CONTROLLED CROSS-POLLINATIONS WITH AMERICAN BEECH TREES THAT ARE RESISTANT TO BEECH BARK DISEASE

Jennifer L. Koch and David W. Carey[†]

ABSTRACT.—American beech tree pollen with viability ranging from 30 to 50 percent was used to perform controlled crosses between resistant parents, a resistant and susceptible parent, and a resistant and intermediate parent. The germinative capacity of the seeds collected from the controlled crosses varied from 12 to 84 percent, possibly indicating mating incompatibilities. The percentage of barren seed ranged from 61 to 89 percent. Open-pollinated seeds were collected from both resistant and susceptible trees, including some of the parental trees used in the cross-pollinations. The germinative capacity and percentage of barren seed were comparable to that found in the cross-pollinated seeds, indicating these numbers were not a result of the pollination bagging process interfering with seed development. Self-pollinated controls indicated a high level of self-sterility. The full- and half-sib families generated will be challenged with scale insect eggs to determine their resistance phenotype and gain insight into the mechanism(s) of inheritance of resistance.

Beech bark disease is initiated when the bark of beech trees is colonized by the beech scale insect, *Cryptococcus fagisuga*, predisposing the tree to subsequent invasion by fungi of the genus *Nectria*. An estimated 1-2 % of beech trees remain disease free in stands long-affected by beech bark disease and challenge trials have shown that they are resistant to the scale insect. Increasing the number of resistant beech trees while reducing the proportion of susceptible trees is currently thought to be the best management approach to minimize the overall impact of beech bark disease (Mielke et al., 1986). However, very little is known about the mechanism(s) of resistance and how resistance is inherited. Studies in European beech (*Fagus sylvatica*) concluded that some beech trees have a genetic predisposition to infestation by the beech scale (Gora et al., 1994; Krabel & Petercord 2000) and a correlation was established between scale infestation and the genotype of the host tree based on isozyme analysis (Krabel & Petercord 2000). No such correlation was found in American beech (*Fagus grandifolia* Ehrh. Houston & Houston, 1994; Houston & Houston, 2000). The inability to correlate resistance with a specific isozyme pattern indicates that a more sensitive marker system such as one that is DNA-based may be required to detect linkage. Furthermore, to gain meaningful insight into the genetic mechanism(s) of resistance, the inheritance of the trait must be studied within a family. To address this, we have initiated a research program to determine the requirements for performing controlled-cross pollinations and to generate both full- and half-sib families in an effort to identify how resistance may be inherited. Such knowledge is a prerequisite to establishing a breeding program and developing seed orchards in order to supply a source of resistant American beech for restorative use and for use in pre-emptive plantings as a way to minimize the impacts of beech bark disease.

Study Area

All of the parent trees used in the cross-pollinations were located in Ludington State Park, Ludington, MI. Ludington State Park provided the ideal setting for this work for several reasons. Of critical importance was the fact that it is the location of a "killing front" of the disease-both heavy scale infestation and *Nectria* are present. Because of the intense disease-pressure at this site, mature beech trees that remain scale-free have a high probability of being truly resistant and not merely "escapes". Even so, trees chosen as putatively resistant parents are currently being tested through insect challenge experiments to confirm their resistance. The estimated minimum age for seed production in American beech is 40 years, and a beech tree of that age can reach heights between 70 and 120 feet (Rudolf &

[†]Research Biologist (JLK) and Biological Technician (DWC), Northeastern Research Station, 359 Main Road, Delaware, OH 43015. JLK is corresponding author: to contact, call (740) 368-0188 or email at jkoch@fs.fed.us.

Leak, 1989). Based on our observations, flowers are most prevalent in areas of the canopy which are in direct contact with sunlight, generally toward the top of the tree. The trees chosen for use as parents in this study had to not only be free of scale and canker in the case of resistant parents, but they also had to be near a road. The paved roadways leading into and out of the campground areas in Ludington State Park provided a way to maneuver a bucket truck close enough to the parent trees to allow access to the flowers. Other areas included in this study were a 150 acre stand in Sebois County, Maine and Delaware State Park, Ohio. The stand in Maine is considered an aftermath forest, and has been heavily affected by beech bark disease. This stand was of particular interest for the collection of seed for half-sib families because all of the susceptible trees have been removed (D. Struble, personal communication) and there is subsequently a high probability of also having a resistant pollen donor. The trees in Delaware State Park were used in self-fertilization experiments and were chosen simply based on proximity to our research facility.

