Factors Affecting Seed and Stem Cutting Propagation of *Stevia rebaudiana* (BERT.)

by

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Abstract

Stevia rebaudiana (BERT.) is a very remarkable plant that is starting to gain ground in the United States. *Stevia rebaudiana* has been used in numerous countries for thousands of years as a source of sweetener that is all natural and will not raise a person's glycemic index, which is especially important for diabetics. Propagation of *Stevia rebaudiana* is problematic with low seed germination rates and limited research done in the United States. *Stevia rebaudiana* has potential to be a prominent crop in the southern United States but growers need to know how to produce the plants efficiently as the demand for natural products continues to rise. The main objective of this work was to identify specific methods of propagation for *Stevia rebaudiana* using readily available materials to produce healthy transplants can be grown.

Seed germination for *Stevia rebaudiana* is typically poor with seeds planted right after harvest, due to a variety of reasons, such as: low viability; fungus produced on pappus bristles of seed coats; and poor pollination in some areas. Little literature is available on *Stevia rebaudiana* seed propagation. In Chapter 2, 3 experiments looking at seed germination with light or without light, in 4 substrate types were completed. Substrates of 50% pine bark:50% peat moss, 100% sand, 100% perlite, and 100% vermiculite by volume were evaluated. Cell pack trays with poly lids either blacked out or left clear were used. Under no-light conditions 100% vermiculite and 100% perlite performed best for germinating *Stevia rebaudiana* seeds. One hundred percent sand:light had maximum germination rates at 14 days after seeding (DAS). Fungal growth on the 50% pine bark:50% peat moss substrate and substrates such as 100% vermiculite:light led to the

lowest germination rates. Seeds germinated under both light conditions and therefore 100% sand:light is recommended since most growers germinate seeds in a greenhouse under light conditions.

Stevia rebaudiana can be 250 to 300 times sweeter than sucrose with no calories. Propagation is usually done by stem cuttings due to seed propagation usually having low germination rates. To produce transplants needed for increasing production demand, more efficient propagation techniques are being tested in order to increase plant production.

In a second study stem cutting propagation in the Southeastern Unites States was evaluated (chapter 3.) This asexual propagation study looked at stem cuttings and the effect that substrate and cutting type played. The stem cuttings were taken from container grown stock plants and planted in 32 cell packs with one cutting per cell. The two node cuttings were placed in one of 4 types of substrates: 1:1 pine bark:sand by volume, 1:1 peat moss:perlite by volume, 100% sand, or 1:1 sand:vermiculite by volume. Two cutting types were evaluated, medial and terminal. Stem and cuttings were placed under mist for 15 seconds every 10 minutes for the first 4 weeks, then 5 seconds every 10 minutes the remaining 4 weeks of the studies. Data analyzed included: foliar color rating of both old and new foliage, shoot breaks over 2.54 cm (1 inch long), and root length was also looked at. No interactions were found between substrate and cutting type were found. Greatest root length occurred in the 1: sand to 1: vermiculite substrate while 1:1 part pine bark:sand substrate had a greater root length. Medial cuttings had more shoot breaks than terminal cuttings regardless of the substrate used. When looking at old and new foliage, medial cuttings had a greater color rating than terminal cuttings on a rating scale of 1-5 (scale of 1-5 with 1-dead, 3-yellow, 5-dark green). Pinebark:sand or sand:vermiculite would be

recommended for use as substrates for liners as root length and new growth are important factors in producing liners. Removing apical dominance in liners is also recommended.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

***Stevia rebaudiana* - Background:**

Historically, items such as honey and fruits have been used for their sweetening properties. Not until the 14th century was sugar refined and considered as a food item of notable importance. While beet sugar *(Beta vulgaris L.*) has provided a small percentage, the origin for sugar has been primarily cane sugar (*Saccharum officinarum* L.). Large amounts of sugar have been shown to have negative effects on human health such as contributing calories to diets, which can lead to obesity, and is a risk factor for some chronic diseases such as *Diabetes mellitus*, cardiovascular disease, and hypertension. The desire for sweetness without health concerns has brought man to look for and discover various alternative sweeteners, which have been able to offer consumers sweetness without calories (Savita, et al., 2004).

One of the advances for alternative sweeteners comes from the plant *Stevia rebaudiana* (Bertoni). *Stevia rebaudiana* is plant native to Paraguay in south-central South America. *Stevia rebaudiana* grows as a perennial in its spp. native area of the Rio Monday Valley of the Amambay Mountain Region on the outline of marshes or in areas of grasslands where shallow water tables exist (Shock, 1982). *Stevia rebaudiana* has an extensive root system along with brittle stems, and small elliptical leaves. *Stevia rebaudiana* flourishes at altitudes between 200 and 500 meters above sea level, having a mean temperature of 23 °C (ranging from -6°C to +43 °C or 21.2°F to 109.4°F), and an annual rainfall from 1500 to 1800 mm (59.05 to 70.86 inches) (Savita et al., 2004).

Stevia rebaudiana, although thought of as a herbaceous perennial in its natural habitat, grown under some environmental conditions and management situations, performs as an annual or mixture of both annual and perennial (Ramesh et al., 2006). One such environmental condition is cold temperature. *Stevia rebaudiana* cannot tolerate frost, therefore, growth ceases during winter (Megeji et al., 2005). Many countries have already been influenced by the sweetening capacity of *Stevia rebaudiana* including: Japan, China, Taiwan, Korea, Mexico, USA, Thailand, Malaysia, Indonesia, Australia, Tanzania, Canada, Georgia, and Russia (Ramesh et al., 2006). Europeans first encountered *Stevia rebaudiana* during World War II because of the need to supplement sugar rations. Japan is a leading country in the production and marketing of *Stevia rebaudiana* sweeteners with extensive uses, including cereals, teas, and soft drinks (Ramesh et al., 2006)

A compound in the leaf of *Stevia rebaudiana*, Rebaudioside A, is one reason that it is being so heavily investigated (Megeji et al., 2005). The Guarani Indians of the Paraguayan Highlands referred to this plant as caà-êhê, meaning “sweet herb.” The extract of *Stevia rebaudiana* can have sweetening power 300 times greater than cane sugar yet it is a zero-calorie sweetener.

Stevia rebaudiana is currently under investigation as an alternative specialty crop for the southern United States. Sugar cane and field corn (*Zea mays* L.), currently dominate a large portion of the sweetener market. *Stevia rebaudiana* has a higher sweetening power compared to sugar cane and field corn, and *Stevia rebaudiana* doesn’t require as much land to produce an equivalent amount of sweetener.

Researchers at Kansas State University executed large trials that produced 1490 kg (3285 lb) dry leaf yield per hectare (2.47 acres) under irrigation. Based on these results, an acre of *Stevia rebaudiana* grown in Kansas could potentially take the place of seven acres of Louisiana sugar cane. With *Stevia rebaudiana* having exceptional sweetening potential, numerous acres of land could be released for other uses such as crops for biofuel or other high demand crops for certain areas. An acre of *Stevia rebaudiana* could produce the equivalent of 17,880.61 kg (39,420 lb) of sugar. *Stevia rebaudiana* also shows promise for producing marketable yields in climates and soils that would not be sufficient for standard row crops. Crops of *Stevia rebaudiana* could also be produced with fewer pesticides and fertilizers when looking at per unit of sweetening power (Rhonda, 2004).

Stevia rebaudiana (Bertoni) being one of the 154 members of the genus *Stevia* is only one of two members of the genus that contains sweet steviol glycosides (Brandle et al., 1998). *Stevia rebaudiana* is a member of the Asteraceae family which also includes plants such as chrysanthemums (*Dendranthema grandiflorum* (Ramat.) Kitamura) and sunflower (*Helianthus annuus L.*) (Megeji et al., 2005).

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Group	Monochlamydae
Order	Asterales
Family	Asteraceae (Compositae formerly)
Subfamily	Asteroideae
Tribe	Eupatorieae
Genus	*Stevia*

Species	*rebaudiana*

Stevia rebaudiana lies in a group that consists of annual and perennial herbs, and shrubs that grow in mountain regions, open forests, along rivers, and dry valleys (Yadav et al., 2010). *Stevia rebaudiana* is a self-incompatible plant, which prevents self-fertilization and is insect pollinated. *Stevia rebaudiana* can be temperamental when first transplanted and will have slow growth initially. Within a month after transplanting, growth should increase if provided optimal environmental conditions (Ramesh et al., 2006).

