brown/buff; *m rk Br,* green/buff; *M rk Br* brown/pink, and *M rk br* pink/pink (Prakken, 1974).

Nutritional value of pinto beans.

Dry legume seeds are important foods in the diets of peoples throughout the world, especially those living in technologically underdeveloped tropical and subtropical areas. Despite relatively low digestibility, dry beans are important source of protein for countries having short supplies of animal protein.

Koehler et al., (1987) reported that pinto beans had the greatest protein quality among the bean cultivars of: White, Navy, Great Northern, Red Kidney, Pink, Red Mexican, and Black Turtle. Koehler et al., (1987) found that UI 114 contained 21.2% protein, and protein quality was 81 RNV (relative nutritive value is defined as the growth of *Tetrahymena* organism supported by the bean protein as compared to growth supported by *casein*). Beans are good sources of thiamine, zinc, iron and potassium, Pinto

bean variety UI 114 contains 6.55mg/100g Fe, 2.8mg/100g Zn, 162mg/100g Ca, 161mg/100g Mg, 1379mg/100g P, 063mg/100g thiamin and .188mg/100g riboflavin (Koehler et al., 1987). The vitamin content of variety UI 114 in mg acid/g protein wet basis are: histidine, 33; iso-leucine, 45; leucine, 79; lysine, 71; total methionine cystine, 21; total aromatic, 96; threonine, 43; and valine, 55. Thus, the nutritional contribution of dry beans has recently become a serious consideration in food science as well as in bean genetics.

MATERIALS AND METHODS

Two preliminary experiments were run during fall '97 and winter '97 to practice growing bean plants and perfecting the water injection method. The fall '97 and winter '97 experiments provided information for use in the winter '98 and spring '98 experiments. The following two experiments, the misting and injection experiments, were conducted during winter '98 and spring '98. Later an experiment involving the effect of moistened filter paper on seed discoloration was conducted in the lab.

1. Misting experiment.

Beans were planted on January 22, 1998 in the Horticulture Research Greenhouses. After three weeks, half of the seedling plants were moved to another greenhouse and placed where the mist equipment was available. Plants were fertilized with 300 ppm of 20N-8.8P-16.6K three times per week until pods formed. After the pods formed, the frequency of the nutritional applications was decreased to once per week. The greenhouse temperature was set at 26°C day / 20°C night. The humidity was not controlled. The soil medium was 27 % vermiculate, 36 % peat, 10 % soil and 27 % sand. The pesticide Marathon™ was applied to all bean seedlings.

The mist treatment was applied daily from March 24 to May 07, 1998. The treatment design was a split-plot. The mist treatment was used as main plot and arranged in rows and columns with 9 replications. The breeding line Pinto NE 94-4, and variety Pinto UI 114 (control) were arranged as the split-plot. The experimental unit consisted of 4 plants in each with 9 replications for a total of 72 plants.

The treatments were applied two weeks before physiological maturity. One of two plots (4 plants) was removed from each replication to the misting area where the plants were misted for 60 minutes per day over a period of 14 days. Thus, half of the experiment, 36 plants, were treated with mist in a special area which was divided with plastic shields so that the moisture could not affect the rest of plants. A total of 302.8 liters of water were applied to the plants during the 60 minutes misting time. After every misting, plants were moved back to their original places. Each pot treated with mist was covered with a plastic bag so that water would not leak into the soil medium. Two days after the completion of treatments the % yellowing of the seed was recorded. Six seeds from 4 pods, taken from the base to the apex, were examined from each plant for a total of 24 seeds.

Mixed model procedure (SAS, 1995) was used to obtain appropriate estimates and to test statistics. To get more reliable information, the mean of 1 to 6 seeds per pod was used.

2. Injection experiment.

For this experiment the plants were grown at the same time and under the same conditions as the misting experiment.

The three treatments were injury (injection only), injection with water and control. Treatments, injury (injection only), injection with water, were applied on March 27, 1998. The treatment design was a randomized complete block design with 12 replications. Pinto UI 114 and breeding line Pinto NE 94-4 were used as blocks. A total of 72 plants were grown in this experiment. Fifteen days before physiological maturity,

the treatments were applied to the pods. Three pods were selected from the base, middle and apex of each plant for each treatment. For the injury treatment (injection only), a hypodermic syringe was used. The hypodermic syringe was inserted into each pod near the pedicel. For injection with water treatment, a hypodermic syringe was used and 5 ml of water was injected into the pod at the pod suture near the pedicel. Fifteen days later % yellowing of seeds were recorded from three pods per plant.

The mixed model analysis procedure (SAS, 1995) was used to obtain appropriate estimates and test statistics. To get more reliable information, the mean of 1 to 6 seeds per pod was used.

3. Moistened filter paper experiment.

This experiment was conducted in the lab (70 $^{\circ}$F) from April 14 to 21 1998. The treatment design was a randomized complete block design with 4 replications. Pinto UI 114 and breeding line Pinto NE 94-4 were the blocks. There were two treatments, moistened filter paper vs. dry filter paper (control). Pods were randomly selected from control plants in the injection experiment. Each pod was opened in the dark, and immediately placed in petri dishes. Twenty seeds were placed on either moist or dry filter paper in a petri dish. Petri dishes were sealed with tape to prevent air exchange, and kept in the dark during the experiment. Seeds were examined daily for % yellowing. Data was taken at day 7. The experiment was terminated when the seeds placed on the moist filter paper started to germinate.

A mixed model procedure was used to obtain statistical values, and the mean of 1 to 20 seeds per petri dish was taken to reduce the complexity of analysis (SAS, 1995).

Measurement of seed coat yellowing.

For all of the above experiments, yellowing was recorded visually by determining the percentage of the yellow area on a bean seed in each experiment. The scale was: 0,10,20, ….., 90,100 % with 10 % intervals.

Identification of the plant pigments on the seed coat of pinto beans.

Twenty-five g seed of well-colored Pinto NE 94-4, discolored (yellowed) Pinto NE 94-4 and Pinto UI 114 (Table 1), were each mixed separately with 50 ml 80 % methanol. The mixture was heated until yellow pigments were extracted from the bean testa. After extraction, the mixture was filtered with Whatman filter paper (No.4). Methanol in the solvent was removed by nitrogen, and the solvent was filtered once more. Finally, the solution was ready for the thin layer paper chromatography. The stationary phase was Whatman chromatography paper (No.1). For flavonoids, the mobile phases were: butanol-acetic acid-water 6:1:2; 15 % aqueous acetic acid, and water (Swain, 1976). For xanthophylls, the mobile phases were: benzene; benzene-ethyl acetate-methanol, 75:20:5; and dicholoromethane-ethyl acetate, 4:1 (Davies, 1976). For carotenes, the mobile phases were: benzene-light petroleum, 90:10; benzene-light petroleum, 50:50; and benzene-light petroleum, 10:90 (Davies, 1976).

The stationary phase, with a concentrated spot of the solution on it, was dipped into each mobile phase. As the mobile phases ascended the chromatography paper it carried each of the pigments with it differently. In this way, the pigments were separated from one another and could be identified by their different colors and position by the R_f

value (the relative distance traveled by the various pigments). For color determination ultraviolet wavelength light was used.

RESULT AND DISCUSSION

Misting experiment.

The treatments, mist vs. control, were designed to simulate different conditions rain and/or sprinkler irrigation that often occur in the field during the last two weeks of the harvest season. No significant differences between the two treatments were observed (Table 2). This could be due to a number of reasons. Either the rain or sprinkler irrigation alone did not affect bean discoloration (yellowing). Therefore, some special conditions were needed to affect bean discoloration. Beans lying down on the ground in the field could have discoloration, particularly if their pods touched the wet ground. A similar condition was observed in an on-farm field trial in Scottsbluff in 1997 where during harvesting of bean plants only half of trial was cut and harvested. The other cut plants were left on the ground in the field and were rained upon. Seeds from the pods of the plants which touched the ground showed more yellowing than seeds from cut plants that were left upright. The variety/line effect and the interaction between variety/line and pod were significant, as expected because breeding line NE 94-4 is susceptible to discoloration (yellowing) and variety UI 114 (Figure 3) is not (Coyne et al., 1994). Pod effects were not significant, and moreover, the interactions of TRT*VAR, TRT*POD, TRT*VAR*POD were not also significant.

Injection experiment.

The injury with a hypodermic syringe, the injection of water with a hypodermic syringe, and control were the treatments in the injection experiment. The injury with the

hypodermic syringe was applied to pods to simulate physical damage such as hail damage, as well as a control for the water injection treatment. The injection of water could simulate the water inside pods after possible physical damage or water into seeds lying on the ground. Treatment effects were significant (Table 3). The injection of water treatment was associated with more testa yellowing and differed form (Figure 4) in that more testa yellowing occurred. The water injected inside pods probably caused this yellowing and may be related to links with Red Kidney bean seed. Hughes and Sansted (1975) reported that 'California Light Red Kidney' bean seed stored at the highest level of relative humidity had further darkening of the seed coat that at lower levels of relative humidity.

Breeding line Pinto NE 94-4 again showed more yellowing than variety Pinto UI 114, as expected. (Figure 4). Pod effects, the interactions of TRT*POD, TRT*VAR, POD*VAR, and TRT*POD*VAR were not statiscally significant.

Moistened filter paper experiment.

The wet filter paper simulated pods lying on the wet ground and possibly a storage unit with high level of relative humidity. The beans placed directly on the wet filter papers started discoloration at day 1 (Figure 7). By three days, discoloration was extensive. The difference between treatments was significant (Table 4) with breeding line NE 94-4 showing more yellowing than variety UI 114 (Figure 5). Both entries, breeding line NE 94-4 and variety UI 114, showed discoloration (yellowing). This could be caused by the high level of moisture content around and/or inside seeds (Morris and Wood,

1955). Bean seeds could be more severely discolored (yellowed) than bean seeds present in pods.

Pigments in the seed coat of Pinto bean seed coat.

Thin layer chromatography (TLC) was used to identify the color pigments on the bean seed testa of variety UI 114 and breeding line NE 94-4. For identification of color pigments (possibly, carotenoids and flavonoids), TLC method was chosen because different mobile phases can be used at the same time, and TLC permits rapid and sharp separation on a small scale (Gross, 1991; Markham, 1975). This screening experiment was done for well-colored NE 94-4 pinto bean seed coat, supplied from an on-farm field trial in Scottsbluff, in winter 1998. Some flavonoids and carotenoids including xanthopylls were identified on well-colored NE 94-4 bean seed coat, as expected. These are listed in Table 5. Smartt (1988) and Timberlake (1975) reported some flavonoids were found on the bean seed coat. Moreover, Gross (1991) indicated that some carotenoids were classified in raw green bean seed.