Methods

Pollen collection

Dormant branches, harvested on May 13, 2002 were approximately 3-4 ft. in length with a diameter of ½". Once a branch was excised from the tree it was placed in a bottle of tap water and parafilm was wrapped around the opening to prevent loss of water during transport. Upon returning to the laboratory the bottom ½ inch of each branch was removed, and the branches were placed in four-liter flasks containing 0.25 X MS media (Murashige & Skoog, 1962). To keep the harvested dormant branches alive long enough in the greenhouse for flower emergence to occur, it was critical to minimize the amount of bacterial and fungal growth in the media. Such contamination results in blockage of the phloem, preventing water and nutrients from being taken up by the cut branch. For this reason, 25 mg/L of rifampicin and 10 mg/L of nystatin were added to the liquid media. Media was changed every 2-3 days, and at each change the bottom portion (¼" to ½") of the branch was cut. The flasks were kept on benches in the greenhouse under ambient light with daytime temperatures of 18° C and nighttime temperatures dropping to 13° C. Once bud break occurred, a 0.75 % solution of sucrose was added to the media. After five days pistillate flowers began to emerge and by nine days staminate flowers were clearly visible. Mature, dehiscing anthers were clipped and ground over a 53 µm nylon sieve to separate the pollen from the flower debris. Sieved pollen was transferred to glass vials sealed with a rubber septa and placed in 50 ml polypropylene tubes along with a packet of silica gel and stored at 4° C (P. Sisco, personal communication).

Pollen Viability

Once pollen was harvested, viability was confirmed by looking at pollen tube formation on artificial media. The pollen germination media contained 1X aspen culture media (Ahua, 1984) and 15 % sucrose, pH 5.6. The media was passed through a 0.45 µM filter prior to adding 20 g/L gelatin. The gelatin was dissolved by incubating the solution in a 55° C water bath. The media was cooled to 40° C and 40 mg/L nystatin and 25 mg/L rifampicin were added. The media was poured into 65 mm x 10 mm petri dishes and stored at 4° C. To test the pollen, a paintbrush was used to pick up pollen grains and lightly tapped to dust pollen onto the gelatinous germination plates. The plates were kept at room temperature in the dark. Pollen grains that had developed germination tubes were counted after a minimum 48 hr. incubation period using a dissecting microscope. To calculate the percent viability, the number of pollen grains that had a visible germination tube out of a minimum of 100 pollen grains observed was determined for each sample.

Controlled Cross-Pollinations

Pollinations bags were made out of Tyvek® home wrap by folding and sealing the edges with Goop® household waterproof adhesive, and reinforcing the folded, sealed edges with staples. Pollination bags were placed over dormant branches on April 22, 2002. Heavy duty outdoor carpet tape and staples were used to seal the edge of the bag that came in contact with the base of the branch. On May 23, 2002 the bags were inspected for defects, and then removed so that pollen could be applied directly to the stigmas of individual flowers using a paintbrush. Following application of the pollen, the bags were immediately put back into place to prevent any other contaminating pollen from entering. Once all

anthers in the immediate vicinity had finished dehiscing, the bags were removed (June 13, 2002) to allow for normal seed development. The bags were again inspected for any damage and any holes or tears were noted.

Seed Collection, Storage and Germination

Two types of seed collection bags were put into place on July 30, 2002, to prevent predators from eating the seeds prior to their harvest and to collect any burrs that may have abscised prematurely. One type of bag was a commercially available mesh cotton laundry bag with a drawstring. A second type of bag was constructed from nylon mesh window screening. The seams of these bags were sealed using strips of Tyvek and Goop® adhesive. The seed collection bags were removed Sept. 5, 2002 at which point seeds were harvested. Burrs resulting from controlled-cross pollinations were removed directly from the branch, placed in plastic baggies and stored on ice for transport. Open-pollinated seed was collected directly from unbagged branches or by removing entire branches and transporting them back to the laboratory in water. At the time of harvest, all burrs were still tightly closed and slightly green in color. The burrs were laid out on flat trays and allowed to air dry overnight, which resulted in the triangular-shaped nuts being released. The seeds were then placed in plastic bags filled with damp peat moss and stored at 4º C for 130-150 days. After the period of cold treatment, seeds were dissected from their seed coats and sowed in flats of Metro Mix 510 amended with micronutrients (90 g per 3 cubic yard bag of Metro Mix) and osmocote (Scott's Corp., Dublin, OH; 300 g per bag). The flats were put in the greenhouse under a 16 h photoperiod with daytime temperatures set at 22º C, and nighttime at 20º C. Drip irrigation was used daily to keep the media moist.