***Stevia rebaudiana* – Sweeteners and Sweetener Safety:**

Leaves of *Stevia rebaudiana* produce stevioside and rebaudioside that can be employed as a natural source of zero calorie sweetener and has been shown to resist changes in its properties when exposed to high temperatures (Lee et al., 1990). *Stevia rebaudiana* extracts have been found to be non-toxic, non-addictive, non-carcinogenic, non-mutagenic, do not affect the growth and development of an embryo or fetus, and show no signs of damaging DNA (Alan, 2002). Another positive attribute for *Stevia rebaudiana* extracts is a lack of effect on blood sugar levels which could help diabetics (Mogra and Dashora, 2009). Diabetics and people who are conscious of their caloric intake may be able to add variety to their diets by using *Stevia rebaudiana* sweeteners rather than sugar or synthetic sweeteners. The American Food and Drug Administration gave approval, in 2008, for Generally Recognized as Safe (GRAS) status to highly purified Rebaudioside A. In 2009, France also approved Rebaudioside A for use in food and beverages (Herranz-lopez et al., 2010). Several other countries in Europe followed suit by approving *Stevia rebaudiana* products by 2011 (Herranz-lopez et al., 2010).

The leaf concentration of glycoside of *Stevia rebaudiana* starts to rise when the plants are grown under long days. Since glycoside synthesis is reduced during flowering, delaying flowering with long days yields more time for glycoside accumulation. The glycoside tends to accumulate in tissues as they age, so that older leaves have more sweetener than younger upper leaves (Singh et al., 2005)

A study was conducted looking at the effect of *Stevia rebaudiana* on a rat population for two years (Xili et al., 1992). The breakdown of the rats were 45 male and 45 female inbred Wistar rats. Zero, 0.2, 0.6 or 1.2% of 85.0% pure stevioside were the levels evaluated. Several factors were examined such as growth, food utilization and consumption, general appearance, and mortality in the rats. These factors were similar over the two year period for both controls and treated groups. No treatment-related changes were observed in rat blood, urinary or clinical biochemical values at any stage of the study.

Japan banned artificial sweeteners about 40 years ago and *Stevia rebaudiana* has been the chosen alternative as a food and beverage sweetener ever since. The Japanese have performed over 40,000 clinical studies and found *Stevia rebaudiana* to be safe. Goyal et al. (2010) reported the use of *Stevia rebaudiana* as safe for diabetics, not affecting blood sugar levels, and no neurological or renal side effects compared with other artificial sweeteners. *Stevia rebaudiana* possesses anti-fungal and anti-bacterial properties in addition to its other versatile uses. Also, mild *Stevia rebaudiana* leaf tea offers excellent relief for an upset stomach (Goyal et al., 2010).

Eight diterpene glycosides have been discovered that contain sweetening properties in the tissue of the *Stevia rebaudiana* leaf. These are unified in the initial stages of plant development by using the same metabolic pathway as gibberellic acid. One of the major glycosides, Rebuadioside A, shows importance in its configuration creating a desired flavor. Stevioside, another major glycoside, differs in interest due to addition of a bitter aftertaste. Stevioside can comprise up 60 or 70% of the total glycoside content in the leaf. Breeding of *Stevia rebaudiana* varieties which have high concentrations of Rebaudioside A along with low stevioside content is a necessary objective in use of *Stevia rebaudiana* varieties (Yadav et al., 2010). Adequate amounts of genetic variability exist to make advances in leaf yields. Genetic variability for leaf yield was shown to be high, and improved selections are possible (Brandle and Rosa, 1992).

A study was conducted in India looking at the availability of specific sweeteners at ten popular medicinal stores in Udaipur City (Mogra and Dashora, 2009). The evaluation of four of the most common sweeteners based on maximum sale and preferences were tasted by consumers who had been selected for the study. Based on the sale of the sweeteners, the study concluded that 70% of the participants preferred sugar-free products, 25% preferred Equal, 10% preferred Sugar-Free Natura, and 5% of consumers used saccharin. Amid the artificial sweeteners Natura was preferred while saccharin was least preferred. *Stevia rebaudiana* extract was concluded to be acceptable, and could aid diabetics and increase weight loss. In conclusion, *Stevia rebaudiana* extract was par or superior when compared to table sugar (Mogra and Dashora, 2009).

Another study conducted by Mogra and Dashora (2009) evaluated the preparation methods of *Stevia rebaudiana* extract to obtain the sweetness from the leaf. Extracts were prepared by boiling 25g of *Stevia rebaudiana* leaf powder in 500 ml of water for one and a half hours. The extracts were then reduced to 150 ml and used as a sweetener for the study. The sweet powder produced from the *Stevia rebaudiana* leaf contained 5 to 10% percent stevioside.

According to Swaminathan (1993), the likeness of the *Stevia rebaudiana* extract sweetness to sugar and other sweeteners was discerned through sensory evaluation by a panel of judges using the Ran Order Test. The sugar solution was used as a control and additional mixtures were prepared by using different sweeteners. The *Stevia rebaudiana* extract was analyzed for its equivalence with the sugar solution. Various food preparations such as milk, milk shake, curd, lemon pepper, lapsi (sweet dalia or a healthy Indian porridge), custard, carrot halwa (carrot and milk dish), tea, and coffee, were prepared by using *Stevia rebaudiana* extract and found to be equal to other sweeteners or even superior for some characteristics compared to table sugar.

***Stevia rebaudiana* - Propagation:**

Stevia rebaudiana propagation can be achieved from either seed or stem cuttings. Germination rates for *Stevia rebaudiana* are generally poor and seedlings are very slow to establish (Brandle et al., 1998). Seed germination is an important factor that limits large-scale cultivation (Geottemoeller and Ching, 1999). Seeds can be found in slender achenes, around 3 mm (0.11 inches) in length. Twenty bristle like structures are found on each seed called pappus bristles (Ramesh et al., 2006). *Stevia rebaudiana* is considered to have two seed types: pale or clear, or a dark, tan color. The clear seeds are considered to be infertile while the dark are considered fertile (Lester, 1999). It is best to grow *Stevia rebaudiana* as an annual or perennial transplant crop (Brandle et al., 1998). Field establishment of healthy, vigorous plants is essential for large scale agricultural production of *Stevia rebaudiana* (Carneiro et al.,1997). Direct seeding is not advised because of small seed size and the high percentage of empty achenes resulting in low germination percentage. Subtropical regions of the United States can grow *Stevia rebaudiana* as a perennial crop, but it must be grown as an annual crop in mid to high latitude areas where temperatures drop below freezing (Ramesh et al., 2006). In the temperate latitudes of the Northern Hemisphere, the production cycle for annual *Stevia rebaudiana* begins with 6 to 7 week old plants as seedlings, in cell packs, from heated greenhouses (Brandle et al., 1998). Once the seedlings are obtained in cell packs they are transplanted to the field in mid- to late-May. Fertilizer is band applied to transplants, and the crop is irrigated as required. *Stevia rebaudiana* is reluctant to establish under Canadian conditions with growth being sluggish until mid-July (Brandle et al., 1998).

In an experiment performed at the Iguatemi Research Station in India, seeds were germinated in multicellular trays and potted using 14 different soil mixtures in a greenhouse where the temperatures ranged from 9˚ to 36˚ C (48.2 ˚F to 96.8˚ F) (Carneiro et al., 1997). The seedlings were watered as needed. Fresh laying hen manure was watered daily for 15 days on a transparent plastic film for aerobic decomposition of the material. The chemical analyses indicated the manure had 186 g kg^{-1} water, 335 g kg^{-1} organic matter, 28 g kg^{-1} total nitrogen, 194 g kg^{-1} total carbon, C/N = 6.1, pH = 7.2, P_2O_5= 3.7% , and K_2O = 2.5%. After natural drying, it was ground and mixed (10% vol/vol) into sand clay loam soil (LEd2) or heavy clay soil (LRd2). Lime was added at 1.4 g kg^{-1} as $CaCO_3$, and 0.35 g kg^{-1} as $MgCO_3$. Certain mixtures were fertilized with 50 or 100 mg dm^{-3} N, and 150 or 300 mg dm^{-3} P, and 75 or 150 mg dm^{-3} K as ammonium sulphate, superphosphate (single), and potassium chloride, respectively.

The 14 different soil mixtures consisted of different mixtures of nitrogen, phosphorus, and potassium along with other nutrients: N_1= 50, N_2 = 100, P_1 = 150, P_2 = 300, K_1 = 75, K_2 = 150 mg dm^{-3}; lime (Li) = 1.4 g CaCO3 + 0.35 g kg^{-1} MgCO3; laying hen manure (LHM) = 10%, vol/vol; LEd_2 = Red Dark Latossol, sandy clay loam soil and LRd2 = Red Latossol, heavy clay soil. The greatest shoot dry weight (168.0 mg) was obtained with the mixture of LEd_2 soil, 10% laying hen manure, and the addition of lime. The physical and chemical analyses of this soil mixture were composed of 420 g coarse sand, 330 g kg^{-1} fine sand, 20 g silt, 230 g clay, showed density = 0.81, porosity = 51%, pH = 6.6, Al^{+3} = 0.1 cmol; H + Al^{+3} = 1.79 cmol; Ca^{+2} + Mg^{+2} = 4.35 cmol; Ca^{+2} = 3.15 cmol; K = 2.58 cmol; P = 660 µg cm^{-3} and 14.7 g carbon. There was no improvement in seedling growth when mixtures containing laying hen manure and sandy clay loam soil (LEd$_2$) were chemically fertilized with 100, 150 and 300 mg dm^{-3} N-P-K. The worst growing medium from this experiment was the heavy clay soil (LRd2) only. These soils would be hard to replicate here in the Southern United States (Carneiro et al., 1997).

