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Bramble Production in Maryland
and
Anticancer Effects of Bramble Fruits on Human Colon Cancer Cells

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Executive Summary

Two locations were chosen as candidate sites for bramble production in the Chesapeake basin, the lower Eastern Shore and the Appalachian Plateau in Garrett Co. MD and Somerset Co. PA. While there were problems in establishment, overwintering and disease using a larger quantity of spring bearing varieties on the Eastern Shore, the Appalachian Plateau planting met expectations. Fruit season, size, firmness and productivity were excellent; annual returns from the top producer, 'Caroline', would be $66,000 per acre. In the years following the establishment of the planting, an additional 5 growers in the area have started their own production facilities. Additional information on cultural methods, including weed control, pest sensitivities and controls, soil water management, and nutrient application were obtained. A series of small newsletters was published to aid novice growers. Two Eastern Shore sites were abandoned, the first due to grower non-cooperation and the second at LESREC due to disease and winter dieback. However, we instructed a private grower in establishing a similar planting in the Salisbury area. Using information gathered from the failed planting at LESREC, the grower was able to obtain returns of $7800 per acre during the first year of production.

The anticancer effects of bramble fruits were measured using human colon cancer cell line HT29 by Dr. Bernadene Magnuson (University of Maryland). Although the amount of potentially active fruit compounds in this assay varied by cultivar, the reduction of cancer cell growth in vitro was consistent above a threshold concentration. Overall, cancer cell inhibition of the juice of the 5 cultivars tested averaged 78% and ranged from 68% to 84%. Wide variation in phenolic content, including the red pigment, anthocyanin, was measured by Dr. Geza Hrazdina (Cornell University) with liquid chromatography and spectrophotometrically. Anthocyanin content for one of the yellow cultivars, Anne, was essentially zero, while total anthocyanins of red-fruited cultivars averaged 5 mg/g dry weight of fruit. Although research has consistently shown that anthocyanins are effective in these types of assays, evidently there are compounds in Anne that are also active in reducing colon cancer cell growth in vitro. Thus, both yellow and red-fruited varieties may be valuable aspects of a healthy diet.

These results redirected the emphasis of our research toward a novel area of food-anticancer research, which is now the subject of several research proposals. In an effort to elucidate the active compounds, research has focused on applying fruit constituents to various assays or fractionating fruit extracts to isolate active fractions. Evidently various compounds act synergistically, or require a food matrix to act. Because fruit varieties have an infinite amount of component concentration combinations, comparing closely related varieties results in confounded results. Which of the 30 different compounds between the two cultivars is active? Instead, we have focused on producing several varieties with altered phenolic contents using gene silencing technology and transformation methodology at the University of Maryland.

Background and Objectives

The University of Maryland has had a bramble breeding program for over 70 years. Through its last 25 years, the program had expanded to a regional effort consisting of 7 seedling fields in 4 states. Several cultivars were produced, including: Caroline, Josephine, Esta, Anne, Alice, Lauren, Jaclyn, Claudia and Chesapeake. Caroline, Josephine, Chesapeake and Jaclyn are being grown on four continents and Caroline and Josephine are commercially produced. Recently, the program privatized and is now called Five Aces Breeding LLC. Five Aces produces and evaluates bramble seedlings for its 7 client companies and 4 grower cooperators. Through these efforts, Five Aces Breeding almost definitely sees more seedlings per year than any other bramble breeding company worldwide.

Although the varieties produced in Maryland have wide adaptability, they lead to no industry growth in the mid-Atlantic region. Profits were improved at local pick your own growers, our main marketing outlet, but acreage was limited by the number of customers visiting the farm. Unfortunately, if we could grow these new varieties well, cooler, more traditional growing areas in Mexico, Chile, the UK, Poland, the Pacific Northwest and California could do better, and they did. Clearly, regional expansion into commercial production would need the firmer and larger fruit that cooler weather produces. That cooler weather can be found in Garrett County MD and Somerset Co. PA, as well as areas on the Appalachian Plateau above 3000 ft elevation in WV and NC. On the Coastal Plain, cooler summers do not exist; however, the April to May and October through December weather is suitable for bramble fruit production. Thus, we proposed early spring and late fall production on the Eastern Shore to compliment mid summer production on the Plateau. The first part of this grant was to set up functional test plantings and “seed operations” in these two areas.