Results

Pollen Collection and Viability

To our knowledge, there are no published reports of successful controlled cross-pollinations in *Fagus grandifolia* and very little is known about flower development, stigma receptivity and the timing of pollen release. In *F. sylvatica* and *F. grandifolia*, both female and male flowers can be found in the same bud (Nielson & De Muckadeli, 1954; Garrison, 1957). For this reason, the branches could not be emasculated at the time the pollination bags were initially put into place by removing male buds. *F. sylvatica* is metandric, meaning that the female flowers are receptive before male flowers on the same tree begin to release pollen. Our observations confirmed this was the case with *F. grandifolia* as well. Female flowers emerged up to five days before male flowers were observed. The onset of female receptivity, which was identified by curvature of the stamen as was reported in *F. sylvatica* (Nielson & De Muckadeli, 1954), occurred prior to pollen release. Although little is known about the frequency of self-fertilization in *F. grandifolia*, reports in *F. sylvatica* indicate that self-fertilization is an infrequent occurrence (Nielson & De Muckadeli, 1954). For this reason and because of the time constraints involved, instead of removing male flowers we chose to minimize self-fertilization by performing the pollinations prior to the occurrence of pollen release. This made it necessary to force pollen production so a viable source of pollen was available during the brief window of time when the female flowers were receptive but the male flowers had not begun to dehisce.

The dormant branches harvested on May 13, 2002 were very close to bud break. Some trees had pistillate flowers already emerging, while others didn't emerge for another day or two after placement in the greenhouse. Staminate flowers emerged between 2 to 5 days after the appearance of the female flowers. Pollen was collected between May 15th and May 19th and was immediately dusted onto germination media prior to being stored at 4º C in a sealed vial along with silica gel packs as a desiccant. Results of the pollen viability assays performed on May 19, 2002 show viability levels between 30 and 50 percent (table 1). The viable pollen was used for pollinations on May 23, 2002 and at this time the female flowers appeared to be in stage 2, meaning they were receptive according to the criteria reported by Nielson & De Muckadeli (1954). None of the parental trees were actively releasing pollen at this time. Pollen viability assays were performed again after the pollinations were complete, on June 24, 2002 (Table 1). At this time, the pollen had been transported on ice, removed from the ice for several hours while it was brushed on individual flowers, and returned to storage at 4º C for one month. A drop in viability was observed, and this reduction in viability varied between

Table 1.—Pollen viability in trees of American beech.

Pollen Donor	Maternal Parent	Viability 5/19/02	Viability 6/24/02	No. of flowers brushed	No. nuts per flower
1501 (I)	1504 (R)	50 %	4 %	69	0.51
1506 (S)	1504 (R)	30 %	16 %	249	0.32
1504 (R)	1506 (S)	50 %	41 %	98	0.97
1504 (R)	1505 (R)	40 %	28 %	115	0.53
1506 (S) @ 4º C	none		51 %	50 %	-
1506 (S) @-20º C	none		54 %	50 %	-

Table 2.—Open-pollinated seedlings derived from seeds of American beech trees.

Parent Tree	No. Seeds	% Full	Germinative Capacity	No. of Plants
1506 (S)	802	35	60 %	168
1504 (R)	2081	28	8.5 %	49
1511 (S)	478	24	2.6 %	3
ME (R)	283	75	53 %	149