Another study looked at the effects of temperature and light on *Stevia rebaudiana* seed germination in Pisa, Italy (Macchia et al., 2006). Five *Stevia rebaudiana* accessions were evaluated. Three from Brazil: B1, B2, and B3, and two from Paraguay P1 and P2. The seeds were placed in petri dishes in a heat controlled cabinet at alternating temperatures and light conditions using cool white fluorescent lamps. Accession P1 had the highest germination percentage regardless of temperature (20/30˚C), with the germination rates only seldom dropping below 70%. Accessions P2 and B3 were among those with the lowest germination rates regardless of temperature .

Light on germinationwas evaluated in 4 different plant in the family asteraceae plants (which is the same family as *Stevia rebaudiana*) *Millotia myosotidifolia* (Benth), *Podotheca gnaphaliodies*, *Podotheca chrysantha* (Steetz), and *Ursinia anthemoides L.*) (Schutz et al., 2002). Germination was determined under light or no light and at 10˚C or 25˚C after 0, 40, and 126 days of storage under controlled conditions. Batches of 50 seeds of each species were placed in 5-cm petri dishes containing filter paper. The paper was wetted with de-ionized water. Dark treatments were immediately wrapped in a double layer of aluminum foil. Tests were carried out in cooled incubators. Seed weight impacted seed germination. *U. anthemoides* and *P. gnapaliodies*, species with the largest seeds, germinated best in the dark. *M. myosotidifolia*, which had the smallest seed of the species tested was almost exclusively confined to germinating in light. *P. gnaphaliodies* had good germination in dark but no germination under light at a constant temperature of 25˚C (Schutz et al., 2002).

A study conducted in Ethiopia produced transplants from stem cuttings of 6-month old *Stevia rebaudiana* plants (Kassahun et al., 2013). This experiment was conducted using two Farmer's Research Groups (FRG's) having 10 members each. The experiment used 3 stem portions (top, middle, and bottom) of *Stevia rebaudiana* and compared node number (three, five, seven, or nine nodes). In both farmers' nursery sites, there were 20 pots per treatment in each replication. Data were collected on cutting survival, percent survival, number of branches per rooted cutting, number of leaves per branch, and number of leaves per liner. Cutting position, node number, and nursery site management influenced stem cutting propagation of *Stevia rebaudiana*. Greatest cutting survival, cutting survival percent, number of branches/seedlings, number of leaves/branches, and number of leaves/seedlings occurred for top position (terminal) cutting position, while lowest were recorded for bottom(medial cuttings) position. Using top (terminal) cutting with three nodes was suggested for the development of quality stem cuttings of *Stevia rebaudiana*.

A study in India looked at different growth regulators on *Stevia rebaudiana* stem cutting production in different types of structures including mist house, polytunnel, shade house, and under open conditions (Ingle, 2008). Three IBA concentrations were evaluated: 300, 400, and 500 ppm as a basal dip. Interactions were noted between the different environment and IBA concentration. Stem cuttings receiving 500 ppm IBA had higher values than other IBA concentrations for all root parameters. Improvements in root characteristics may have been caused by tissue sensitivity and higher rooting numbers by increased internal free IBA generating an increase in the number of roots. Stem cuttings in the mist house and 500 ppm IBA had the highest root parameters followed by shaded polytunnel combined and treated with 500 ppm IBA.

Stevia rebaudiana **- Photoperiod:**

Some work has been done on *Stevia rebaudiana* evaluating optimal photoperiod. *Stevia rebaudiana* is a short day plant based on work looking at short day interrupted light treatments (Hamner and Bonner, 1938). Growing in its native habitat, *Stevia rebaudiana* flowers from January to March in the Southern Hemisphere and from September to December in the Northern Hemisphere. Plants with 4 to 12 pairs of leaves can be induced to flower with photoperiods shorter than 13 hours, but remain vegetative in photoperiods longer than 14 hours (Valio and Rocha, 1977). A minimum of 2 inductive short-day cycles are necessary for flower induction. This can be important due to the amount of steviosides at different vegetation and reproductive stages (Valio and Rocha, 1977).

In a study conducted by Healy and Graper (1992), *Stevia rebaudiana* plants grown under photoperiods of less than 12 hours produced generative umbels that were branched. When the photoperiod exceeded 12 hours, flowering was delayed, while 14 or 16 hour photoperiods inhibited floral initiation as indicated by the number of nodes that extended past the terminal flower.

***Stevia rebaudiana* – Planting Method:**

Stevia rebaudiana natively grows in infertile, acid sand or muck soils yet it will grow well on a wide range of soils given a consistent supply of moisture and adequate drainage (Ramesh et al., 2006). In a Brazilian study (Carneiro et al., 1997), shoot dry weight of seedlings (*Stevia rebaudiana* plants grown in the experimental field at Iguatemi Research Station were germinated in multicellular trays, with cell size of 112 cm^3) performed better in mixtures containing a sandy clay loam soil as the principal component. Greatest shoot dry weight was obtained with the mixture of this soil type and 10% laying hen manure by volume amended with lime. The same study showed laying hen manure was a suitable substitute for chemical fertilizer in the production of *Stevia rebaudiana* transplants, but needed special care with the water supply to the transplants due to an apparent decrease in the root/shoot ratio.

With a study conducted in Bangalore, India, the effects of planting method (flat bed versus ridge and furrow) and N-P-K fertilizer rate on foliage yield and uptake of nutrients by *Stevia rebaudiana* growing on a sandy clay loam soil were evaluated (Chalapathi et al., 1997). The ridge and furrow method of planting gave marginally higher yields and nutrient uptake than the flat bed method, but commercial differences were not observed. Nutrient uptake increased with increasing application rates of N-P-K. The highest uptake of N, P, and K was 190.1, 8.83 and 161.75 kg/ha, respectively.

In the country of Georgia, a study was conducted on growing methods in a territory known as Abkhazia, which lies on the eastern coast of the Black Sea

(Gvasaliya et al., 1990). Winter protection was looked at along with medium type for rooting. Cuttings under two layers of polyethylene had the highest survival rate for stem cuttings of 75% survival. Rooting percentage was highest in perlite and lowest in a krasnozem soil. Krasnozem soils are classified as Australian soils due to their high free iron oxide content. Also, krasnozem soils in good condition have loose tilth, high permeability of both air and water, reasonable plant-available water content, and low soil strength when moist. Current year's stem cuttings taken from the leaf axils performed the best while growing in a perlite/krasnozem substrate.

***Stevia rebaudiana* – Hydroponic Production:**

Some research has been conducted raising seedlings of *Stevia rebaudiana* hydroponically. In China, seedlings of *Stevia rebaudiana* were raised on matrices of sand or slag and sprinkled periodically with three different nutrient solutions (Yulin et al., 1992). Seedlings grown on the matrix of sand and sprinkled with Knop nutrient solution (a nutrient solution used in plant research and containing precise amounts of calcium nitrate, potassium nitrate, magnesium sulfate, monobasic potassium phosphate, and potassium chloride dissolved in water) had a stronger, well-developed root system, obvious spindle-shaped root tubers, less plant diseases, and no insect pests or weeds compared to other treatments. This method proved better than the control.

***Stevia rebaudiana* – Environmental Impacts:**

When considering biotic stresses for this plant, Thomas (2000) reported diseases like powdery mildew, dampening off, and stem rot were significant for *Stevia rebaudiana*. Many different diseases have been seen around the world for this plant. Some nurseries in Northern Italy grow this plant in pots. In February 2008, plants that were three months old started showing signs of an unknown disease (Garibaldi et al., 2009). Plants were in plastic pots in a glasshouse with benches that were heated. Plants exhibited brown spots that spread across the surface of the leaf. The crown and stem showed signs of infection, and the pathogen developed ample amounts of soft, gray mycelium on the leaf surface and stems. The plants also began to show the myceliol growth in the center of the heads which was determined to be a gray mold brought on by *Botrytis cinera*.

In Canada, Septoria Leaf Spot (*Septoria lycopersici*) has been observed on *Stevia rebaudiana* (Reeleder, 1999). The disease was observed in fields and in research plots in Ontario and British Columbia. The plants had angular, shiny grey lesions that grew fast and were encompassed by a chlorotic circle. Leaves became necrotic and dropped from the plants soon after. The disease showed an upward progression through the plant.