To further develop this industry, the second area of research involved developing a marketing advantage for these growers by investigating the reported anticancer effects of bramble fruits. Ultimately, a major goal of this type of research is to identify active compounds and either fortify processed food with those compounds, or select for varieties or growing conditions which produce high levels of beneficial compounds. Thus, the second objective for this research was to survey our raspberry varieties for the content of compounds potentially active in preventing colon cancer. However, there is a wide gap between what we think is potentially active and demonstrating activity at preventing cancer in humans. Fortunately, during this process, a research team was developed which was able to expand this research to initiate identification of active compounds using human cancer cells in vitro, thereby allowing us to get information closer to what occurs in the human body.

Methodology

Two demonstration plantings were set in the spring of 2002, one at the University of Maryland's Lower Eastern Shore Research and Education Center in Salisbury, MD using spring bearing varieties, and one at Dan Yoder's Farm at 2600 ft. in Oakland, MD which used "fall bearing" varieties which do not have to overwinter above ground and survive temperatures commonly lower than -10F. Both plantings were set with plants produced at the University of Maryland and on landscape fabric. Growth was poor and surviving canes on all red raspberries, except PCS-2, two black raspberries, two blackberries and two hybrid blackberries, were winterkilled to the ground. The planting was not studied afterward. The Yoder planting was covered with a DeCloet tunnel (plastic greenhouse) of 31 x 100 ft. Plants were fertilized with 30 lbs and 60 lbs per acre of N (half the amount was fertigated during summer) in the first and second years of production, respectively. Pesticides were applied as necessary, and water was applied by trickle tube at the rate necessary to supplement to 1.5 inches or rain per week, especially during fruiting. The tunnel sides were kept closed during the winter and left open when temperatures exceeded approximately 65F outside. Plants were hand harvested and fruit and total harvest weight recorded.

On three dates, samples of the fruit were refrigerated and transported on ice to College Park. The fruit was either juiced and frozen or flash frozen with liquid N. Thawed fruit samples were dissolved in water (other solvents were found to be unnecessary), purified through a C18 miniprep column and concentrated in vacuo. A concentrated sample of the fruit was added to cell culture media and a measured aliquot of human colon cancer cell line HT 29. Cell growth was measured at 24 hours and 48 hours and the results were expressed as percent reduction of growth as compared to a control of just the extraction buffer (no fruit). A dosage curve was used with the red cultivars of 10, 25, 50, 100, 250 and 500 mg/l of anthocyanins (as measured at 525 nm). Total phenolics were estimated using a commercial colorimetric test and used to calibrate the tests for the yellow fruited cultivars since they do not contain significant amounts of anthocyanins. An aliquot of these samples was carried frozen on ice to Geneva, NY for a phenolics profile on liquid chromatography. These samples were thawed and pressed through 0.45 µm screens before injecting onto a Sephadex C18 column eluted in 0.1 M acetic acid in water with increasing gradient concentrations of 0.1 M acetic acid in acetonitrile to 100%. Various gradients were used. Visualization of eluants was at 272 nm for phenolic compounds and 525 nm for anthocyanins.

Results

Production at Yoder's Farm during the first year of production was not substantial due to a later planting date necessitated by land preparation in a wet year. The soil types in Garrett Co., while suitable for most agriculture, are heavy and do not drain quickly. Thus, fall site preparation is preferred. During first winter of production, a 50-inch snowfall collapsed the tunnel used in this experiment as well as two new tunnels erected by other growers who became interested in production because of the initial results at Yoder's, specifically upon observing the fruit size and growth. Second year yield was substantial, but since the tunnels were not rebuilt until mid-summer, no yield comparisons between outside and tunneled plants were possible. Third year data were taken from July 25 to September 15, when production had nearly stopped. The results were, in lbs of fruit sold per acre:

Variety	In the Tunnel	Outdoors
Alice	7382	1012
Anne	8362	2270
Caroline	22910	9146
Jaclyn	11760	6697
Josephine	9800	1584
Average All Varieties	12043	4142

The tunnel in Mid July:

Berry size of a subsample of fruit always averaged over 3 grams per fruit; although typically Josephine averaged over 5 grams (90 fruit per pound) and Caroline, Anne and Jaclyn were over 4 grams per fruit (120 fruit per pound). Caroline is typically a 2 gram fruit in the Washington, DC area. Fruit prices ran from a low of $ 1.87 per pound to a high of $3.20 at auction. Farm market retail price was consistently at $3.35 per pound. Subjective observations of quality indicated a high flavor was achieved on all varieties and consumer acceptance at the Oakland Farm Market was brisk. Sellouts were within one hour of commencing sales. Firmness was very acceptable, but not as high as expected, perhaps due to the higher temperatures and production in the tunnel.