individual samples (Table 1). It was encouraging to note that the pollen retained viability after being in the uncontrolled field environment for such a long period of time. It is also interesting to note that the pollen collected from tree 1504, which exhibited the smallest drop in viability also resulted in the highest number of filled seed being produced per flower, 0.97. In contrast, 1501 pollen had a drop in viability from 50 percent to 4 percent, yet still produced 0.51 nuts per flower. A portion of the pollen collected from 1506 was not used in any of the pollinations and was instead aliquoted and stored at either 4º C or -20º C to study how well viability was maintained at these two temperatures over a period of time. The starting viability was determined on May 19th and viability was assayed again on June 24th (table 1). There were no differences in viability between 1506 pollen that had been stored at 4º C and 1506 pollen that had been stored at -20º C. In addition, viability of pollen stored at both of these temperatures did not decrease from the initial test date to the final test date, over a month later. Some samples of pollen were freeze-dried overnight, and this resulted in a complete loss of viability (data not shown). All of the pollen samples shown in Table 1 were again assayed for viability after one year in storage at 4º C and all had dropped to 1 percent or below. Although we did not have enough pollen to do extensive studies on optimal long-term storage conditions, a batch of 1503 pollen (not used for pollinations) was aliquoted and half stored at -20º C for one year and the other half stored at -80º C. After one year, the viability of the 1503 pollen stored at -20º C had dropped from 50 percent to 1.5 percent, but the pollen stored at -80º C retained a level of 47 percent viability (data not shown). In the future, studies will be dedicated to working out optimal conditions for long-term storage and will look at not only storage temperature but moisture content as well.

Open-Pollinated Seed

Open-pollinated seed (table 2) was obtained from two of the parents used in the cross-pollinations study, the resistant tree 1506 and the susceptible tree 1504. Open-pollinated seed was also collected from the susceptible tree 1511 and from a tree located in Sebois County, Maine-ME. Between 24-35 percent of the seeds collected from trees at Ludington State Park (1506, 1504, and 1511) were full (table 2). This figure is only slightly higher than the reported 13 - 29 percent of sound nuts collected from 20 trees in East Lansing, MI (Gysel, 1971). A greater degree of variability in the percent sound seed was reported by Sain and Blume (1981) for American beech seed collected from trees in the Great Smokey Mountains National Park (GSMNP). In this report, the number of full seed ranged from 0.8 to 95 percent. Comparisons of trees in high elevations with trees in low elevations found no correlation between elevation and the production of sound nuts. The percentage of seeds collected from the ME tree that were sound was much higher (75 percent) than those collected from Ludington State Park.

Table 3.—Controlled cross-pollinated seed from American beech trees.

Cross (female x male)	Full	Seeds Germinated	Rotten	Empty	Total	% Full	Germinative Capacity	Total No. Plants
1506 (S) x 1504 (R)	11	84	0	146	241	39	81 %	77
1504 (R) x 1506 (S)	49	31	10	585	675	13	12 %	11
1504 (R) x 1501 (I)	35	0	0	98	133	26	37 %	13
1505 (R) x 1504 (R)	28	33	0	170	231	26	84 %	51

This value was comparable to the values reported by Leak & Graber (1993) for seed collected from beech in the White Mountain National Forest. Over a 6-year period of time seeds from this area were consistently between 75 and 88 percent sound. Studies in *F. sylvatica* also reported variability in the number of seed produced that were full, ranging from 3 to 92 percent (Nielsen & De Muckadeli, 1954). Nielsen & De Muckadeli (1954) also reported an experiment in which branches were emasculated prior to pollen release and then bagged to prevent pollen from entering. These branches still produced large amounts of nuts, but all of them were barren thus proving that *F. sylvatica* has the ability to produce fruit lacking a seed in the absence of fertilization, a phenomenon known as parthenocarpy. Observations by H.J. Garrison (1957) on developing *F. grandifolia* fruit led her to conclude that in the absence of pollen a seedless fruit develops and it is similar in size to fruit containing a seed. This would suggest that the variability in the quantities of empty seed produced may be dependent on pollen production and staminate flower development as well as weather conditions such as high winds and heavy rainfall which may interfere with pollinations.

Cross-Pollinated Seed

The results of the controlled cross-pollinations are listed in Table 3. Overall, the germinative capacity (the percent of sound seed that germinated) was variable, ranging from 12 to 84 percent. However, compared to open-pollinated seeds from this study, the average germinative capacity of cross-pollinated seeds was greater. This was also the case in *F. sylvatica* (Nielsen & De Muckadeli, 1954). The percent of barren seed was similar in both the cross-pollinations and the open-pollinations, with the exception of the 1504 female x 1506 male cross which produced a slightly lower number of 13 percent sound seeds. This similarity between cross-pollinated and open-pollinated seed production provides an indication that the pollination bagging process did not negatively affect seed development. The production of a lower percentage of sound seed in the 1504 female x 1506 male cross could possibly be attributed to an incompatibility between the parents or perhaps 1504 does not produce vigorous pistillate flowers. Open-pollinated 1504 flowers produced seed with a low germinative capacity and the two controlled crosses that used 1504 as the maternal parent produced seed with lower germinative capacities compared to seeds from crosses that used 1505 or 1506 as the maternal parent.