Ten specimen plants were isolated (five from each Canadian providence) and compared in respect to their conidia size. Prior to this work, *Septoriae steviae* had previously only been reported in Japan. The Canadian specimens were grown on potato dextrose and V-8 agar medium. They began to produce colonies with brownish-grey margins and within the colonies. White mycelium grew that, as the colony aged, turned a grey-olive color. The isolates from Japan and Canada showed signs of being similar and it was determined that the Canadian specimens belonged to *Septoriae steviae.*

Research Objectives

With *Stevia rebaudiana*, much more research is needed to optimize the potential production of this plant as a specialty crop in the Southern United States. *Stevia rebaudiana* is likely to become a major source of high potency sweetener for the growing world food market (Dubey and Haider, 2007). *Stevia rebaudiana* has been looked at extensively all over the world, but more information on how to produce the plant in the southern United States is needed. In December 2012, an article by Southeast Farm Press Magazine told of very successful *Stevia rebaudiana* production in both Georgia and North Carolina by the company, Sweet Green Fields Inc. The company mentioned wanting to see 1,000 acres produced commercially in the southeast in the next few years (Robertson, 2012). Production begins with propagation methods, both seed and stem cuttings, and will be the first step to seeing how the plant behaves under common practices used in the southern United States. Objectives of this work were to look at the effects of light or substrate on seed germination of *Stevia rebaudiana* and to determine the best method to root healthy stem cuttings based on cutting type and rooting medium.

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CHAPTER II

EFFECT OF PHOTOTROPIC RESPONSE AND SUBSTRATE TYPE ON SEED GERMINATION OF *STEVIA REBAUDIANA* (BERT.)

Abstract:

Seed germination for *Stevia rebaudiana* (BERT.) is typically poor with seeds planted right after harvest. Due to a variety of reasons, such as, low viability, fungus produced on pappus bristles of seed coats, and poor pollination in some areas. Little literature is available on *Stevia rebaudiana* seed propagation. Three experiments looking at seed germination with light or without light, in 4 substrate types were completed. Substrates of 50% pine bark:50% peat moss, 100% sand, 100% perlite, and 100% vermiculite by volume were evaluated. Cell pack trays with poly lids either blacked out or left clear were used. In Experiment 1, only substrate influenced seed germination with the sand substrate having higher germination than 100% vermiculite and 50%:50% pine bark:peat moss treatments. Experiments 2 and 3 had interactions between light and substrate. Under no light conditions, 100% vermiculite and 100% perlite performed best for seed germination for *Stevia rebaudiana* seeds. One hundred percent sand/light consistently had the highest germination rates at experiment termination, 14 days after seeding (DAS). Fungal growth on the pine bark: peat moss substrate and substrates such as 100% vermiculite/light had the lowest germination rates. Seeds germinated under both light and no light conditions. One hundred percent

sand/light is recommended since most growers germinate seeds in a greenhouse under light conditions

Index Words: dark, cotyledons, light levels, propagation

Significance to the Nursery Industry:

Stevia rebaudiana has the potential to become a major specialty crop in the southern United States because of the growing conditions and interest in *Stevia rebaudiana* as a major source of high potency sweetener for the growing world food market (Himanshu and Haider, 2007). With interest in *Stevia rebaudiana* rising in the United States, growers need quick, inexpensive ways to produce this plant. *Stevia* needs a long growing season, minimal frost, high light intensity, and warm temperatures (Shock, 1982). One way to maximize *Stevia rebaudiana* production is to improve seed germination to produce transplants. Germination substrates in this study were chosen to resemble soils that *Stevia rebaudiana* natively grows in and components that growers have access to in the southern United States. The cost reduction nurserymen and farmers would receive germinating their own seedlings versus either buying micro-propagated plants or micro-propagating plants themselves could be substantial. Results indicate that the industry could produce suitable seedlings with substrate already available such as sand and perlite. Sand was consistently one of the best substrates and it is available and cheap. This information enables growers to produce *Stevia rebaudiana* from seed more efficiently.

Introduction:

Poor seed germination is an important factor that limits large-scale cultivation of *Stevia rebaudiana* by seed (Geottemoeller and Ching, 1999). *Stevia rebaudiana* seeds are found in slender achenes, around 3 mm (0.11 inches) in length. Twenty bristle like structures are on each seed called pappus bristles (Ramesh et al., 2006). *Stevia rebaudiana* is considered to have two seed types: pale or clear, and the second, a dark, tan color. Clear seeds are considered to be infertile while the dark, tan seeds are considered fertile (Lester, 1999). It is best to grow *Stevia rebaudiana* as an annual or perennial transplant crop (Brandle et al., 1998). Field establishment of healthy, vigorous plants is essential for large scale agricultural production of *Stevia rebaudiana* (Carneiro et al.,1997). Direct seeding is not recommended because of small seed size and the high percentage of empty achenes.

A germination study was conducted using two lots of seeds collected from cloned *Stevia rebaudiana* plants in China (Goettemoeller and Ching, 1999). Seed were divided into two lots based on color, black versus tan. Once the seeds were separated, germination was tested utilizing 100 seeds from each lot. The seeds were placed between paper towels in a nursery flat, covered by a plastic dome. The temperature for all tests was 24°C (75.2°F). Fluorescent lights were placed 15 cm (5.90 inches) above the seed outside the plastic domes. Twelve days after seeding, germination was determined. Black seeds had higher germination percentages than tan seeds, and light had an effect increasing the germination percentage of black seeds, but not tan seeds.

In a second experiment within the same work, Geottemoeller and Ching (1999) looked at 5 pollination treatments: cross-pollination by bumblebees in a cage, cross pollination by hand, cross pollination by wind from a fan, self-pollination by hand, and a control group isolated from other genotypes. They found that pollination required some active manipulation of the blossoms and increased seed germination of black seeds.

Another study looked at the effects of temperature and light on *Stevia rebaudiana* seed germination (Macchia et al., 2006). Five *Stevia rebaudiana* accessions were evaluated. Three from Brazil: B1, B2, and B3, and two from Paraguay P1 and P2. The seeds were placed in petri dishes in a heat-controlled cabinet at alternating temperatures and light conditions using cool white fluorescent lamps. Within the temperatures tested, accession P1 had the highest germination percentage, with the rates seldom dropping below 70% germination. Accessions B3 and P2 were among those with the lowest germination percentages. The highest germination rate for B3 was 44%, with temperatures of 20°C in presence of light after pre-chilling, while P2 had a maximum germination rate of 35%, achieved at the alternating temperature of 15 to 25°C in the presence of light without pre-chilling. Based on this work we wanted to look at using sunlight in a greenhouse versus a cool white fluorescent lights.

Medium aeration also plays a role in seed germination and is impacted by substrate type. Gas exchange between germination medium and embryo is essential for rapid and uniform germination. Oxygen is essential for respiratory processes in germinating seed. Oxygen uptake by seed can be measured shortly after imbibition begins. The rate of oxygen uptake is an indicator of germination progress and has been suggested as a measure of seed vigor. Oxygen supply is limited where there is excessive water in the substrate. Carbon dioxide is a product of respiration and under conditions of poor aeration can accumulate in the soil. At deeper soil depths increased CO_2 may inhibit seed germination to some extent, but usually plays a minor role in maintaining dormancy (Hartmann and Kester, 1997)

In an experiment performed in India, *Stevia rebaudiana* seeds were germinated in multicellular trays using 14 different substrates under greenhouse conditions where temperatures ranged from 9 to 36˚C (48.2° F to 96.8° F) (Carneiro et al., 1997). Seedlings were watered as needed. The 14 different soil mixtures consisted of different mixtures of the following: N1= 50, N2 = 100, P1 = 150, P2 = 300, K1 = 75, K2 = 150 mg; lime (Li) = 1.4 g CaCO3 + 0.35 MgCO3; laying hen manure (LHM) = 10%, vol/vol; LEd2 = Red dark latossol, sandy clay loam soil and LRd2 = Red latossol, heavy clay soil. The greatest shoot dry weight (168.0 mg) was obtained with the mixture of LEd2 soil, 10% laying hen manure, and lime. The physical and chemical analyses of this mixture was 420 g coarse sand, 330 g fine sand, 20 g silt, 230 g clay, showed density = 0.81, porosity = 51%, pH = 6.6, Al+3 = 0.1 cmol; H + Al+3 = 1.79 cmol; Ca+2 + Mg+2 = 4.35 cmol; Ca+2 = 3.15 cmol; K = 2.58 cmol; P = 660 µg and 14.7 g carbon. The worst growing substrate from this experiment was the heavy clay soil. This steered us away from using anything similar in our experiments.

As interest in natural products continues to rise, the demand for *Stevia rebaudiana* should increase as well. The southern part of the United States has environmental conditions that should be suitable for production of *Stevia rebaudiana* for natural sweetener production if quality transplants are available. However little information is available for producers on the best practices to start and grow seedlings for this crop. The total market value of *Stevia rebaudiana* as a sweetener in Japan is estimated to be around 2 to 3 billion yen/yr or 21.5 to 32.3 million in U.S. dollars. The objective of this study was to evaluate 4 reproducible substrates and to determine whether light is required for germination of *Stevia rebaudiana* seeds.