During the summer of 1998, another experiment was performed for the identification of yellow pigments. This time, well-colored and yellowed NE 94-4, and UI 114 bean seed coats were used. The beans were supplied from the three previous experiments. In this experiment, well-colored and yellowed NE 94-4 had some of the same flavonoids, which are flavonols and flavanones (Table 6 and table 7). The different flavonids identified in this experiment were: *apigenin* on the seed coat of well-colored NE 94-4, *sulphuretin* on the seed coat of yellowed NE 94-4. This could be explained by some changes in constituents throughout the seed (Hughes and Sansted, 1975). Sulphuretin is one of aurones, called 'anthoclar' pigments (Bohm, 1975). Anthoclar

means a group of yellow pigments which turn red in the presence of alkali (Bohm, 1975). Bohm (1975) reported aurones color to be a yellow-golden color. This yellow-golden aurone could explain the yellowing. Both of the entries showed the same carotenes as listed in tables 5 and 6. None of them showed xanthophylls. The following flavonoids were identified: flavones, flavonols, flavanons and isoflavones on the seed coat of variety Pinto UI 114 (Table 8). The same carotenes identified on the seed coat of well-colored and yellowed NE 94-4 were also found on the seed coat of UI 114, but no xanthophylls were classified. The main difference for the flavonoids between variety UI 114 seed coat and breeding line NE 94-4 including well-colored and yellowed types was that variety UI 114 seed testa carries some innocuous isoflavonoids (isoflavones) *daidzein* and *genistein* (Table 8). In contrast, the other flavonoids, isoflavonoids have estrogenic, insecticidial, piscicidal and anti-fungal properties (Wong, 1975). This could be why variety UI 114 is always used for the control bean among pinto bean varieties and breeding lines. The seed color pattern of UI 114 is highly desired by consumers and processors because it maintains brighter color pattern over a wide range of environmental conditions due to its chemical constituents.

CONCLUSIONS

The moisture content either around the seed or inside the seed is probably the biggest factor responsible for discoloration when the seed completes its physiological development. The moistened filter paper experiment supports this idea. On the other hand, the pod could protect the seed against discoloration (yellowing) as long as the seed and pod are physiologically connected to each other. This idea is supported by the injection of water treatment in the injection experiment. Therefore, additional moisture studies are needed on dry bean seeds to verify this (Morris and Wood, 1955; Hughes and Sansted, 1975).

The relative humidity (moisture content) probably involves some changes in constituents throughout the seed (Hughes and Sansted, 1975). This idea was supported by the presence of sulphuretin found only on the seed coat of yellowed NE 94-4. Most likely, the aurones could be causing the bean yellowing.

The seed coat of Pinto UI 114 showed less discoloration than breeding line Pinto NE 94-4. This is possibly due to the presence of innocuous genistein and daidzein. The isoflavonoids in UI 114 could have innocuous estrogenic, insecticidial, piscicidal, anti-fungal properties (Wong, 1975). On the other hand, Pinto 94-4 was bred for resistance to pests, fungal diseases, white mold, root rot and the bacterial disease common blight.

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Table 1. Seed samples, their lots and number of times thin layer paper chromatography (TLC) was run for identification of the plant pigments (flavonoids, carotenes and xanthophylls) in the seed coat of Pinto beans.

Seed lots	Seed samples	The TLC runs
Scotsbluff	well-colored NE 94-4	2
Ogallala	well-colored NE 94-4	2
	discolored NE 94-4	2
	well-colored UI 114	2
Lincoln	well-colored NE 94-4	3
(Horticulture greenhouses)	discolored NE 94-4	3
	well-colored UI 114	3
	discolored UI 114	3

Table 2. ANOVA for the misting experiment.

Class level information

Class	Levels	Value
Replication	9	
Column	6	1 2 3 4 5 6 7 8 9
Treatment	2	1 2 3 4 5 6
Variety/Line	2	mist vs. control
Plant	2	UI 114 NE 94-4
Pod	4	1 2
		1 2 3 4

Covariance parameter estimates (RML)

Cov Parm	Estimate
REP*COL*TRT	0.000000000
PLANT(REP*COL*TRT*VAR)	42.36616509
Residual	33.61913123

Tests of fixed effects

Source	NDF	DDF	Type III F	Pr > F
REP	6	5	0.46	0.8158
COL	3	5	1.09	0.4334
TRT	1	5	1.31	0.3043
VAR	1	52	6.70	0.0125
TRT*VAR	1	52	0.89	0.3495
POD	3	204	0.53	0.6631
TRT*POD	3	204	1.80	0.1485
VAR*POD	3	204	2.63	0.0512
TRT*VAR*POD	3	204	0.80	0.4977

Table 3. ANOVA for the injection experiment.

Class level information

Class	Levels	Value
Replication	12	1 2 3 4 5 6 7 8 9 10 11 12
Treatment	3	control injury in.water
Variety/LinePod	2	UI 114 NE 94-4
	3	1 2 3

Covariance parameter estimates (REML)

Cov Parm	Estimate
REP*TRT*VAR	4.84682941
Residual	18.8185910

Tests of fixed effects

Source	NDF	DDF	Type III	Pr > F
REP	11	55	1.91	0.0582
TRT	2	55	4.01	0.0238
VAR	1	55	5.40	0.0239
TRT*VAR	2	55	0.44	0.6440
POD	2	132	0.52	0.5955
TRT*POD	4	132	1.01	0.4057
VAR*POD	2	132	0.31	0.7376
TRT*VAR*POD	4	132	0.62	0.6521

Table 4. ANOVA for the moistened seed experiment.

Class level information

Class	Levels	Value
Replication	4	1 2 3 4
Variety/Line	2	UI 114 NE 94-4
Treatment	2	Control vs. moistened

Covariance parameter estimates (REML)

Cov Parm	Estimate
Residual	47.09265176

Tests of fixed effects

Class	NDF	DDF	Type III F	Pr > F
REP	3	313	2.78	0.0413
VAR	1	313	2534.39	0.0001
TRT	1	313	2943.37	0.0001
VAR*TRT	1	313	2518.01	0.0001

Table 5. Yellow pigments identified in well-colored NE 94-4 bean seed coat testa (greenhouse-grown) in winter 1998.

Flavonoids	Carotenes	Xanthopylls
flavones	β-cryptoxanthin	ε-carotene
apigenin	canthaxanthin	β-carotene
luteolin	lutein	γ-carotene
flavonols	violaxanthin	
quercetin		
kaempferol		
flavanones		
naringenin		
chalkones		
butein		

Table 6. Yellow pigments identified in well-colored NE 94-4 bean seed coat testa (greenhouse-grown) in summer 1998.

Flavanoids	Carotenes	Xanthopylls

<u>flavones</u>
  apigenin
<u>flavonols</u>
  quercetin
  kaemferol
<u>flavonones</u>
  naringenin
	γ-carotene
	δ-carotene
	lycopene
		none

Table 7. Yellow pigments identified in yellowed NE 94-4 bean seed coat testa (greenhouse-grown) in summer 1998.

Flavanols	Carotenes	Xanthophylls

<u>flavonols</u>
  quercetin
  kaemferol
<u>flavonones</u>
  naringenin
<u>aurones</u>
  sulphuretin
	γ-carotene
	δ-carotene
	lycopene
		none

Table 8. Yellow pigments identified in UI 114 bean seed coat testa (greenhouse-grown) in summer 1998.

Flavanoids	Carotenes	Xanthopylls
flavones	γ-carotene	none
apigenin	δ-carotene	
flavonols	lycopene	
quercetin		
kaemferol		
flavonones		
naringenin		
isoflavones		
daidzein		
genistein		

Figure 1. Map of on-farm field trial sites.
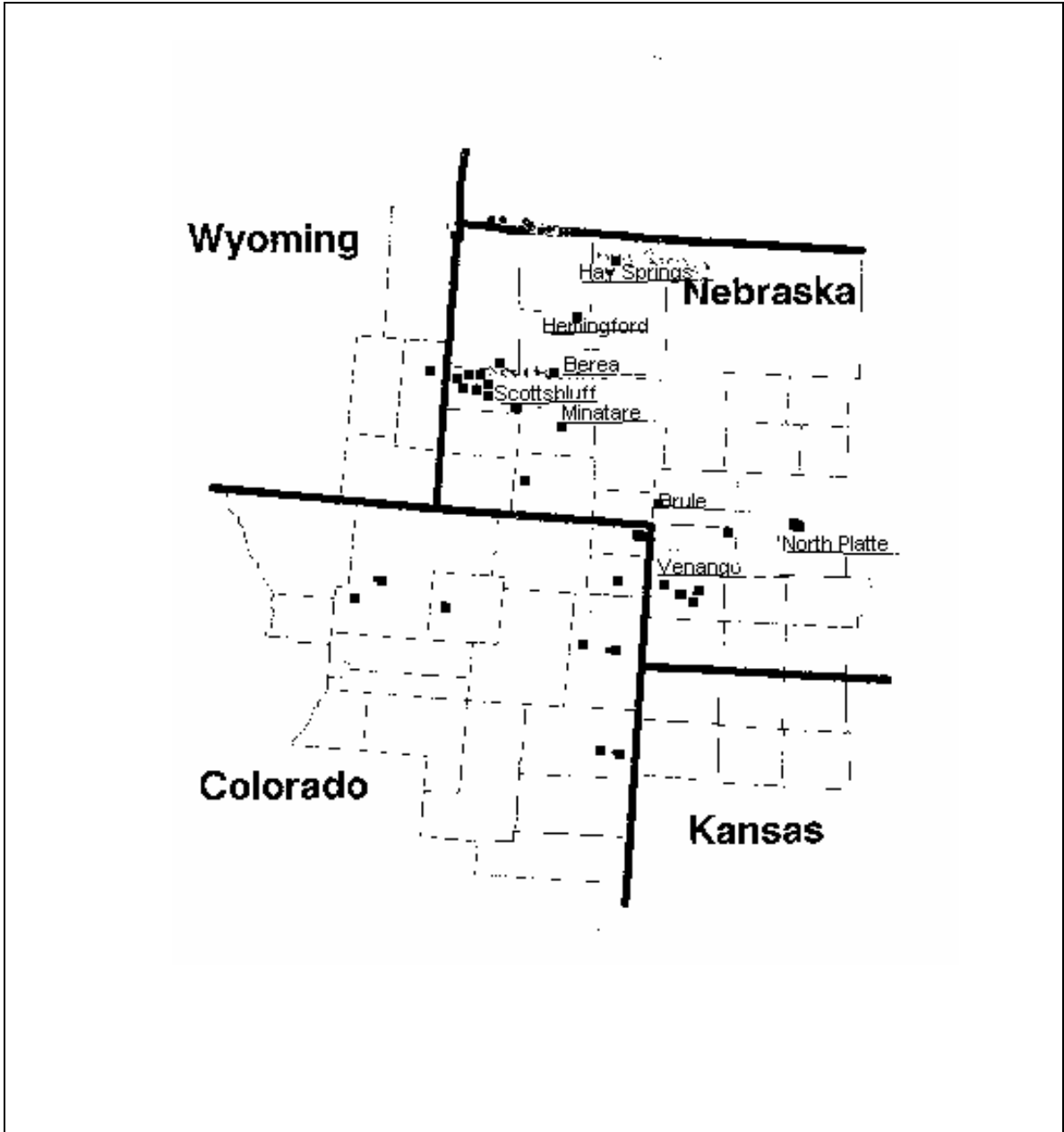
Figure 2. Percentage of seed coat yellowing of 8 field-grown pinto bean varieties/lines (1997).