American beech are monoecious, having both staminate and pistillate flowers on the same plant, making it possible to perform reciprocal crosses. However, due to other limiting circumstances such as the quantity of pollen harvested and the number of branches that could be reached for bagging, only one set of reciprocal crosses was performed in this study. The susceptible tree 1506 was used as both a pollen donor and a maternal parent along with the resistant tree 1504. Interestingly, when 1504 was used as a pollen donor with both 1506 and the resistant tree 1505, seeds with a high germinative capacity (81 and 84 percent, respectively) were produced. But when 1504 was used as a maternal parent with either 1506 pollen or 1501 pollen, the seeds produced had germinative capacities of 12 and 37 percent, respectively. For tree 1506, the opposite pattern was observed; this tree was more successful as a maternal parent than a pollen donor. Differences in compatibility between pairs of parents is not uncommon and identifying compatible combinations is an important part in developing seed orchards for tree improvement (Lambeth, 1993).

Self-Fertilization

Virtually nothing is known about the extent of self-fertilization in the American beech. Self-pollination experiments that were done in European beech demonstrated that *F. sylvatica,* for the most part has a

Table 4. Self-fertilization in trees of American beech.

	Self-Pollinated Controls			Open-Pollinated		
Tree	Total Seed	Full Seed	% Barren	Total Seed	Full Seed	% Barren
1504 (R)	32	0	100	2081	583	72
1501 (I)	44	10	75	NA	NA	NA
DSP-1973	111	0	100	89	12	86
DSP-RDC	62	0	100	60	4	93
DSP-FAY	7	0	100	134	24	82

high degree of self-sterility (Nielsen & De Muckadeli, 1954). However, there was a significant amount of variability of self-fertilization between individual trees and from year to year in the same individual. Rates as high as 40 percent were reported, although the average was 13.6 percent and the majority of trees included in the study had between 0 and 5 percent self-fertilized nuts. It is possible that the higher rates of self-fertilization reported were actually due to a contaminating pollen source. During the time this study was performed, the molecular techniques that would be required to rule out a pollen contaminant had not yet been developed. To address the question of self-fertilization in *F. grandifolia*, self-pollination control experiments were set up by placing the pollination bag over the branch and instead of removing it to apply pollen, the bag was kept in place until after pollen release was complete. The only pollen that would be available to fertilize the flowers would be from the staminate flowers on the same branch. Only two of the parents used in the cross-pollinations, 1504 and 1501, had enough accessible branches to set up additional self-fertilization experiments. Therefore, to try to get an idea of the self-fertility in *F. grandifolia* in general, three trees located in Delaware State Park were included in these studies, DSP-1973, DSP-RDC, and DSP-FAY. No occurrence of beech scale has ever been reported at Delaware State Park, so the resistant or susceptible phenotype of these trees is unknown, although they are most likely susceptible. Of the five trees tested for the ability to self-fertilize, only one tree, 1501, produced any full seed (Table 4). Furthermore, of the 10 full seeds obtained from 1501, only 2 germinated (data not shown). The remaining 4 trees produced 100 % barren seed. In each case, open-pollinated seed from the same tree yielded at least a portion of sound seed. In the case of 1504, the open-pollinated seed was 28 percent full. The open-pollinated seed from the trees in Delaware State Park produced between 7 and 18 percent sound seed (Table 4). Our observations of the self-fertilization experiments revealed an abundance of pollen was available for fertilization within the pollination bags. The extremely low occurrence of sound seed in the self-fertilization experiments, especially when compared to open-pollinated seed, is therefore likely due to a high degree of self-sterility in the American beech.