Josephine fruit:

Tunnels of this size would cost approximately $22,000 per acre, including landscape fabric and irrigation tubing. Obviously, the costs of tunneling outweigh the expenses. The tunnel effects could be due to increased heat in the day with no effect on night time temperature (since the sides are open during the whole summer). This higher temperature obviously leads to increased growth but did not reduce fruit size. It would be interesting to run controlled temperature experiments on day, night and day/night temperature differentials to determine the effects on growth, yield, firmness and size.

The use of landscape fabric, at $2000 per acre, is initially expensive, but the system worked well on the Plateau. On the Eastern Shore, the fabric prevented weeds, but lead to premature soil warming and death of overwintering canes at LESREC and a similar planting in Blackstone, VA. Complete covering with landscape fabric is not recommended outside the Appalachian Plateau. Several other growers have used landscape fabric on the Plateau and have had similar success controlling weeds and maintaining canes within their rows. Plant holes in the fabric should be established or enlarged by late March as later holes are not fully occupied by new canes. This is new and unexpected information about bramble growth and will be included in the mid-Atlantic Production Guide.

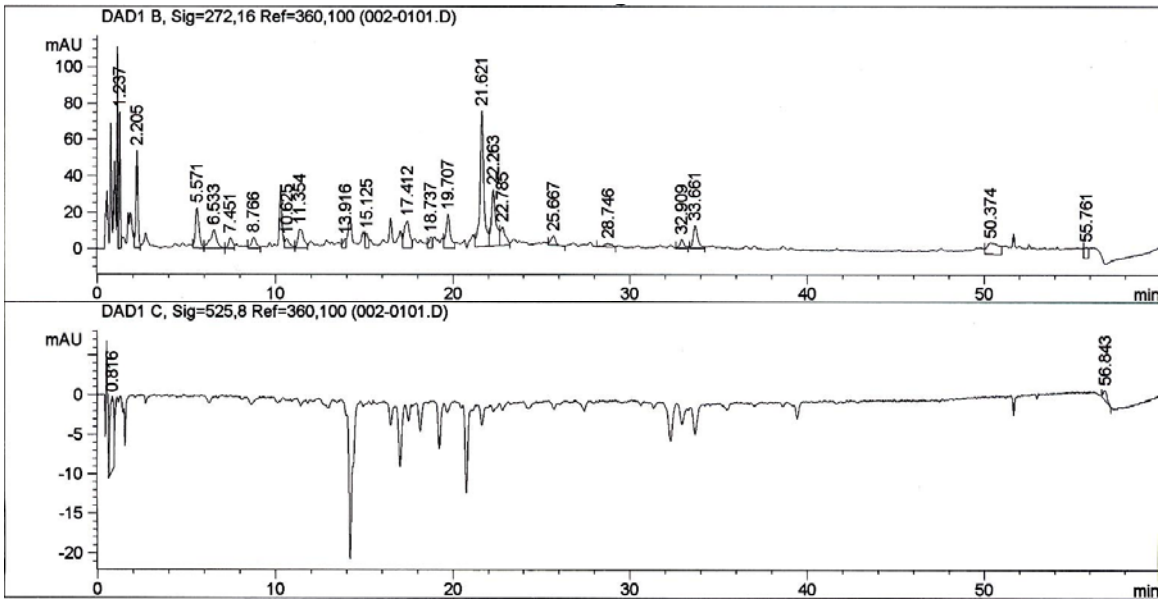
One of the expectations of using a tunnel is a drastic reduction in pest management. From previous experiences with raspberries in tunnels or greenhouses, we expected powdery mildew and mites to be the primary concerns. We have had success with predator mites in cooler climates and overhead water application, or sublimated sulfur, can be used to control mildew. Organic products, such as Oxidate (peroxide), are also effective if used as indicated on the label.