Material and Methods:

There were three experiments conducted in the work. Experiment 1 was conducted at the Paterson Greenhouse Complex (Latitude: 32.600147, Longitude: -85.488011) and Experiments 2 and 3 at the Plant Sciences Greenhouse Complex (Latitude: 32.588153, Longitude: -85.489009). Both locations were on the campus of Auburn University. Seeds were purchased through Horizon Herbs (Williams, Oregon). Trays contained one of four germination substrates under light or no light conditions. The four germination substrates used were 50% pine bark:50% peat moss, 100% sand, 100% perlite, and 100% vermiculite with no amendments added. All experiments used 32 cell pack trays. The seeds usually have around 20 persistent pappus bristles, that may inhibit germination, were removed before being shipped. The seeds were counted into lots of 12 and then put into vials for sowing. The seed in each vial were placed into a single cell of a 32 cell pack tray. The trays were misted three days before sowing using a mist nozzle until the medium surface had standing water. Once seeds were sown they were misted once a day for the duration of each experiment. Clear tops were used for the trays receiving light and those to receive no-light were painted black for Experiment 1. Black trash bags were used to cover the tops in Experiments 2 and 3 for no-light treatments because the painted tops began to contort in experiment 1. Analog meat thermometers were put into the bottom of each tray to record substrate temperatures daily. Seeds were considered germinated when cotyledons first appeared in each experiment. Data were subjected to ANOVA and means separated by Tukeys Studentized Range test ($p \leq 0.05$) in a statistical software package (SAS ® JMP 10.0.2 Cary, N.C.).

Results and Discussion:

Experiment 1

Experiment 1 began on January 26 and was terminated February 9, 2011. Seed germination began, regardless of treatment, 3 days after seeding (DAS) and by 6 DAS all treatments experienced some seed germination (Table 2.1). Tray covers were removed 12 DAS for all treatments. There was no interaction between substrate and light on seed germination during the experiment, therefore only main effects of substrate and light will be discussed. Maximum seed germination for all treatments occurred by 12 DAS. Light treatment only affected seed germination on 12 DAS, with the no-light treatment having greater seed germination than the light treatment.

Substrate impacted seed germination on all observation dates 3 DAS to termination of the study (Table 2.1) The 100% sand treatment consistently had the highest seed germination, but was similar on all dates to germination in the 100% perlite treatment. Seed germination for the 50% pine bark:50% peat moss and 100% vermiculite was lowest on all observations dates 3 DAS and beyond with the exceptions of 12 and 14 DAS where seed germination was similar to the 100% perlite treatment.

Experiment 2

The second experiment began on April 25 and was terminated on May 23, 2011. As in Experiment 1, seed germination began, by 3 DAS regardless of treatment (Table 2.2). Tray covers were not removed for the duration of this study. There was an interaction between substrate and light on all observation dates after 1 DAS. Maximum seed germination for all treatments occurred by 14 DAS. On 3, 7, and 8 DAS the 100% vermiculite/no light treatment and the 100% sand/light treatment had greater seed germination than the 100% perlite/light and vermiculite/light treatments. However, by 14 DAS all treatments were similar expect for the 100% vermiculite/light treatment. The vermiculite/light treatment had the worst germination for *Stevia rebaudiana* seeds.

When comparing Experiment 1 to Experiment 2 , 100% sand/light resulted in a higher germination rate for *Stevia rebaudiana* seed even though there was no interaction. Experiment 2 occurred during the warmer months, April and May. The material used for the no light treatments was different as well. These parameters had an effect on the germination and therefore an interaction could be seen with substrate and light conditions. Also, seeds germinated one day earlier in Experiment 2 with warmer temperatures in the greenhouse. Average substrate temperatures of trays receiving no light were: perlite - 80.8˚F, vermiculite – 81˚F, peat/pinebark - 80.4˚F, sand – 83.7˚F. Average substrate temperatures of trays receiving light were: perlite – 80.9˚F, vermiculite – 84.8˚F, 50:50 peat:pinebark – 83.3˚F, Sand – 83˚F.

Experiment 3

Experiment 3 began on June 13 and was terminated July 23, 2011. By 7 DAS, every treatment had some seed germination (Table 2.3). Tray covers were not removed for the duration of this study. There was an interaction between the main effects of substrate and light on all observation dates other than 1 DAS.Ccompared to all other treatments, and the 100% perlite/no light and 100% sand/ light had the greatest seed germination 4 DAS to termination. One hundred percent vermiculite/light treatment had the least germination. When looking at this study compared to Experiment 2, both studies had an interaction between substrate and light. Experiment 3 was conducted during warmer months of the year, June and July, which had more hours of sunlight each day. These parameters had an effect on the germination and therefore an interaction could be seen with substrate and light conditions for germination. Average substrate temperatures of trays receiving no light: 100% perlite - 87.5˚F, 100% vermiculite - 86.6˚F, pinebark:peat - 87.9˚F, and 100% sand - 83.8˚F. Average substrate temperatures of trays receiving light: 100% perlite – 85.6˚F, 100% vermiculite – 86.9˚F, 50% pine bark:50% peat moss – 88.5˚F, and 100% sand – 89.6˚F. Compared to Experiment 2, all average temperatures were about 6 ˚F higher except 100% sand/no light. Lower seed germination counts were seen across all treatments in Experiment 3 compared to Experiment 2.

Conclusions:

One hundred percent sand and 100% perlite consistently had the best germination in experiment 1 and 2. Because of the interactions, and the fact that the highest germination occurred in a treatment with and without light the seed does not appear to need a specific light treatment to germinate. Germination in cooler months in a greenhouse in 100% sand yielded the best germination of *Stevia rebaudiana* seed. Light versus no-light did not have an effect so this will not matter for germination during this time of year. One hundred percent vermiculite/light had the lowest germination in both studies. The 100% perlite treatment does not hold water as well as other substrates no light will cut down on water loss and might be best used if the seeds would be under a timed irrigation once or twice a day.

In Experiments 2 and 3, 100% sand substrate yielded the greatest germination of seeds occurred at 12 to 14 DAS. With perlite, the greatest germination rates occurring in between 8 to 14 DAS. When looking at the two substrates impacted by light, 100% sand had the best germination rate in between 12 and 14 DAS. Based on all of the experiments looked at maximum germination occurred in between 10 to 14 DAS. Perlite reached its germination threshold between 8 and 10 DAS.

In the long term germination study, Experiment 2, 100% sand with light resulted in greater seedling viability than the other 3 substrates. This could help growers hold longer before transplanting . One hundred percent sand had better germination with light than without. One hundred percent sand/light was consistently the better treatment for seed germination in *Stevia rebaudiana*. One hundred percent perlite and 100% vermiculite had highest germination numbers with no light. Seeds did germinate under both light conditions. In a standard propagation protocol, 100% sand/light is recommended since most producers germinate seeds with light conditions rather than without. Using a rock based type of substrate is best to cut down on microbial and fungal growth, said to hinder germination of seeds, which was seen with the pine bark: peat moss. Fungal growth was seen on the pine bark: peat moss mix during all three experiments.

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Table 1. January 26 - Influence of 4 substrates and 2 light levels on germination of Stevia rebaudiana^{z}.

		1 DAS	2 DAS	3 DAS	4 DAS	6 DAS	7 DAS	8 DAS	10 DAS	12 DAS	14 DAS
Light	*Light*^{x}										
	No light	0.0	0.0	0.6	2.8	5.2	6.4	6.9	7.4	8.5 a	8.1
	Light	0.0	0.0	1.1	3.6	5.6	6.2	6.6	6.9	7.3 b	7.7
Substrate											
50:50 pine bark: peat moss		0.0	0.0	0.1 b	0.7 b	2.1 b	3.1 b	3.6 b	4.3 b	5.8 c	6.6 b
100% sand		0.0	0.0	1.4 a	5.1 a	8.5 a	8.9 a	9.8 a	9.4 a	10.3 a	9.8 a
100% perlite		0.0	0.0	2.0 a	5.9 a	8.2 a	8.6 a	9.5 a	9.0 a	8.8 ab	8.8 ab
100% vermiculite		0.0	0.0	0.1 b	0.8 b	2.7 b	4.3 b	4.8 b	5.9 b	6.8 bc	6.7 b
Substrate	*Light*^{x}										
50:50 pine bark: peat moss	No light	0.0	0.0^{w,ns}	0.0	0.0	1.6	4.0	4.4	5.4	8.0	7.3
100% sand	No light	0.0	0.0	1.5	4.4	8.1	8.3	9.6	8.8	8.7	10.1
100% perlite	No light	0.0	0.0	0.9	5.1	7.8	8.5	9.3	9.4	9.0	9.0
100% vermiculite	No light	0.0	0.0	0.1	1.3	3.1	4.8	5.1	6.0	6.8	8.0
50:50 pine bark: peat moss	light	0.0	0.0	0.1	1.2	2.5	2.6	2.8	3.1	4.6	4.4
100% sand	light	0.0	0.0	1.3	5.8	8.9	9.6	9.9	10.0	10.8	10.4
100% perlite	light	0.0	0.0	3.1	6.6	8.6	8.8	9.7	8.6	8.6	8.6
100% vermiculite	light	0.0	0.0	0.0	0.4	2.3	3.8	4.5	5.8	6.5	5.6
ANOVA											
					p-value						
Substrate*light		0.0	0.0	0.21	0.45	0.73	0.54	0.74	0.35	0.25	0.18
Light		0.0	0.0	0.12	0.21	0.54	0.78	0.63	0.47	0.02	0.55
Substrate		0.0	0.0	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.04

[z] Seeds were considered germinated when cotyledons appeared.