Discussion

Both half-sib and full-sib families were successfully generated for use in genetic studies. The seedlings are currently being tested for their resistant/susceptible phenotype through the use of insect challenge experiments (Houston, 1982). Based on estimates of between 1-2 % of beech trees displaying resistance to the scale insect, the half-sib family from the ME parent might yield one or two resistant trees. It is possible that the percent of resistant trees may be higher in controlled crosses between resistant parents. For thorough genetic mapping studies, a larger full-sib population will be required. The data presented here have provided the fundamental information needed to increase the numbers of controlled cross-progeny. Our data also indicate that there is a high degree of self-sterility in American beech. However, the degree of self-sterility can vary between individuals so it is possible that higher levels of self-fertilization can occur. Because we did not emasculate the branches used in our controlled cross-pollinations, some of the seed that was obtained may be from self-fertilization. It is also possible that the two plants obtained from the self-fertilization of 1501 were the result of contaminating pollen entering the bag. In order to confirm the parentage of each of our cross-progeny, and to more definitively address the issue of self-sterility, we are currently developing DNA-based markers. These markers will also allow a more definitive approach to determining the percent of self-fertilization that may have occurred in our half-sib and self-fertilized progenies.

Acknowledgment

The authors thank the Michigan Department of Natural Resources and the Maine Department of Forestry for their cooperation. This work was funded in part through the USDA Forest Service FHM Evaluation Monitoring Program.

Literature Cited

Garrison, H.J. 1957. **Floral morphology and ontology of *Fagus grandifolia* Ehrh.** Dissertation. The Pennsylvania State University. 101p.

Gora, V.; Starke, R.; Ziehe, M.; Konig, J.; Muller-Starck, G.; Lunderstadt, J. 1994. **Influence of genetic structures and silvicultural treatments in a beech stand (*Fagus sylvatica*) on the population dynamics of beech scale (*Cryptococcus fagisuga*).** Forest Genetics. 1: 157-164.

Gysel, L.W. 1971. **A 10-year analysis of beechnut production and use in Michigan.** Journal of Wildlife Management. 35(3): 516-519.

Houston, D.R. 1982. **A technique to artificially infest beech bark with the beech scale, *Cryptococcus fagisuga* (Lindinger).** Research Paper NE-507. U.S. Department of Agriculture Forest Service, Northeastern Forest Experiment Station. 8p.

Houston, D.B.; Houston, D.R. 1994. **Variation in American beech (*Fagus grandifolia* Ehrh.): Isozyme analysis of genetic structure in selected stands.** Silvae Genetica. 43: 277-284.

Houston, D.B.; Houston D.R. 2000. **Allozyme genetic diversity among *Fagus grandifolia* trees resistant or susceptible to beech bark disease in natural populations.** Canadian Journal of Forest Research. 30: 778-789.

Krabel, D.; Petercord, R. 2000. **Genetic diversity and bark physiology of the European beech (*Fagus sylvatica*): a coevolutionary relationship with the beech scale (*Cryptococcus fagisuga*).** Tree Physiology. 20:485-491.

Lambeth, C.C. 1993. **Overview of pollen management in tree breeding**. In: Advances in pollen management. Agriculture Handbook 698. Washington, D.C.: U.S. Department of Agriculture, Forest Service: 97-99.

Leak, W.B.; Graber, R.E. 1993. **Six-year beechnut production in New Hampshire**. Res. Pap. NE-677. Radnor, PA: U.S. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station. 6p.

Mielke, M.E.; Houston, D.R.; Bullard, A.T. 1986. **Beech bark disease management alternatives.** In: Proceedings, integrated pest management symposium for northern forests; 1986 March 24-27; Madison, WI: University of Wisconsin, Cooperative Extension Service: 272-280.

Murashige T; Skoog F. 1962. **A revised medium for rapid growth and bioassays with tobacco tissue cultures.** Physiologia Plantarum. 15: 473-497.

Nielson P.; de Muckadeli M.S. 1954. **Flower observation and controlled pollination in *Fagus.*** Silvae Genetica 3: 6-17.

Sain, R.E.; Blum, K.E. 1981. **Seedling production in the high-elevation beech (*Fagus grandifolia* Ehrh.) forest of the Great Smokey Mountains National Park.** Castanea. 46: 217-224.

Rudolf, P.O.; Leak, W.B. 1989. ***Fagus* L. Beech.** In: Seed of woody plants in the United States. Handbook No. 450. Washington, D.C. USDA: 401-405.