Our expectations were correct. Fruit Botrytis (mold or "rot") was minimal without pesticide application. Late season rust, which defoliated a couple outdoors plants of susceptible varieties (Jaclyn, Caroline, Alice), was found in the tunnel, but it did not need treatment. Other cane and leaf diseases, such as anthracnose, spur blight, cane blight and cane botrytis were not observed in the tunnel. Insects were not affected by the tunnel. Aphids and leafhoppers had to be treated with insecticidal soap, while Japanese beetles were few enough to be removed by hand. Mites were the only pests that required a pesticide application. Infestations were highest during the hottest periods at Yoder's. Experience at companion plantings in Garrett and Somerset Cos. indicated sufficient watering, and improved ventilation, perhaps to lower temperatures, perhaps to allow access for predators, reduces mite pressure significantly. Altogether, tunnels with landscape fabric offer a real opportunity to produce organic fruit, especially since weed control is accomplished without the use of residual or contact herbicides.

Analysis of Fruit Components

Rehydrated or juiced samples gave similar concentrations of anthocyanins, thus results are comparable for that class of phenolics. Multiple liquid chromatographs were obtained from samples of fruit from Anne, Josephine, Caroline, Jaclyn and three cultivars which were added for future study, 'Royalty', 'Heritage' and its anthocyanin reduced sport (mutant), 'Kiwigold'. The following chromatograms indicate the presence of several compounds of interest, including anthocyanin peaks at approximately 21 minutes elution time for 'Royalty' only. 'Anne', the yellow fruited cultivar has no peaks there in the red pigment detecting 525 nm wavelength. The small negative peaks are unknowns, but note the scale is very sensitive. The identification of some of the peaks is ongoing at Cornell, but note the complexity of the phenolic profile of the yellow fruited 'Anne' compared to the dark red fruited 'Royalty'.

Anne



Royalty

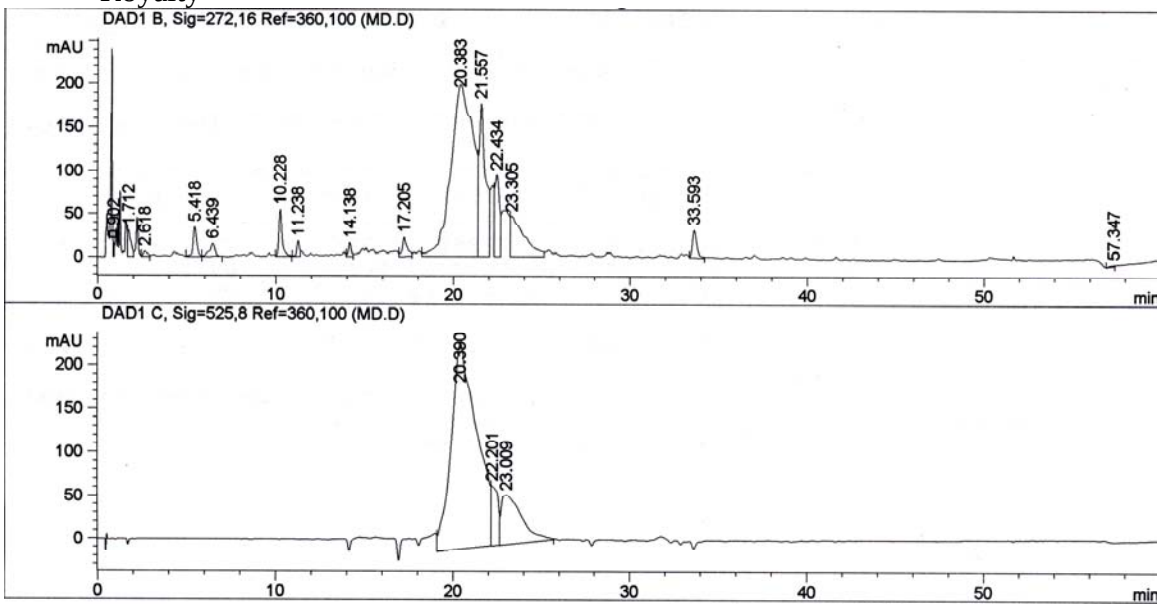


Figure 1. Liquid Chromatograms on C18 columns of Royalty and Anne raspberry fruits at 272 nm (top) and 525 nm (bottom). Observations are in milliabsorbance units.