LSD .05=17.55
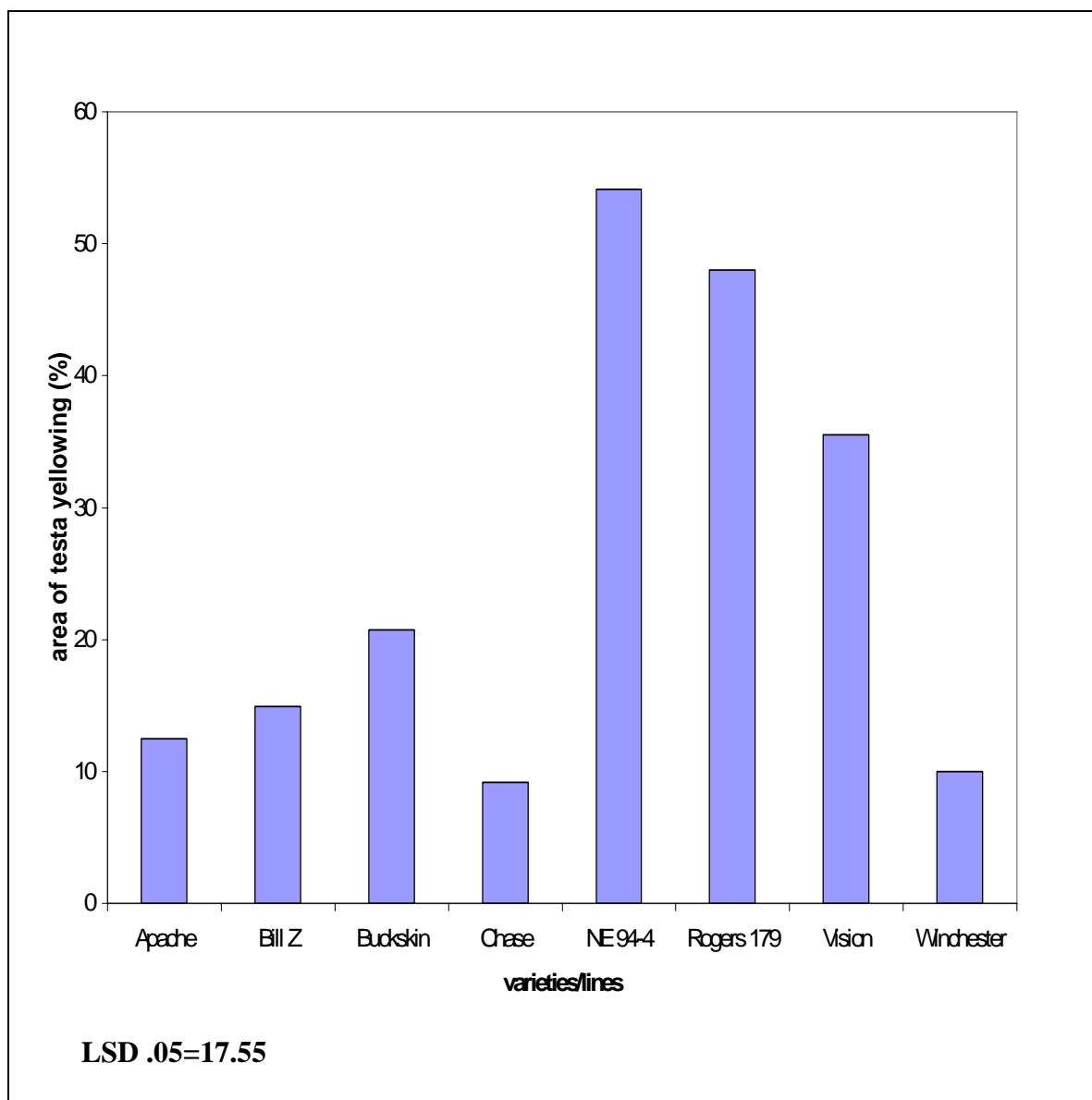
Figure 3. **Percentage of seed yellowing of seed coat of two bean entries under different treatments in the misting experiment.**
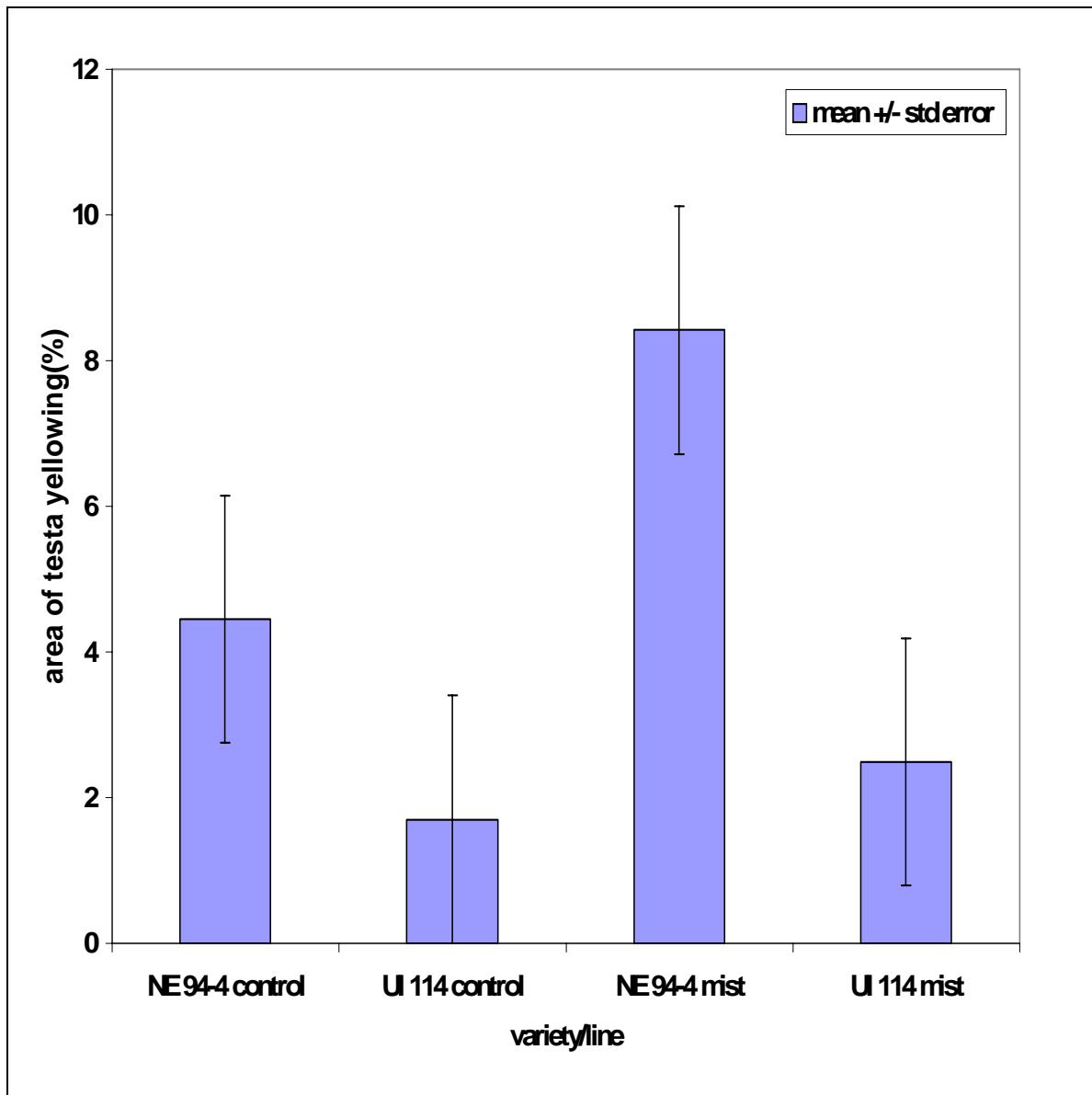
Figure 4. Percentage of seed yellowing of seed coat of bean entries under different treatments in the injection experiment.
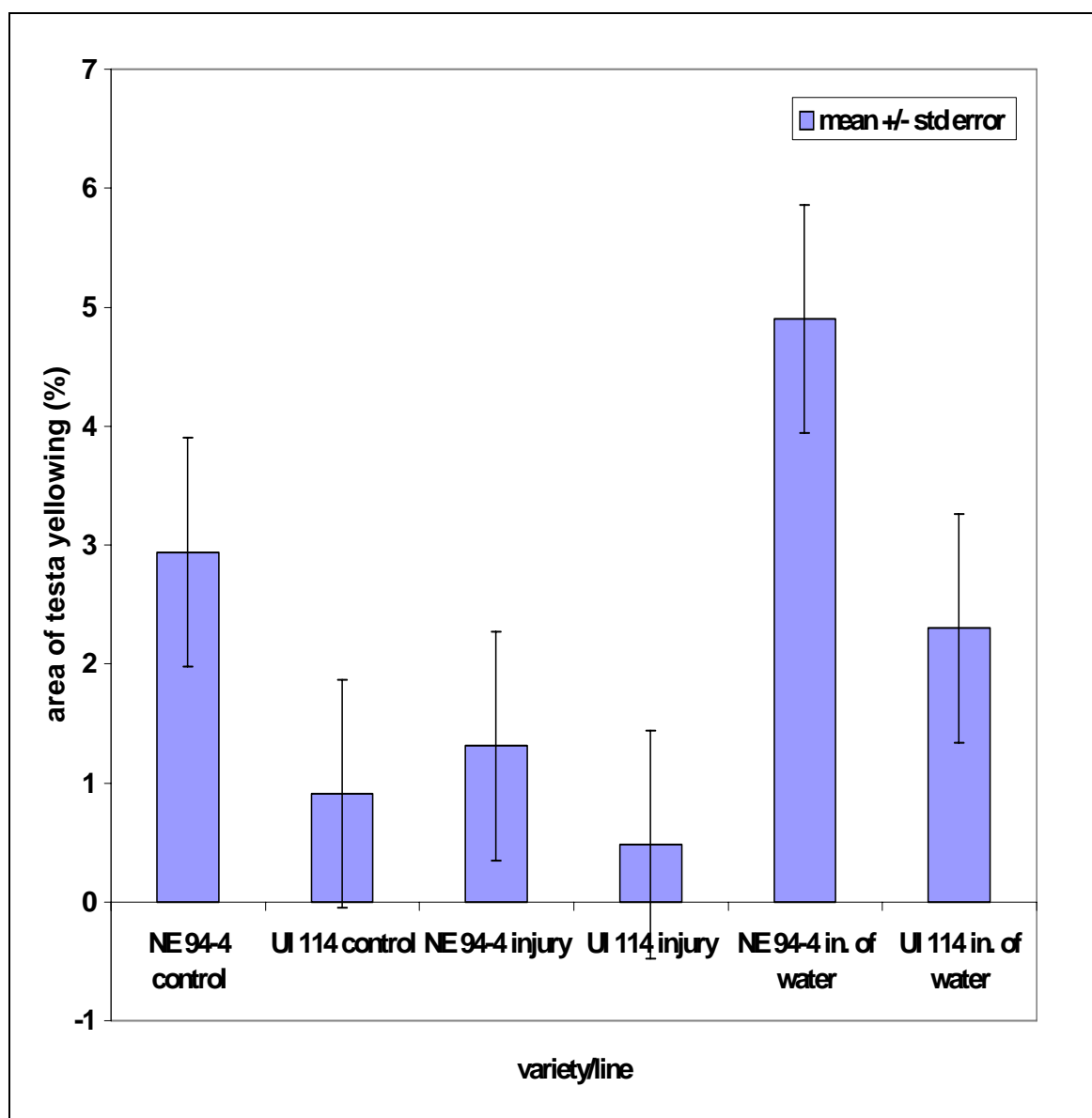
Figure 5. Percentage of seed yellowing of seed coat of two bean entries under different treatments in the moistened filter paper experiment.
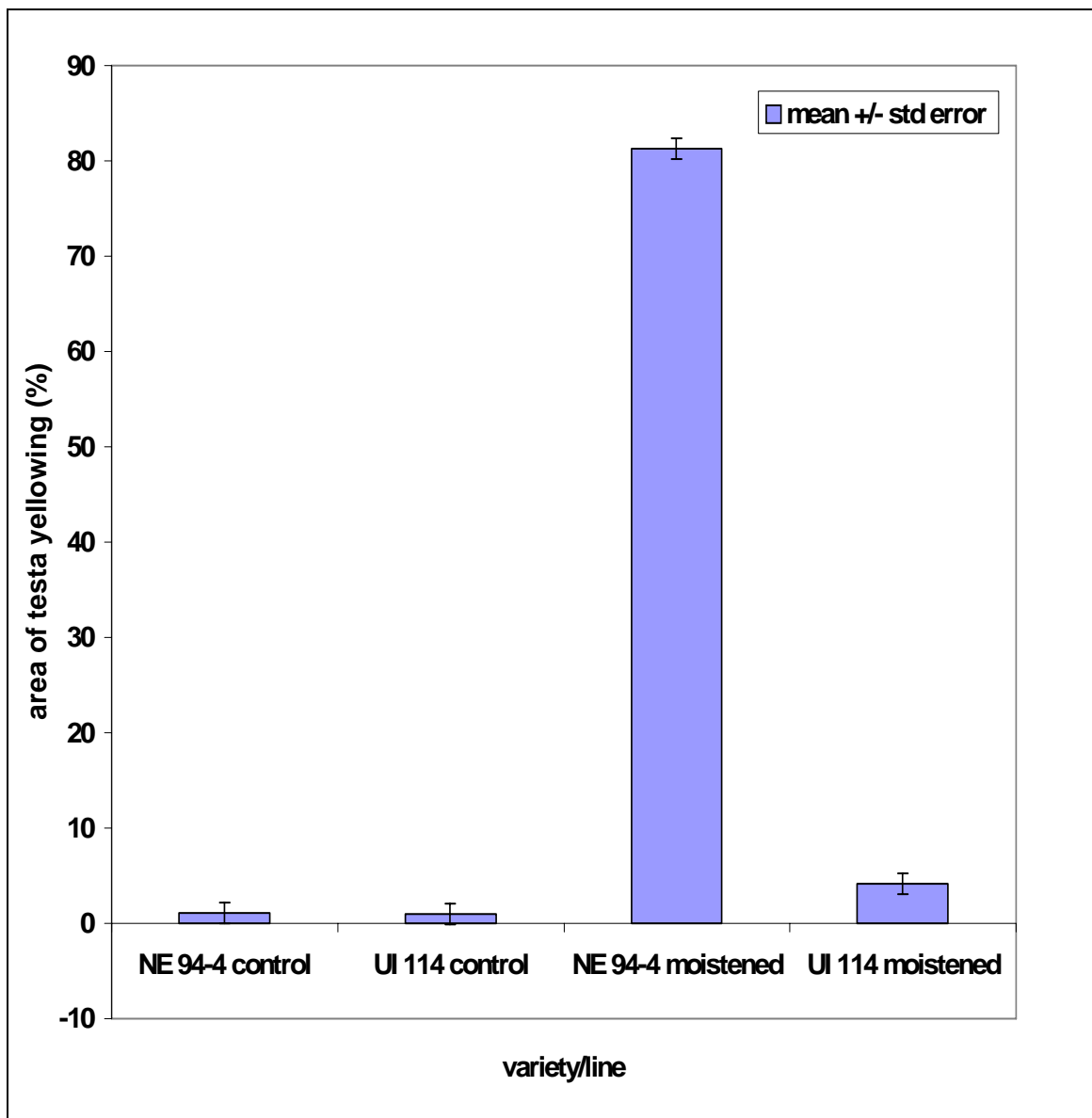
Figure 6. Pinto UI 114 and NE 94-4 from the moistened filter paper experiment.