[y] DAS = Days After Sowing.

[x] All trays were covered with clear plastic lids, those receiving the no-light treatment were painted black.

[w] Means within column followed by the same letter are not different based on Tukey's Studentized Range Test at α = 0.05 (n=8).

[ns] Means in row not significantly different.

Table 2. April 25 - Influence of 4 substrates and 2 light levels on germination of Stevia rebaudiana[z].

		1 DAS	2 DAS	3 DAS	4 DAS	7 DAS	8 DAS	9 DAS	14 DAS
Light	*Light*[x]								
	No light	0.0	1.7	4.8 a	5.9 a	7.4 a	8.0 a	8.2 a	8.2 a
	Light	0.0	1.0	3.1 b	4.1 b	4.9 b	5.5 b	5.4 b	6.2 b
Substrate									
50:50 pine bark: peat moss		0.0	1.1	4.4	5.2 ab	6.8 ab	7.6 ab	7.5 a	7.6 a
100% sand		0.0	1.6	4.9	6.8 a	7.6 a	8.5 a	8.3 a	9.4 a
100% perlite		0.0	1.1	3.2	4.0 b	5.4 ab	6.1 bc	6.7 ab	7.7 a
100% vermiculite		0.0	1.4	3.3	3.9 b	4.8 b	4.8 c	4.6 b	4.1 b
Substrate	*Light*[x]								
50:50 pine bark: peat moss	No light	0.0 [w,ns]	1.2 ab	3.2 abc	3.5 abc	5.3 ab	6.7 ab	7.0 ab	7.7 a
100% sand	No light	0.0	0.0 b	3.7 abc	5.3 ab	6.9 ab	7.7 ab	8.0 ab	9.0 a
100% perlite	No light	0.0	2.3 a	5.3 ab	6.4 a	7.4 ab	7.6 ab	8.3 ab	8.1 a
100% vermiculite	No light	0.0	2.9 a	6.5 a	7.9 a	9.5 a	9.5 a	9.3 a	8.1 a
50:50 pine bark: peat moss	Light	0.0	1.1 ab	5.4 ab	6.5 a	8.9 ab	8.3 ab	7.9 ab	7.5 a
100% sand	Light	0.0	1.1 a	5.9 a	8.1 a	8.3 a	9.1 a	8.6 ab	9.8 a
100% perlite	Light	0.0	1.1 b	1.1 bc	1.6 bc	3.5 bc	4.5 b	5.1 b	7.3 a
100% vermiculite	Light	0.0	1.1 b	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c	0.1 b

ANOVA								
			p-value					
Substrate*light	0.0	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001
light	0.0	0.1319	0.0306	0.0177	0.002	0.0004	< 000.1	0.0002
Substrate	0.0	0.8494	0.2889	0.0243	0.04	0.0009	0.0006	<.0001

[z] Seeds were considered germinated when cotyledons appeared.

[y] DAS = Days After Sowing.

[x] All trays were covered with clear plastic lids, those receiving the no-light treatment were covered with black plastic.

[w] Means within column followed by the same letter are not significantly different based on Tukey's Studentized Range Test at α = 0.05 (n=8).

[ns] Means in row not significanlty different.

Table 3. June 13 - Influence of 4 substrates and 2 light levels on germination of Stevia rebaudiana[z].

		1 DAS	3 DAS	4 DAS	6 DAS	7 DAS	8 DAS	9 DAS	10 DAS
Light	*Light*[x]								
	No light	0.0	0.9 a	2.1 a	3.3	3.8 a	3.6 a	3.7 a	3.8
	Light	0.0	0.2 b	0.8 b	2.5	2.7 b	2.7 b	2.6 b	3.1
Substrate									
50:50 pine bark: peat moss		0.0	1.4	2.2	2.3 b	2.3 b	2.4 b	1.8 b	2.5 b
100% sand		0.0	1.3	3.8	4.2 a	4.2 a	3.9 a	4.5 a	4.7 a
100% perlite		0.0	2.3	4.1	4.9 a	4.9 a	4.9 a	4.9 a	5.1 a
100% vermiculite		0.0	1.1	1.5	1.9 b	1.9 b	1.7 b	1.6 b	1.7 b
Substrate	*Light*[x]								
50:50 pine bark: peat moss	No light	0.0 [w,ns]	0.1 c	1.0 bc	1.8 cd	1.6 de	1.6 cd	2.0 cde	2.5 b
100% sand	No light	0.0	0.5 bc	1.5 b	3.0 bc	3.6 bc	3.3 bc	3.6 bc	3.6 b
100% perlite	No light	0.0	1.6 a	3.9 a	5.3 a	6.3 a	6.1 a	6.1 a	6.0 a
100% vermiculite	No light	0.0	1.3 ab	2.1 b	3.0 bc	3.6 bc	3.4 b	3.0 cd	3.0 b
50:50 pine bark: peat moss	light	0.0	0.4 c	1.7 b	2.6 c	2.9 cd	3.0 bc	1.6 de	2.4 b
100% sand	light	0.0	0.1 c	1.1 bc	4.6 ab	4.7 ab	4.4 ab	5.2 ab	5.7 a
100% perlite	light	0.0	0.0 c	0.0 c	0.0 bc	0.1 bcd	0.0 bc	0.0 cd	3.8 b
100% vermiculite	light	0.0	0.0 c	0.0 c	0.0 d	0.1 e	0.0 d	0.3 e	0.4 c
ANOVA									
				p-value					
Substrate*light		0.0	0.0104	<0.0001	0.001	<0.0001	<0.0001	0.001	0.0007
Light		0.0	0.0010	<0.0001	0.096	0.015	0.0293	0.014	0.1035
Substrate		0.0	0.2605	0.258	0.001	<0.0001	<0.0001	<.0001	<0.0001

[z] Seeds were considered germinated when cotyledons appeared.

[y] DAS = Days After Sowing.

[x] All trays were covered with clear plastic lids, those receiving the no-light treatment were covered with black plastic.

[w] Means within column followed by the same letter are not significantly different based on Tukey's Studentized Range Test at $\alpha = 0.05$ (n=8).

[ns] Means not significantly different.

CHAPTER III

PROPAGATION OF *STEVIA REBAUDIANA* STEM CUTTINGS INFLUENCED BY SUBSTRATE AND CUTTING TYPE

Abstract:

An herb from Paraguay, *Stevia rebaudiana* (BERT.) is becoming popular in the natural sweetener market. *Stevia rebaudiana* can be 250 to 300 times sweeter than sucrose while containing no calories. Propagation is usually done by stem cuttings due to low germination rates with seed propagation. To produce plants that meet increasing demand, more efficient propagation techniques are being tested in order to generate the sufficient plants for transplanting.

To look at healthy plant propagation in the Southeastern United States an experiment was conducted to evaluate dealing cutting propagation. This first experiment looked at cuttings and the effect that substrate and cutting type have on successful propagation. The cuttings were taken from container grown stock plants and planted in 32 cell packs with one cutting per cell. Two node cuttings were placed in one of 4 types of substrates: 1:1 pine bark: sand by volume, 1:1 peat moss: perlite by volume, 100% sand, or 1:1 part sand: vermiculite by volume. Medial and terminal cutting types were evaluated. The cuttings were uniform with 2 nodes and cuttings were placed under mist for 15 seconds every 10 minutes for the first 4 weeks, then 5 seconds every 10 minutes the remaining 4 weeks. Data collected included: foliar color rating of both old and new foliage, shoot breaks over 2.54 cm (1 inch long), and root length. No interactions were found between substrates and cutting type therefore only main effects will be discussed. Greatest root length occurred in 1:1 sand: vermiculite by volume while 1:1 pine bark:sand had a greater root length yet was similar to other treatments. Medial cuttings had more shoot breaks than terminal cuttings regardless of the substrate used. Old and new foliage were given a a rating of 1-5 (scale of 1-5 with 1-dead, 3-yellow, 5-dark green). Pinebark:sand or sand:vermiculite would be recommended for use as substrates for liners as root length and new growth are important factors in producing liners. Removing apical dominance in liners would also be recommended.