Overall: the anthocyanin concentrations for cultivars used in this study were (in mg/g dry weight of tissue):
Caroline: 3.924, Heritage 3.892, Josephine 2.889, Kiwigold 0.994 and Anne 0.

Overall: the total phenolic concentrations for cultivars used in this study were (in mg/g dry weight of tissue):
Caroline: 14.48, Heritage 11.32, Josephine 14.36, Kiwigold 8.36 and Anne 7.98.

A separate trial was used to measure the phenolic content differences between tunnel and outdoor grown fruit and between fruit from the north or south side of the plant canopy. Using Josephine, a 10% decrease in anthocyanins occurred in the tunnel. North side grown fruit had 11% less anthocyanins. Apple fruit grown in the sun has a similar reaction, however, the solid red color of ripe raspberry fruit hides this difference. A 14% decrease in total phenolics content occurred in the tunnel. North side grown fruit had 12% less phenolics. The differences due to the tunnel may be due to the 10% reduction in light due to the poly covering.

Extracts of these varieties were also fed to human colon cancer cell line HT29 in vitro. Fruit extract concentrations were normalized to 5 to 500 mg/l of anthocyanin in the case of red pigmented varieties, or to 5 to 500 mg/l phenolics content for the yellow fruited varieties. The average reduction in cell growth after 48 hours exposure to fruit extract was measured. Significant reduction in cancer cell growth was obtained at concentrations of 50 mg/l and higher. Higher concentrations gave positive results in all three tests. At 500 mg/l reduction was consistently above 68%, with Josephine having the lowest effect. The range of reduction was 68% to 84%, with Caroline having insignificantly greater effect than the red fruited ‘Heritage’, the orange colored ‘Kiwigold’ and the yellow colored ‘Anne’. The average reduction of 78% is considered noteworthy for fruits.

Conclusions

Specific Recommendations for Garrett Co.

Plant the fall bearing varieties: Jaclyn for early fruit, Caroline for midseason production and Josephine to finish in the fall or for long distance shipping.

Use tunnels: even when planting on lighter soils, tunnels allow for precise water management (Garrett Co gets 50 inches of rain per year), a two month expansion of the harvest season and a pest reduced environment. Once summer has arrived (>65F), open the sides of the tunnels to reduce temperature and pest pressure.

Use landscape fabric: for $2000 per acre investment, a 15 year weed control program is achieved.

From other experiences, we have found 1 lb actual N and K per acre per week sufficient summer fertigation for production during the first three years of production. No other nutrient deficiencies were observed or measured. Raised beds are not required in the tunnels; however, the area around the tunnels should be trenched or similarly drained.

Recommendations for the Eastern Shore

Plant the fall bearing variety: Josephine, it resists late leaf rust, produces 3 gram berries in the first year in Wicomico Co., and will not be harmed by the winter temperatures on the coastal plain. Grow black raspberries, hybrid berries and blackberries, as they seem to be winter hardy, or wait until more information on the performance of the promising advanced selections from the breeding program (there are 3 that do survive, but cannot be disclosed at this time).

Do not plant on continuous landscape fabric: abnormal heating of the root zone may be causing premature growth and subsequent winter injury. Use landscape fabric to mulch 6 inch high raised beds and cut holes of 5 to 6 inches in diameter to plant.

Continuing Fruit Constituent Research

Raspberry fruit, either reconstituted after freeze drying or as a juice (which can be frozen), is a very powerful inhibitor of colon cancer cells in vitro. About two-thirds of the oxygen radical absorbance capacity of raspberry fruits was thought to have come from the red color anthocyanins, even though anthocyanins represent about one third of the total phenolic content of raspberries. In this research, even though testing is not complete, a yellow fruited variety and an anthocyanin reduced variety had activity against cancer cells in vitro. This result can not yet be explained with any subsequent data and will be the subject of future research with fruit collected this fall. The phenolic profiles of yellow fruit indicate they have a wide variety of other phenolics, some of which are not abundant in red fruited varieties. We do not know which, if any, of these compounds are suitable substitutes for anthocyanins.

Given the complexity of the differences between the cultivars, we will concentrate mostly on trying to develop transgenic clones with only slight differences in fruit constituents. Genetic transformation has been initiated and will be the subject of two other grants. Initial trials indicate a highly successful protocol has been adapted for raspberries using a standard transformed cell selection system, a multiple stage subculturing and plantlet recovery system and all varieties used in this research.