APPENDICES

Appendix 1. The mixed procedure of SAS™, least squares means, frequency tables and analysis for the misting experiment.

```
                      The MIXED Procedure

                   Class Level Information

        Class      Levels  Values

        REP            9  1 2 3 4 5 6 7 8 9
        COL            6  1 2 3 4 5 6
        TRT            2  CONTROL MIST
        VAR            2  94-4 UI-114
        PLANT          2  1 2
        POD            4  1 2 3 4


            REML Estimation Iteration History

    Iteration  Evaluations    Objective     Criterion

         0             1  1453.3916474
         1             2  1362.1466914    0.00000000


                 Convergence criteria met.

          Covariance Parameter Estimates (REML)

          Cov Parm                  Estimate

          REP*COL*TRT              0.00000000
          PLA(REP*COL*TRT*VAR)    42.36616509
          Residual                33.61913123

           Model Fitting Information for YELLOW

          Description                      Value

          Observations                   288.0000
          Res Log Likelihood             -920.916
          Akaike's Information Criterion  -923.916
          Schwarz's Bayesian Criterion   -929.263
          -2 Res Log Likelihood          1841.833


                 Tests of Fixed Effects

       Source        NDF   DDF  Type III F  Pr > F

       REP             6     5        0.46  0.8158


                 Tests of Fixed Effects

       Source        NDF   DDF  Type III F  Pr > F

       COL             3     5        1.09  0.433
```

```
TRT              1    5      1.31  0.3043
VAR              1    52     6.70  0.0125
TRT*VAR          1    52     0.89  0.3495
POD              3    204    0.53  0.6631
TRT*POD          3    204    1.80  0.1485
VAR*POD          3    204    2.63  0.0512
TRT*VAR*POD      3    204    0.80  0.4977
```

Least Squares Means

Effect	TRT	VAR	POD	LSMEAN	Std Error	DF	t
TRT*POD	CONTROL		1	1.62557870	1.48285086	204	1.10
TRT*POD	CONTROL		2	3.76446759	1.48285086	204	2.54
TRT*POD	CONTROL		3	4.63946759	1.48285086	204	3.13
TRT*POD	CONTROL		4	2.96817130	1.48285086	204	2.00
TRT*POD	MIST		1	6.28182870	1.48285086	204	4.24
TRT*POD	MIST		2	5.30497685	1.48285086	204	3.58
TRT*POD	MIST		3	5.01331019	1.48285086	204	3.38
TRT*POD	MIST		4	4.55034722	1.48285086	204	3.07
VAR*POD		94-4	1	6.81944444	1.45282576	204	4.69
VAR*POD		94-4	2	5.22685185	1.45282576	204	3.60
VAR*POD		94-4	3	7.98611111	1.45282576	204	5.50
VAR*POD		94-4	4	5.73611111	1.45282576	204	3.95
VAR*POD		UI-114	1	1.08796296	1.45282576	204	0.75
VAR*POD		UI-114	2	3.84259259	1.45282576	204	2.64
VAR*POD		UI-114	3	1.66666667	1.45282576	204	1.15
VAR*POD		UI-114	4	1.78240741	1.45282576	204	1.23

Least Squares Means

```
Pr > |t|
  0.2743
  0.0119
  0.0020
  0.0466
  0.0001
  0.0004
  0.0009
  0.0024
  0.0001
```

Least Squares Means

```
Pr > |t|
  0.0004
  0.0001
  0.0001
  0.4548
  0.0088
  0.2526
  0.2213
```

Differences of Least Squares Means

Effect	TRT	VAR	POD	_TRT	_VAR	_POD	Difference

```
TRT*POD  CONTROL         1    CONTROL         2    -2.13888889
TRT*POD  CONTROL         1    CONTROL         3    -3.01388889
TRT*POD  CONTROL         1    CONTROL         4    -1.34259259
TRT*POD  CONTROL         1    MIST            1    -4.65625000
TRT*POD  CONTROL         1    MIST            2    -3.67939815
TRT*POD  CONTROL         1    MIST            3    -3.38773148
TRT*POD  CONTROL         1    MIST            4    -2.92476852
TRT*POD  CONTROL         2    CONTROL         3    -0.87500000
TRT*POD  CONTROL         2    CONTROL         4     0.79629630
TRT*POD  CONTROL         2    MIST            1    -2.51736111
TRT*POD  CONTROL         2    MIST            2    -1.54050926
TRT*POD  CONTROL         2    MIST            3    -1.24884259
TRT*POD  CONTROL         2    MIST            4    -0.78587963
TRT*POD  CONTROL         3    CONTROL         4     1.67129630
TRT*POD  CONTROL         3    MIST            1    -1.64236111
TRT*POD  CONTROL         3    MIST            2    -0.66550926
TRT*POD  CONTROL         3    MIST            3    -0.37384259
TRT*POD  CONTROL         3    MIST            4     0.08912037
TRT*POD  CONTROL         4    MIST            1    -3.31365741
TRT*POD  CONTROL         4    MIST            2    -2.33680556
TRT*POD  CONTROL         4    MIST            3    -2.04513889
TRT*POD  CONTROL         4    MIST            4    -1.58217593
TRT*POD  MIST            1    MIST            2     0.97685185
TRT*POD  MIST            1    MIST            3     1.26851852
TRT*POD  MIST            1    MIST            4     1.73148148
TRT*POD  MIST            2    MIST            3     0.29166667
TRT*POD  MIST            2    MIST            4     0.75462963
TRT*POD  MIST            3    MIST            4     0.46296296
VAR*POD          94-4   1          94-4     2     1.59259259
VAR*POD          94-4   1          94-4     3    -1.16666667
VAR*POD          94-4   1          94-4     4     1.08333333
VAR*POD          94-4   1          UI-114   1     5.73148148
```