Index words: medial stem cuttings, terminal stem cutting, rooting medium

Significance to the Nursery Industry:

Much of the ongoing work that is being conducted regarding *Stevia rebaudiana* is dealing with in vitro and micro propagation work. For farmers and nursery growers, this type of work is expensive to conduct and most of the time it is not feasible. Setting up a laboratory to conduct such work and also having the skill and labor for most producers cannot be justified. For a crop that could eventually be very beneficial in the south, more inexpensive yet equally successful propagation methods of seed and/or stem cuttings will have to be developed. By using substrates common to the Southern United States, the best substrate to use for stem cuttings can be identified. Seed germination can have low germination rates while cuttings can be slow to establish. This research examined different strengths of light for seed germination along with different substrates that could play a more helpful role in propagation of *Stevia rebaudiana*.

Introduction:

Propagation of *Stevia rebaudiana* is often done by stem cuttings, rooting easily, but requiring high labor inputs. For some varieties or selections, stem cuttings are the only way of multiplication for the plant (Yadav et al., 2010). Cuttings from new shoots of *Stevia rebaudiana* can be propagated successfully (Lee et al. 1979). Gvasaliya et al. (1990) reported that 98 to 100% rooting can be obtained for stem cuttings, when the current year's cuttings are taken from leaf axils. Cuttings from the top part of the main shoot with four nodes generally give the best results (Tirtoboma, 1988). The number of leaf pairs for the cutting, as well as the season of the year determines rooting percentage and growth. Cuttings with four pairs of leaves rooted poorly, especially in February. Cuttings with two pairs of leaves rooted best in February and those with three pairs of leaves in April (Zubenko et al., 1991).

When rooting stem cuttings, the substrate should be considered. There is not one type of substrate that is accepted universally or is an ideal rooting substrate for all stem cuttings. A suitable propagation substrate depends on several different factors including: plant species, cutting type, season, and mist system. As Smitha and Umesha (2009) discussed for *Stevia rebaudiana,* information on naturally available, cost effective rooting substrates, bio inoculants, and their interaction with the growing conditions for multiplication of *Stevia rebaudiana* has been scarce. Two big items to consider are the availability of the substrate components for an area and their cost.

A study was conducted in Ethiopia looking at producing liners using stem cuttings of *Stevia rebaudiana*. Six month old *Stevia rebaudiana* plants were used as stock plants from the Wondo Genet Agricultural Research Center. This experiment looked at three parts of the stem of *Stevia rebaudiana* plants as cuttings: the top, middle, and bottom portions. Also the number of nodes were examined, 3, 5, 7 and 9 per cutting. Results from this work indicate that the position of the stem cutting, node number per cutting, and nursery site management influenced stem propagation of *Stevia rebaudiana* (Kassahun et al., 2013).

A study in India looked at different growth regulators for *Stevia rebaudiana* stem cuttings production in different environments (Ingle, 2008). Three IBA concentrations as a basal dip were evaluated: 300, 400, and 500 ppm. Environments were mist house, polytunnel, shade house, and open conditions. Interactions between the IBA concentrations and different environments were observed. Stem cuttings receiving 500 ppm had higher values for all root parameters. Stem cuttings in the mist house treated with 500 ppm IBA had the highest root values. Plants in the shaded polytunnel combined with 500 IBA ppm had the next best results.

Survival count, survival percentage, number of branches/rooted cuttings, number of leaves/branches, and number of leaves/rooted cutting, on terminal cutting positions had the highest values while the lowest values were recorded for the bottom cutting position. Top cutting position were suggested for obtaining quality *Stevia rebaudiana* liners using stem cuttings in this work (Kassahun et al., 2013).

Vegetative propagation of *Stevia rebaudiana* was evaluated in Karanataka, India where 2 different environmental conditions and 4 substrates types (Smitha and Umesha, 2012). The environmental conditions were natural shade of a Singapore cherry (*Muntingia calubura* L.) and greenhouse conditions with intermittent mist (average temperature 30˚C and relative humidity at 85%). Substrates were: soil:sand (1:1), soil:sand:FYM (1:1:1), soil:sand:vermicompost (1:1:1), and soil:sand:coirdust (1:1:1). Six growth regualtors were also looked at along with bio-inoculants (control, IBA 500 ppm, IBA 1000 ppm, IBA 2000 ppm, *Trichoderma viride*, and cow's urine). The *Trichoderma viride* was mixed using 0.5 kg of culture in 0.5 liters of water. Fresh cow urine was diluted with water at 1:10 ratio. Three node cuttings were used by taking mature basal protions from current years stems. Cuttings were dipped (1 to 2 cm) in growth regulator solution for 10 seconds. Percent rooting, root length, and thickness were recorded by destructive sampling. When looking at the greenhouse treatment, early sprouting (20 days), maximum bud sprout (79%), maximum sprout length (23 cm), highest number of leaves per rooted cutting (27), highest dry weight of the shoot (3.04 g), highest rooting (77%), maximum number of roots (14), longest root length (8.02 cm), root girth (0.7 mm), and maximum root dry weight (0.37 g) were observed. Faster sprouting could be due to warmer temperatures and a higher relative humidity inside the structure. *T. viride* produced better results than the other growth regulators and bio-inoculents. *T. viride* showed the capacity to increase uptake of nutrients by releasing enzymes that make soil nutrients soluble. Stem cuttings of *Stevia rebaudiana* that were treated with *T.viride* and also planted in soil:sand:vermicompost substrate under greenhouse conditions produced the highest number of leaves (49.67) and maximum root length (12.29 cm).

A study conducted in Sirsi, India at the College of Forestry examined the affect of growth regulators on propagation of *Stevia rebaudiana* stem cuttings (Koppad et al., 2006). Different concentrations of IAA (100, 300, and 500 ppm) and coumarin were used on 10-15 cm stem cuttings were used in the study. Stem cuttings were defoliated up to 3 nodes and then slant cuts were used at the base of each cutting. Cuttings were dipped in each concentration of regulator for 5 minutes before sticking. The cuttings were planted in 4” x 6” polyethylene bags containing a substrate of 2:1:1 by volume ratio of soil, sand, and farm yard manure, respectively. Cuttings were kept in a glass house for 35 days. Percent rooting and root length were observed at 15, 25, and 35 days after sticking. The number of roots was highest for *Stevia rebaudiana* stem cuttings dipped in 500 ppm IAA followed by a 300 ppm coumarin dip. Thirty-five days after planting, IAA 500 ppm had the greatest root length (7.40 cm) followed by coumarin 500 ppm (7.00 cm).

During the past ten years a trend in America for more organic produce and less sugar, and fewer calories in many of the foods we enjoy. *Stevia rebaudiana* has become nationally recognized in the form of Truvia, a zero calorie sugar that is currently on the market made from *Stevia rebaudiana* as well as many other brand names incorporating *Stevia rebaudiana* are starting to hit the market. As *Stevia rebaudiana* gains more recognition, more work will have to be done to find the best ways to propagate and grow this plant. This study examines the effects of cutting type (medial versus terminal) and substrate type on rooting and growth of the *Stevia rebaudiana*. Two of the four substrates used in this study are sand based because the native soil in Paraguay is rich in sand. Pine bark is a substrate component that is used heavily in the Southeastern United States because of its cost and availability and we will also investigate this substrate component of stem propagation for *Stevia rebaudiana* will be investigated.

Material and Methods:

Container grown *Stevia rebaudiana* plants, located at the Paterson Greenhouse Complex on Auburn University's campus, were grown from a single seed source and used as stock plants for stem cuttings used in this study. On June 22, 2011, stem cuttings were taken with sterilized pruners, placed into plastic bags and sealed, placed on ice in a cooler, and transported to the Plant Sciences Research Facility on Auburn University's campus. A mist system was constructed previously for the cuttings with plastic drapes to maintain high humidity. The cuttings were placed into 32 cell pack trays and with each of the 4 substrates randomly arranged in each tray. The 4 substrates were: 1:1 pine bark:sand by volume, 1 peat moss:perlite by volume, 100% sand, or 1:1 sand:vermiculite by volume. Both medial and terminal stem cuttings were present in each tray. The experiment was a 4 x 2 factorial with 4 substrates and 2 cutting types. There were eight replications, all placed under mist. The study ran for eight weeks with mist times changed once during the study. For the first 4 weeks the cuttings were misted for 15 seconds every 10 minutes. On July 20, 2011, the mist system was adjusted to 5 seconds every 10 minutes. After eight weeks, stem cuttings were observed for foliar color of old foliage (foliage present when cuttings were taken) and foliar color of new foliage (new foliage that was produced during the eight week study). A subjective rating scale of 1 to 5 where 1 was dead, 3 was yellow, and 5 was dark green was used for both foliar evaluations. Number of shoot breaks while cuttings were in the study, over one inch long and root length were also counted. The longest root for each cutting in Experiment 1 was measured. The second experiment was put in July 3, 2012. This study lasted eight weeks and was terminated on August 29 2012. The second experiment looked at the average of the three longest root lengths per cutting was collected in Experiment 2.. Growth indices [(height + width1 +width2)/3] (cm) were measured at the end of each study.