Differences of Least Squares Means

```
Effect   TRT     VAR     POD  _TRT     _VAR     _POD    Difference

VAR*POD          94-4    1          UI-114   2     2.97685185
VAR*POD          94-4    1          UI-114   3     5.15277778
VAR*POD          94-4    1          UI-114   4     5.03703704
VAR*POD          94-4    2          94-4     3    -2.75925926
VAR*POD          94-4    2          94-4     4    -0.50925926
VAR*POD          94-4    2          UI-114   1     4.13888889
VAR*POD          94-4    2          UI-114   2     1.38425926
VAR*POD          94-4    2          UI-114   3     3.56018519
VAR*POD          94-4    2          UI-114   4     3.44444444
VAR*POD          94-4    3          94-4     4     2.25000000
VAR*POD          94-4    3          UI-114   1     6.89814815
VAR*POD          94-4    3          UI-114   2     4.14351852
VAR*POD          94-4    3          UI-114   3     6.31944444
VAR*POD          94-4    3          UI-114   4     6.20370370
VAR*POD          94-4    4          UI-114   1     4.64814815
VAR*POD          94-4    4          UI-114   2     1.89351852
VAR*POD          94-4    4          UI-114   3     4.06944444
```

```
VAR*POD          94-4    4              UI-114   4       3.95370370
VAR*POD          UI-114  1              UI-114   2      -2.75462963
VAR*POD          UI-114  1              UI-114   3      -0.57870370
VAR*POD          UI-114  1              UI-114   4      -0.69444444
VAR*POD          UI-114  2              UI-114   3       2.17592593
VAR*POD          UI-114  2              UI-114   4       2.06018519
VAR*POD          UI-114  3              UI-114   4      -0.11574074
```

           Differences of Least Squares Means

```
           Std Error    DF        t  Pr > |t|

           1.36664901   204    -1.57    0.1191
           1.36664901   204    -2.21    0.0285
           1.36664901   204    -0.98    0.3271
           2.13868683   204    -2.18    0.0306
           2.13868683   204    -1.72    0.0869
           2.13868683   204    -1.58    0.1147
           2.13868683   204    -1.37    0.1730
           1.36664901   204    -0.64    0.5227
           1.36664901   204     0.58    0.5608
           2.13868683   204    -1.18    0.2405
           2.13868683   204    -0.72    0.4722
           2.13868683   204    -0.58    0.5599
           2.13868683   204    -0.37    0.7137
           1.36664901   204     1.22    0.2228
           2.13868683   204    -0.77    0.4434
```

           Differences of Least Squares Means

```
           Std Error    DF        t  Pr > |t|

           2.13868683   204    -0.31    0.7560
           2.13868683   204    -0.17    0.8614
           2.13868683   204     0.04    0.9668
           2.13868683   204    -1.55    0.1228
           2.13868683   204    -1.09    0.2758
           2.13868683   204    -0.96    0.3401
           2.13868683   204    -0.74    0.4603
           1.36664901   204     0.71    0.4756
           1.36664901   204     0.93    0.3544
           1.36664901   204     1.27    0.2066
           1.36664901   204     0.21    0.8312
           1.36664901   204     0.55    0.5814
           1.36664901   204     0.34    0.7351
           1.36664901   204     1.17    0.2452
           1.36664901   204    -0.85    0.3943
           1.36664901   204     0.79    0.4289
           2.05460589   204     2.79    0.0058
           2.05460589   204     1.45    0.1489
           2.05460589   204     2.51    0.0129
           2.05460589   204     2.45    0.0151
           1.36664901   204    -2.02    0.0448
```

```
                    1.36664901    204    -0.37    0.7098
                    2.05460589    204     2.01    0.0453
                    2.05460589    204     0.67    0.5012
                    2.05460589    204     1.73    0.0846
                    2.05460589    204     1.68    0.0952
                    1.36664901    204     1.65    0.1012
                    2.05460589    204     3.36    0.0009
                    2.05460589    204     2.02    0.0450
                    2.05460589    204     3.08    0.0024
                    2.05460589    204     3.02    0.0029
                    2.05460589    204     2.26    0.0247
                    2.05460589    204     0.92    0.3578
                    2.05460589    204     1.98    0.0490
                    2.05460589    204     1.92    0.0557
                    1.36664901    204    -2.02    0.0452
                    1.36664901    204    -0.42    0.6724
                    1.36664901    204    -0.51    0.6119
                    1.36664901    204     1.59    0.1129
                    1.36664901    204     1.51    0.1332
                    1.36664901    204    -0.08    0.9326
```

```
---------------------------- TRT=CONTROL ------------------------------


Frequency

   60  ┤                         **
                                 **
                                 **
                                 **
                                 **
                                 **
   50  ┤                         **
                                 **
                                 **
                                 **
                                 **
   40  ┤                         **
                                 **
                                 **
         **                      **
         **                      **
   30  ┤ **                      **
         **                      **
         **                      **
         **                      **
         ** **                   **
   20  ┤ ** **                   **
         ** **                   **
         ** **                   **
         ** **                   **
         ** ** **                **
   10  ┤ ** ** **                **
         ** ** **                ** **
         ** ** **                ** **
         ** ** ** **             ** **         **
         ** ** ** ** **      **  ** ** ** ** ** **
        └──────────────────────────────────────────────
```
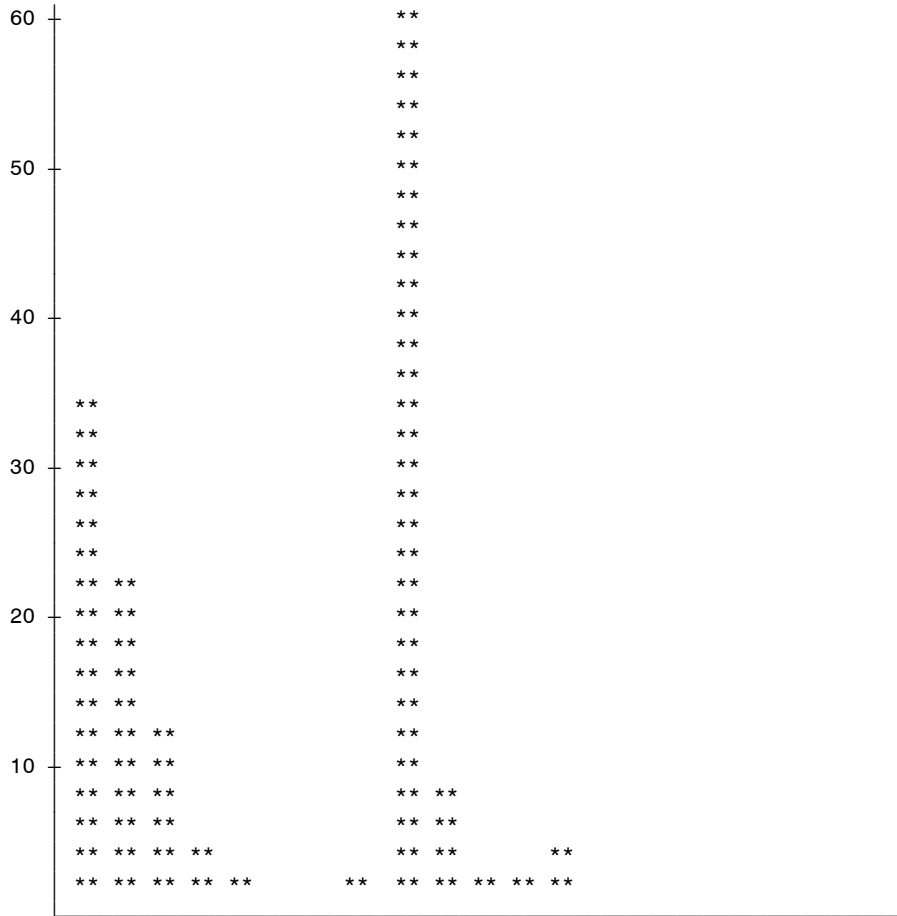
```
        0  5 10 15 20 25 30 35   0  5 10 15 20 25 30 35  YELLOW Midpoint

        |——————— 94-4 ———————|  |——————— UI-114 ———————|  VAR



------------------------------ TRT=MIST --------------------------------

Frequency

      |                         **
      |                         **
      |                         **
   60 _ **                      **
      |  **                      **
      |  **                      **
      |  **                      **
      |  **                      **
   50 _ **                      **
      |  **                      **
      |  **                      **
      |  **                      **
      |  **                      **
   40 _ **                      **
      |  **                      **
      |  **                      **
      |  **                      **
      |  **                      **
   30 _ **                      **
      |  **                      **
      |  **                      **
      |  **                      **
      |  **                      **
   20 _ **                      **
      |  **                      **
      |  **                      **
      |  **                      **
      |  **                      **
   10 _ ** **                   **
      |  ** **                   ** **
      |  ** **                   ** **
      |  ** **                   ** **
      |  ** ** ** **     **    ** ** **
      |_____

        6 18 30 42 54 66 78 90   6 18 30 42 54 66 78 90  YELLOW Midpoint

        |——————— 94-4 ———————|  |——————— UI-114 ———————|  VAR




                       Analysis Variable : YELLOW


------------------------- TRT=CONTROL VAR=94-4 -------------------------


              N          Mean        Std Dev       Variance
```
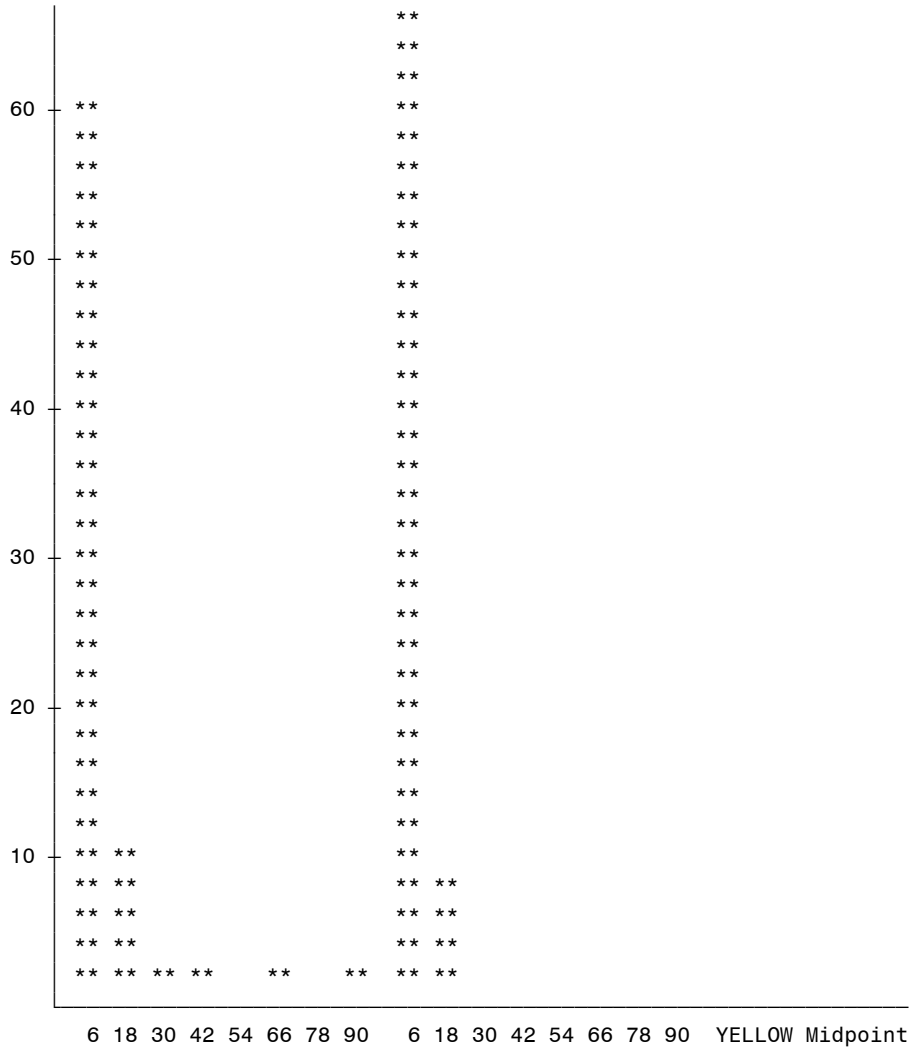
```
                ----------------------------------------------
                72     4.4629630    6.0546689    36.6590158
                ----------------------------------------------
```

```
----------------------- TRT=CONTROL VAR=UI-114 ----------------------
```

```
            N         Mean      Std Dev      Variance
            ----------------------------------------------
            72     1.7013889    4.6438862    21.5656788
            ----------------------------------------------
```

```
------------------------- TRT=MIST VAR=94-4 -------------------------
```

```
            N         Mean      Std Dev      Variance
            ----------------------------------------------
            72     8.4212963   14.4517700   208.8536559
            ----------------------------------------------
```

```
------------------------- TRT=MIST VAR=UI-114 -----------------------
```

```
            N         Mean      Std Dev      Variance
            ----------------------------------------------
            72     2.4884259    4.7295666    22.3688000
            ----------------------------------------------
```

Appendix 2. The mixed procedure of SAS™, least squares means, frequency tables and analysis for the injection experiment.

```
                        The MIXED Procedure

                     Class Level Information

       Class     Levels  Values

       REP           12  1 10 11 12 2 3 4 5 6 7 8 9
       TRT            3  CONTROL INONLY INWATER
       VAR            2  94-4 UI-114
       POD            3  1 2 3


                REML Estimation Iteration History

    Iteration  Evaluations     Objective      Criterion

           0             1  848.14738265
           1             1  841.34022594    0.00000000

                   Convergence criteria met.


           Covariance Parameter Estimates (REML)

           Cov Parm          Estimate

           REP*TRT*VAR      4.84682941
           Residual        18.81859100


            Model Fitting Information for YELLOW

           Description                       Value

           Observations                   216.0000
           Res Log Likelihood             -592.512
           Akaike's Information Criterion  -594.512
           Schwarz's Bayesian Criterion   -597.743
           -2 Res Log Likelihood          1185.023


                    Tests of Fixed Effects

           Source       NDF   DDF  Type III F  Pr > F

           REP           11    55        1.91  0.0582
           TRT            2    55        4.01  0.0238
```

```
VAR              1    55          5.40  0.0239
TRT*VAR          2    55          0.44  0.6440
POD              2   132          0.52  0.5955
TRT*POD          4   132          1.01  0.4057
```

Tests of Fixed Effects

```
Source          NDF   DDF  Type III F  Pr > F

VAR*POD           2   132         0.31  0.7376
TRT*VAR*POD       4   132         0.62  0.6521
```

Least Squares Means

Effect	TRT	VAR	POD	LSMEAN	Std Error	DF	t
TRT*POD	CONTROL		1	1.61111111	0.99300513	132	1.62
TRT*POD	CONTROL		2	2.66666667	0.99300513	132	2.69
TRT*POD	CONTROL		3	1.49305556	0.99300513	132	1.50
TRT*POD	INONLY		1	1.43055556	0.99300513	132	1.44
TRT*POD	INONLY		2	1.05555556	0.99300513	132	1.06
TRT*POD	INONLY		3	0.20833333	0.99300513	132	0.21
TRT*POD	INWATER		1	4.59027778	0.99300513	132	4.62
TRT*POD	INWATER		2	2.43750000	0.99300513	132	2.45
TRT*POD	INWATER		3	3.76388889	0.99300513	132	3.79
VAR*POD		94-4	1	3.20833333	0.81078529	132	3.96
VAR*POD		94-4	2	2.90740741	0.81078529	132	3.59
VAR*POD		94-4	3	3.04166667	0.81078529	132	3.75
VAR*POD		UI-114	1	1.87962963	0.81078529	132	2.32
VAR*POD		UI-114	2	1.19907407	0.81078529	132	1.48
VAR*POD		UI-114	3	0.60185185	0.81078529	132	0.74

Least Squares Means

Pr > |t|

```
0.1071
0.0082
0.1351
0.1521
0.2897
0.8341
0.0001
0.0154
0.0002
0.0001
0.0005
0.0003
0.0220
0.1415
0.4592
```

```
                      Differences of Least Squares Means

Effect    TRT       VAR    POD   _TRT      _VAR     _POD    Difference

TRT*POD   CONTROL          1     CONTROL            2      -1.05555556
TRT*POD   CONTROL          1     CONTROL            3       0.11805556
TRT*POD   CONTROL          1     INONLY             1       0.18055556
TRT*POD   CONTROL          1     INONLY             2       0.55555556
TRT*POD   CONTROL          1     INONLY             3       1.40277778
TRT*POD   CONTROL          1     INWATER            1      -2.97916667
TRT*POD   CONTROL          1     INWATER            2      -0.82638889
TRT*POD   CONTROL          1     INWATER            3      -2.15277778
TRT*POD   CONTROL          2     CONTROL            3       1.17361111
TRT*POD   CONTROL          2     INONLY             1       1.23611111
TRT*POD   CONTROL          2     INONLY             2       1.61111111
TRT*POD   CONTROL          2     INONLY             3       2.45833333
TRT*POD   CONTROL          2     INWATER            1      -1.92361111
TRT*POD   CONTROL          2     INWATER            2       0.22916667
TRT*POD   CONTROL          2     INWATER            3      -1.09722222
TRT*POD   CONTROL          3     INONLY             1       0.06250000
TRT*POD   CONTROL          3     INONLY             2       0.43750000
TRT*POD   CONTROL          3     INONLY             3       1.28472222
TRT*POD   CONTROL          3     INWATER            1      -3.09722222
TRT*POD   CONTROL          3     INWATER            2      -0.94444444
TRT*POD   CONTROL          3     INWATER            3      -2.27083333
TRT*POD   INONLY           1     INONLY             2       0.37500000
TRT*POD   INONLY           1     INONLY             3       1.22222222
TRT*POD   INONLY           1     INWATER            1      -3.15972222
TRT*POD   INONLY           1     INWATER            2      -1.00694444
TRT*POD   INONLY           1     INWATER            3      -2.33333333
TRT*POD   INONLY           2     INONLY             3       0.84722222
TRT*POD   INONLY           2     INWATER            1      -3.53472222
TRT*POD   INONLY           2     INWATER            2      -1.38194444
TRT*POD   INONLY           2     INWATER            3      -2.70833333
TRT*POD   INONLY           3     INWATER            1      -4.38194444
TRT*POD   INONLY           3     INWATER            2      -2.22916667
TRT*POD   INONLY           3     INWATER            3      -3.55555556
TRT*POD   INWATER          1     INWATER            2       2.15277778
TRT*POD   INWATER          1     INWATER            3       0.82638889
TRT*POD   INWATER          2     INWATER            3      -1.32638889
VAR*POD             94-4   1               94-4      2       0.30092593
VAR*POD             94-4   1               94-4      3       0.16666667
VAR*POD             94-4   1               UI-114    1       1.32870370
VAR*POD             94-4   1               UI-114    2       2.00925926
VAR*POD             94-4   1               UI-114    3       2.60648148
VAR*POD             94-4   2               94-4      3      -0.13425926
VAR*POD             94-4   2               UI-114    1       1.02777778
VAR*POD             94-4   2               UI-114    2       1.70833333
VAR*POD             94-4   2               UI-114    3       2.30555556
VAR*POD             94-4   3               UI-114    1       1.16203704
VAR*POD             94-4   3               UI-114    2       1.84259259
```

Differences of Least Squares Means

Effect	TRT	VAR	POD	_TRT	_VAR	_POD	Difference
VAR*POD		94-4	3		UI-114	3	2.43981481
VAR*POD		UI-114	1		UI-114	2	0.68055556
VAR*POD		UI-114	1		UI-114	3	1.27777778
VAR*POD		UI-114	2		UI-114	3	0.59722222

Differences of Least Squares Means

Std Error	DF	t	Pr > |t|
1.25228428	132	-0.84	0.4008
1.25228428	132	0.09	0.9250
1.40432132	132	0.13	0.8979
1.40432132	132	0.40	0.6930
1.40432132	132	1.00	0.3197
1.40432132	132	-2.12	0.0358
1.40432132	132	-0.59	0.5572
1.40432132	132	-1.53	0.1277
1.25228428	132	0.94	0.3504
1.40432132	132	0.88	0.3803
1.40432132	132	1.15	0.2534
1.40432132	132	1.75	0.0823
1.40432132	132	-1.37	0.1731
1.40432132	132	0.16	0.8706
1.40432132	132	-0.78	0.4360
1.40432132	132	0.04	0.9646
1.40432132	132	0.31	0.7559
1.40432132	132	0.91	0.3619
1.40432132	132	-2.21	0.0292
1.40432132	132	-0.67	0.5024
1.40432132	132	-1.62	0.1083
1.25228428	132	0.30	0.7651
1.25228428	132	0.98	0.3309
1.40432132	132	-2.25	0.0261
1.40432132	132	-0.72	0.4746
1.40432132	132	-1.66	0.0990
1.25228428	132	0.68	0.4999
1.40432132	132	-2.52	0.0130
1.40432132	132	-0.98	0.3269
1.40432132	132	-1.93	0.0559
1.40432132	132	-3.12	0.0022
1.40432132	132	-1.59	0.1148
1.40432132	132	-2.53	0.0125
1.25228428	132	1.72	0.0879
1.25228428	132	0.66	0.5105
1.25228428	132	-1.06	0.2915
1.02248583	132	0.29	0.7690

Differences of Least Squares Means

Std Error	DF	t	Pr > |t|

```
                    1.02248583     132     0.16     0.8708
                    1.14662356     132     1.16     0.2486
                    1.14662356     132     1.75     0.0820
                    1.14662356     132     2.27     0.0246
                    1.02248583     132    -0.13     0.8957
                    1.14662356     132     0.90     0.3717
                    1.14662356     132     1.49     0.1386
                    1.14662356     132     2.01     0.0464
                    1.14662356     132     1.01     0.3127
                    1.14662356     132     1.61     0.1104
                    1.14662356     132     2.13     0.0352
                    1.02248583     132     0.67     0.5068
                    1.02248583     132     1.25     0.2136
                    1.02248583     132     0.58     0.5602
```

```
---------------------------- TRT=CONTROL ------------------------------

   Frequency

                                    **
                                    **
                                    **
                                    **
     30 -                          **
                                    **
         **                        **
         **                        **
         **                        **
     25 - **                        **
         **                        **
         **                        **
         **                        **
         **                        **
     20 - **                        **
         **                        **
         **                        **
         **                        **
         **                        **
     15 - **                        **
         **                        **
         **                        **
         **                        **
         **                        **
     10 - **                        **
         **                        **
         **                        **
         **                        **
         **                        **
      5 - **                        **
         ** **                      **
         ** **                      **
         ** ** **                   **
         ** ** **      **      **   ** ** **
         _____

           1  1  2  2  3          1  1  2  2  3   YELLOW Midpoint
```
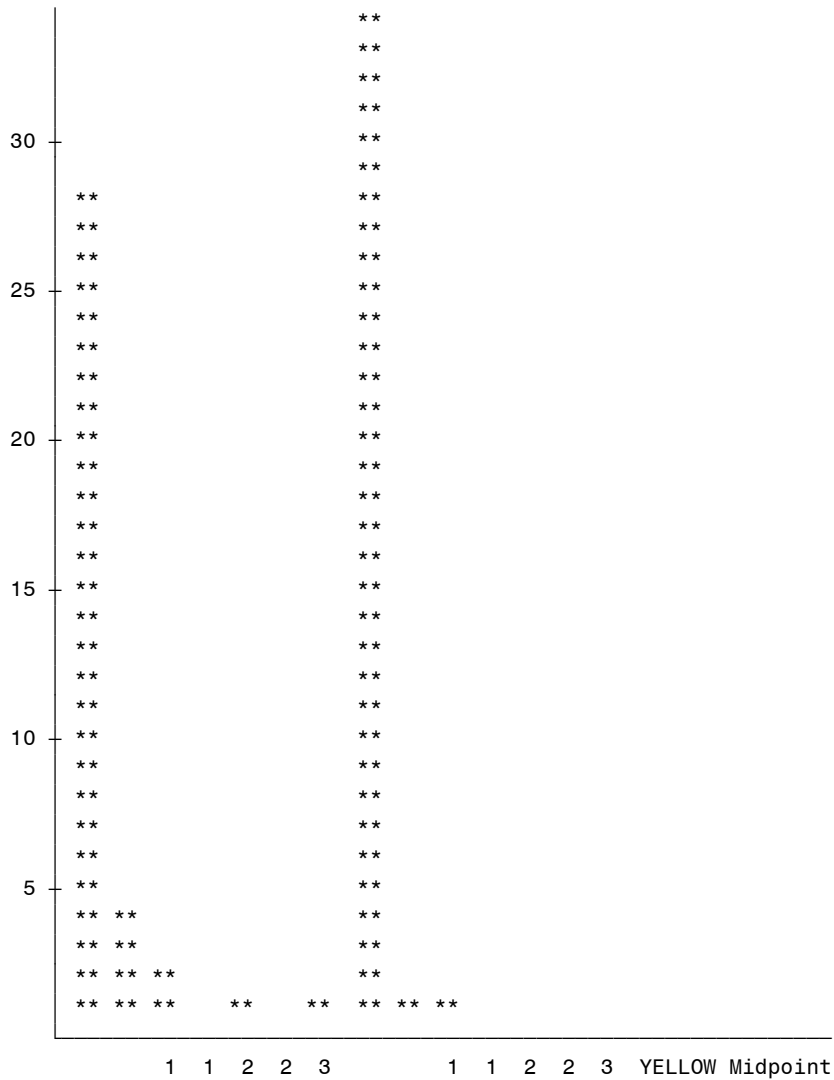
```
--------------------------- TRT=INWATER -----------------------------

   Frequency

                                        **
                                        **
      25 -                               **
                                        **
                                        **
                                        **
                                        **
      20 -                               **
                                        **
                                        **
         **                             **
         **                             **
      15 - **                             **
         **                             **
         **                             **
         **                             **
         **                             **
      10 - ** **                          **
         ** **                          **
         ** **                          **
         ** **                          **
         ** **                          **
       5 - ** **                          ** **
         ** **        **                 ** **
         ** ** ** **                    ** ** **
         ** ** ** **                    ** ** **
         ** ** ** ** **      **          ** ** **              **

          0  5 10 15 20 25 30 35    0  5 10 15 20 25 30 35  YELLOW Midpoint

         |———— 94-4 ————|   |———— UI-114 ————|  VAR
```
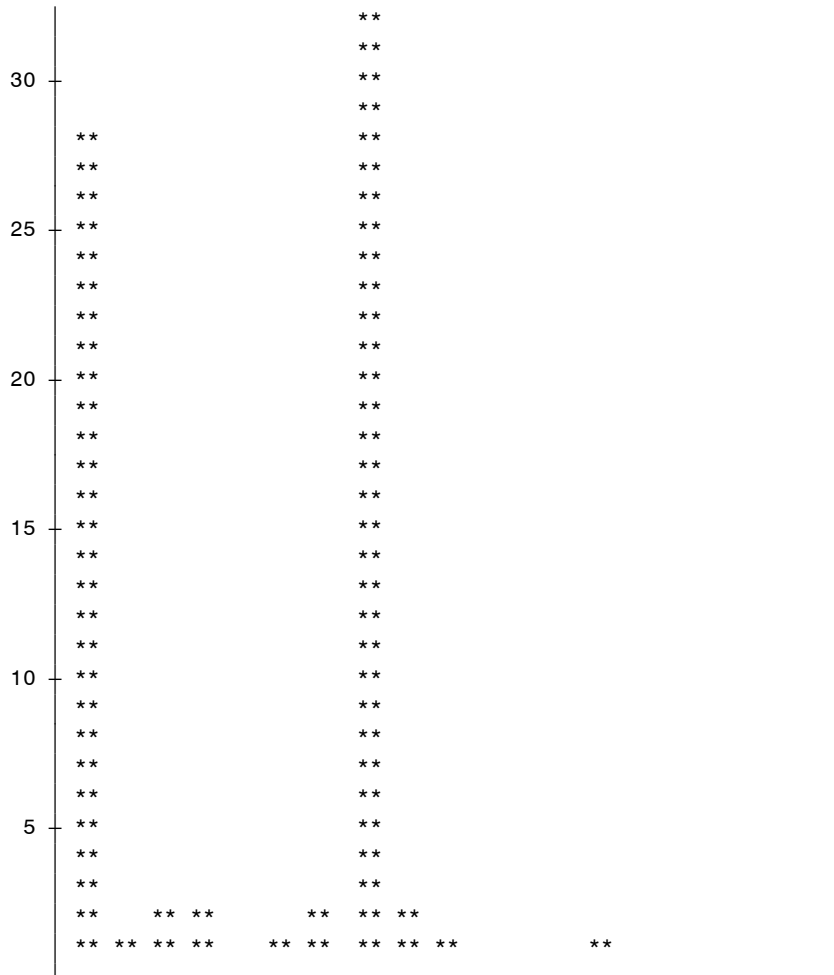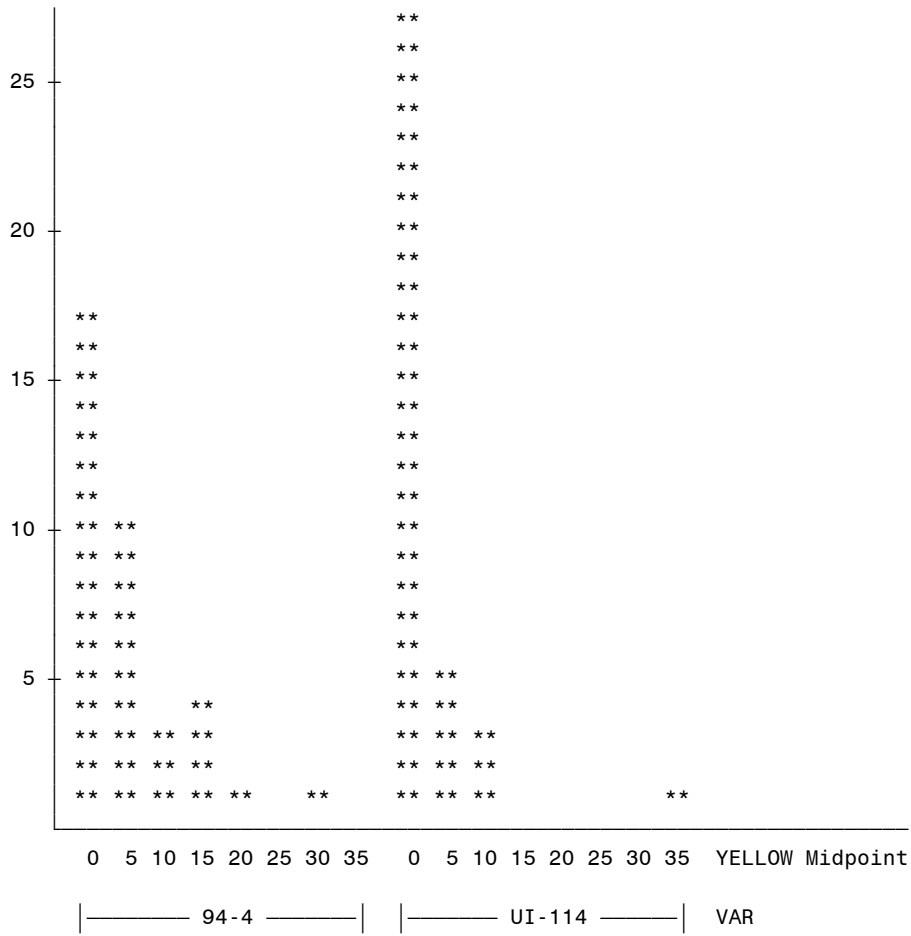
Analysis Variable : YELLOW

```
------------------------ VAR=94-4 TRT=CONTROL ------------------------


      N          Mean        Std Dev       Variance
      -------------------------------------------------
      36       2.9398148     6.6323106     43.9875441
      -------------------------------------------------

------------------------ VAR=94-4 TRT=INONLY ------------------------


      N          Mean        Std Dev       Variance
      -------------------------------------------------
      36       1.3148148     2.8640569      8.2028219
```

```
                    -------------------------------------------

----------------------- VAR=94-4 TRT=INWATER -----------------------


          N         Mean        Std Dev       Variance
        -------------------------------------------
         36      4.9027778     6.8136622    46.4259921
        -------------------------------------------


----------------------- VAR=UI-114 TRT=CONTROL -----------------------


          N         Mean        Std Dev       Variance
        -------------------------------------------
         36      0.9074074     2.6251060     6.8911817
        -------------------------------------------


----------------------- VAR=UI-114 TRT=INONLY -----------------------


          N         Mean        Std Dev       Variance
        -------------------------------------------
         36      0.4814815     1.7789679     3.1647266
        -------------------------------------------


----------------------- VAR=UI-114 TRT=INWATER -----------------------


          N         Mean        Std Dev       Variance
        -------------------------------------------
         36      2.2916667     6.2530469    39.1005952
        -------------------------------------------
```

Appendix 3. The mixed procedure of SAS™, least squares means, frequency tables and analysis for the moistened filter paper experiment.

```
                  The MIXED Procedure

             Class Level Information

     Class     Levels  Values

     REP          4  1 2 3 4
     VAR          2  94-4 UI-114
     TRT          2  CONTROL MOISTED


   Covariance Parameter Estimates (REML)

   Cov Parm       Estimate

   Residual    47.09265176


    Model Fitting Information for YELLOW

  Description                      Value

  Observations                   320.0000
  Res Log Likelihood             -1061.63
```

```
              Akaike's Information Criterion   -1062.63
              Schwarz's Bayesian Criterion     -1064.50
              -2 Res Log Likelihood            2123.256
```

```
                        Tests of Fixed Effects


              Source      NDF   DDF   Type III F   Pr > F


              REP          3    313        2.78    0.0413
              VAR          1    313     2534.39    0.0001
              TRT          1    313     2943.37    0.0001
              VAR*TRT      1    313     2518.01    0.0001
```

```
                        Least Squares Means


   Effect    VAR     TRT         LSMEAN      Std Error     DF        t


   VAR       94-4            41.18750000   0.54252104    313     75.92
   VAR       UI-114           2.56250000   0.54252104    313      4.72
   TRT               CONTROL   1.06250000   0.54252104    313      1.96
   TRT               MOISTED  42.68750000   0.54252104    313     78.68
   VAR*TRT   94-4   CONTROL    1.12500000   0.76724061    313      1.47
   VAR*TRT   94-4   MOISTED   81.25000000   0.76724061    313    105.90
   VAR*TRT   UI-114 CONTROL    1.00000000   0.76724061    313      1.30
   VAR*TRT   UI-114 MOISTED    4.12500000   0.76724061    313      5.38
```

```
                     Least Squares Means


                        Pr > |t|


                         0.0001
                         0.0001
                         0.0511
                         0.0001
                         0.1436
                         0.0001
                         0.1934
                         0.0001
```

```
                  Differences of Least Squares Means


   Effect    VAR     TRT      _VAR    _TRT      Difference     Std Error


   VAR       94-4            UI-114           38.62500000    0.76724061
   TRT               CONTROL         MOISTED  -41.62500000    0.76724061
   VAR*TRT   94-4   CONTROL  94-4    MOISTED  -80.12500000    1.08504207
   VAR*TRT   94-4   CONTROL  UI-114  CONTROL    0.12500000    1.08504207
   VAR*TRT   94-4   CONTROL  UI-114  MOISTED   -3.00000000    1.08504207
   VAR*TRT   94-4   MOISTED  UI-114  CONTROL   80.25000000    1.08504207
   VAR*TRT   94-4   MOISTED  UI-114  MOISTED   77.12500000    1.08504207
   VAR*TRT   UI-114 CONTROL  UI-114  MOISTED   -3.12500000    1.08504207
```

Differences of Least Squares Means

DF	t	Pr > |t|
313	50.34	0.0001
313	-54.25	0.0001
313	-73.85	0.0001
313	0.12	0.9084
313	-2.76	0.0060
313	73.96	0.0001
313	71.08	0.0001
313	-2.88	0.0043

---------------------------- TRT=CONTROL -----------------------------

Frequency

```
                                     *
        *                            *
70 +    *                            *
        *                            *
        *                            *
        *                            *
        *                            *
60 +    *                            *
        *                            *
        *                            *
        *                            *
        *                            *
50 +    *                            *
        *                            *
        *                            *
        *                            *
        *                            *
40 +    *                            *
        *                            *
        *                            *
        *                            *
        *                            *
30 +    *                            *
        *                            *
        *                            *
        *                            *
        *                            *
20 +    *                            *
        *                            *
        *                            *
        *                            *
        *                            *
10 +    *                            *
        *          *                 *
        *          *                 *
        *          *                 *          *
        *          *        *   *         *           *
```
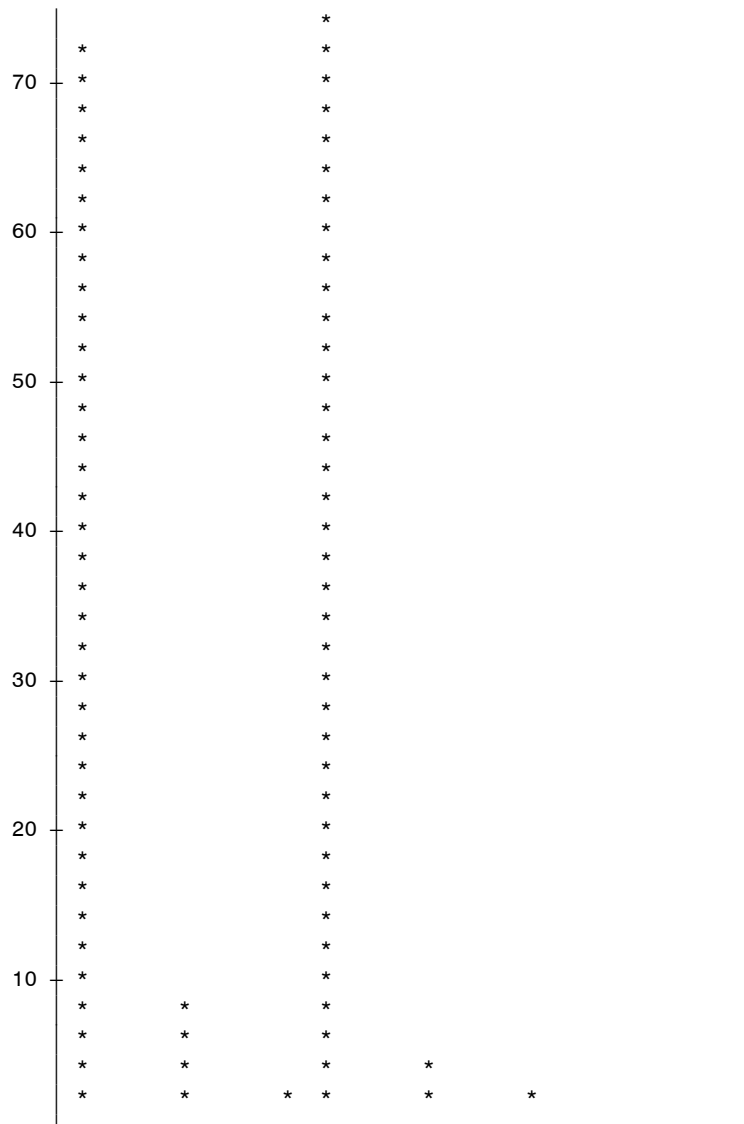
```
                1 1 1 1 2         1 1 1 1 2   YELLOW Midpoint
        0 2 5 7 0 2 5 7 0  0 2 5 7 0 2 5 7 0
        . . . . . . . . .  . . . . . . . . .
        0 5 0 5 0 5 0 5 0  0 5 0 5 0 5 0 5 0

        |—— 94-4 ——|   |—— UI-114 ——|  VAR
```

---------------------------- TRT=MOISTED ------------------------------

```
    Frequency

                            *
                            *
                            *
                            *
        50 ┼                *
                            *
                            *
                            *
                            *
                            *
        40 ┼                *
                            *
                        *   *
                        *   *
                        *   *
        30 ┼            *   *
                        * * *
                        * * *
                        * * *
                        * * *
        20 ┼            * * *
                        * * *
                    * * *   * *
                    * * *   * *
                    * * *   * *
        10 ┼        * * *   * *
                    * * *   * *
                    * * *   * * *
                    * * *   * * *
            *       * * *   * * * *
           └────────────────────────────────
            1 2 3 4 6 7 8 9  1 2 3 4 6 7 8 9  YELLOW Midpoint
            0 2 4 6 8 0 2 4 6  0 2 4 6 8 0 2 4 6

            |—— 94-4 ——|   |—— UI-114 ——|  VAR
```
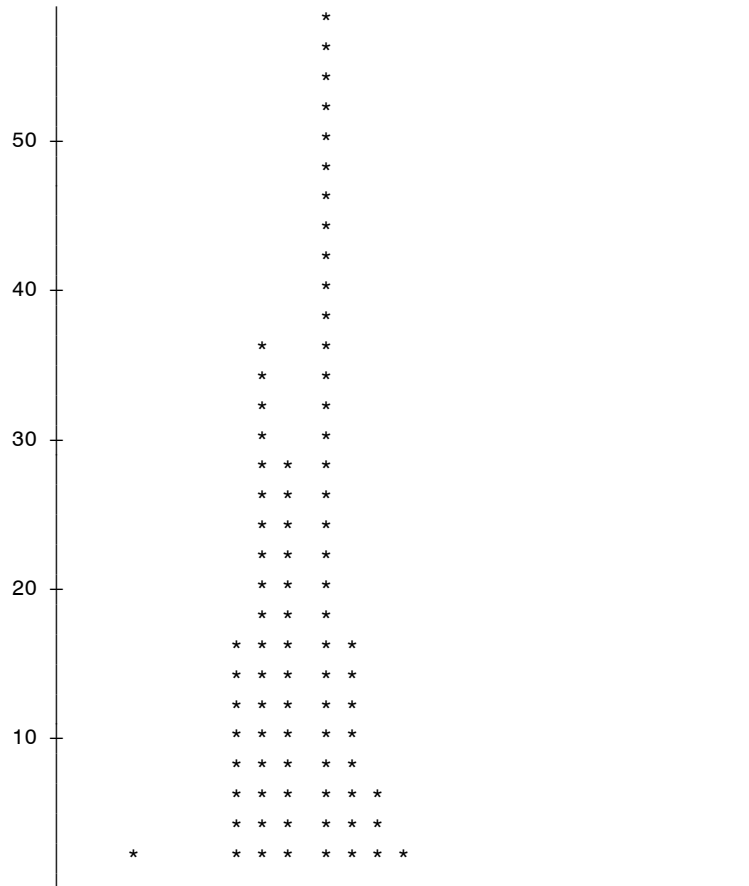
Analysis Variable : YELLOW

------------------------- VAR=94-4 TRT=CONTROL -------------------------

```
        N        Mean       Std Dev      Variance
       ---------------------------------------------
       80      1.1250000    3.5556160    12.6424051
       ---------------------------------------------
```

```
------------------------- VAR=94-4 TRT=MOISTED -----------------------


          N         Mean       Std Dev      Variance
         ---------------------------------------------
          80    81.2500000    10.4790327   109.8101266
         ---------------------------------------------


----------------------- VAR=UI-114 TRT=CONTROL ----------------------


          N         Mean       Std Dev      Variance
         ---------------------------------------------
          80     1.0000000     3.7652643    14.1772152
         ---------------------------------------------


----------------------- VAR=UI-114 TRT=MOISTED ----------------------


          N         Mean       Std Dev      Variance
         ---------------------------------------------
          80     4.1250000     7.4108627    54.9208861
         ---------------------------------------------
```