Results and Discussion:

Experiment 1 was initiated June 22 and terminated August 19, 2011. The ambient temperature for this study ranged from 76-81˚F (24-27˚C). There were no interactions between substrate and cutting type for root length, number of shootbreaks, or new foliar color (Table 4).Pine bark:sand was the better substrate for root length, sand:vermiculite was the best for shootbreaks and sand:vermiculite, peat moss:perlite and pine bark:sand were similar for new foliar color. Stem cutting type showed medial stem cuttings worked better for all parameters. Medial cuttings had a greater root length, more shootbreaks, and a better new foliar color rating than terminal cuttings. Substrate also affected root length, number of shootbreaks, and foliar color ratings. Cuttings propagated in pine bark:sand substrate had the greatest root length when compared to other substrates. There were more shootbreaks for stem cuttings in sand:vermiculite substrate compared to those in other substrates.

During Experiment 2, the ambient temperature ranged from 76-82˚F (24-27˚C). For the first 4 weeks the cuttings were misted 15 seconds every 10 minutes and then 5 seconds every ten minutes for the remainder of the study. There were no interactions between substrate and cutting type for any parameter evaluated in the study and only main effects will be discussed (Table 5). When examining main effects, substrate showed to have an effect with only shootbreaks. One hundred percent sand showed to be better when looking at number of shootbreaks. Stem cutting type showed to only have an effect with old foliar color with medial cutting type showing to be better.

When comparing Experiment 1 and Experiment 2, using medial cutting type for propagation was the better method in both studies. There was only an interaction between substrate and cutting type in Table 4 where no interaction between substrate and cutting type was seen in Table 5. For shootbreaks, in both studies there was an interaction and 100% sand and sand:vermiculite showed to be the better substrates.

Pinebark:sand or sand:vermiculite would be recommended for use as substrates as root length and new growth are important factors in producing liners. Removing apical dominance in liners would also be recommended.

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Table 4. June 22nd. Influence of 4 substrates and 2 cutting types on stem propagation of *Stevia rebaudiana* ^{z}.

		Root Length^{y}	Shootbreaks^{x}	New Foliar Color^{w}	Old Foliar Color^{v}
Substrate					
Pine Bark:Sand		20.9 a	0.67 b	3.39 a	3.39 a
Peat moss:Perlite		15.8 c	0.47 b	3.23 a	3.18 a
100% Sand		17.1 bc	0.56 b	2.93 b	2.70 b
Sand:Vermiculite		17.9 b	1.14 a	3.16 a	3.12 a
Cutting type	*Cutting type*^{u}				
	Medial	18.8 a	1.2 a	3.34 a	3.32 a
	Terminal	16.8 b	0.2 b	3.01 b	2.87 b
Substrate					
Pine Bark:Sand	Medial	20.6	1.06	3.62	3.62 a
Peat moss:Perlite	Medial	16.3	0.84	3.28	3.28 ab
100% Sand	Medial	19.2	0.96	3.18	3.15 ab
Sand:Vermiculite	Medial	19.3	1.80	3.28	3.21 abc
Pine Bark:Sand	Terminal	21.1	0.28	3.15	3.15 abc
Peat moss:Perlite	Terminal	13.9	0.09	3.18	3.09 abc
100% Sand	Terminal	14.9	0.15	2.64	2.25 c
Sand:Vermiculite	Terminal	16.7	0.43	3.03	3.03 bc

ANOVA					
			p-value		
Substrate*cutting type		0.299	0.093	0.3806	<.0001
Cutting type		0.019	<.0001	0.0009	<.0001
Substrate		0.000	<.0001	0.0092	0.0527

[z] All cuttings had 2 nodes per cutting.

[y] The longest root was measured.

[x] Shootbreaks over an inch lonf were counted.

[w] New foliar color was foliage produced after cuttingd were stuck. Foliar ratings 1 to 5 scale (1 =dead, 3 = yellow, 5 = green foliage; no yellowing).

[v] Foliage existing as cuttings were taken. Foliar ratings 1 to 5 scale (1 =dead, 3 = yellow, 5 = green foliage; no yellowing).

Table 5. July 3. Influence of 4 substrates and 2 cutting types on stem propagation of *Stevia rebaudiana*^{z}.

		Average Root Length^{y}	**Shootbreaks**^{x}	**New Foliar Color**^{w}	**Old Foliar Color**^{v}
Substrate					
Pine Bark:Sand		10.9	0.04 b	2.70	2.95
Peat moss:Perlite		12.1	0.04 b	3.07	2.89
100% Sand		12.7	0.18 a	2.75	2.85
Sand:Vermiculite		12.7	0.14 ab	2.59	2.82
Cutting type	Cutting type^{u}				
	Medial	11.7	0.1	2.87	3.12 a
	Terminal	12.5	0.1	2.68	2.64 b
Substrate					
Pine Bark:Sand	Medial	10.6	0.06	2.85	3.12
Peat moss:Perlite	Medial	11.6	0.03	3.18	3.09
100% Sand	Medial	11.9	0.18	2.93	3.31
Sand:Vermiculite	Medial	12.6	0.15	2.50	2.96
Pine Bark:Sand	Terminal	11.1	0.03	2.53	2.78
Peat moss:Perlite	Terminal	12.7	0.06	2.96	2.68
100% Sand	Terminal	13.5	0.18	2.56	2.40
Sand:Vermiculite	Terminal	12.8	0.12	2.68	2.68
ANOVA					
		p-value			
Substrate*cutting type		0.797	0.947	0.3898	0.2204
Cutting type		0.120	0.856	0.1472	<.0001
Substrate		0.061	0.046	0.0518	0.8919

[z] All cuttings had 2 node per cuttings.

[y] The three longest roots were measured.

[x] Shootbreaks over an inch long were counted.

[w] New foliar color was folaige produced after cutting were stuck. Foliar ratings 1-5 scale (1 =dead, 3 = yellow, 5 = green foliage; no yellowing).

[v] Foliage existing as cuttings were taken. Foliar ratings 1-5 scale (1 =dead, 3 = yellow, 5 = green foliage; no yellowing).

Chapter IV

Final Discussion

The purpose of this study was to evaluate different propagation methods such as seed and stem cuttings for the potential specialty crop growers In the southeastern0 United States. A shift in consumers buying habits has shifted in the past ten years to include more natural, chemical free components in their diets. Stevia is one of those components as it is a naturally, zero calorie sweetener. Sugar has also been a major factor with the increase of diabetes in the United States. Both of these markets are showing favor towards using this sweetener. Interest has been growing along with demand and companies are starting to show up in the southeast such as Sweet Green Fields show major promise for this crop.

The ambient temperature Experiment 1 ranged from 76-81˚F. There was no interaction between substrate and cutting type for root length, number of shootbreaks or new foliar color (Table 3.1). Medial cuttings had a greater root length, more shootbreaks, and a better new foliar color rating than terminal cuttings. Substrate also affected root length, number of shootbreaks, and old and new foliar color rating. Cuttings in the substrate pine:bark sand had the greatest root length when compared to other substrates.Number of shootbreaks for cuttings in the sand:vermiculite have more shootbreaks over an inch long than the other substrates.

During Experiment 2 the ambient temperature ranged from: 76-82˚F (24-27˚C). For the first 4 weeks the cuttings were misted for 15 seconds every 10 minutes, and cuttings began to be misted for 5 seconds every ten minutes for the remainder of the study.

There was no interaction between substrate and cutting type for any parameter evaluated in the study and only main effects will be discussed (Table 3.2). Cutting type had no affect on average root length, shootbreaks or new foliar color rating. Medial cuttings were better than terminal for old foliar color rating. Results for substrate for substrate on average root length showed pinebark:sand to be best and peat:moss:perlite prodeuced the least average root length. Shootbreaks showed that sand:vermiculite was better than other treatments. Both foliar color ratings were worst for sand.

One hundred percent sand and 100% perlite consistently showed to be the best for all three experiments other than in Experiment 3. Because of the interactions, and the fact that the highest germination occurred in a treatment with light and without light the seed does not appear to need a specific light treatment. Germination in cooler months in a greenhouse in 100% sand yielded the best results regarding germination rate of *Stevia rebaudiana* seeds. Light versus no-light did not have an effect so this will not matter for germination during this time of year. As temperature starts to increase outside and the length of days begins to increase light did start to have an effect on the seeds germination. One hundred percent vermiculite with light had the lowest germination in both studies. The 100% perlite treatment does not hold water as well so this substrate in combination with no light will cut down on water loss and might be best used if the seeds would be under a timed irrigation once or twice a day.

Based on Experiments 2 and 3, I 100% sand had the greatest germination rate of seeds occurred at 12 to 14 DAS. With perlite, the greatest germination rates occurred in between 8 to 14 DAS. When looking at the two substrates impacted by light, 100% sand showed to have the best germination rate in between 12-14 DAS. Based on all of the experiments looked at maximum germination occurred in between 10-14 DAS. Perlite reached its germination threshold in between 8-10 DAS.

In the long term germination study, Experiment 2, 100% sand with light also showed that it could keep more seedlings alive longer than the other 3 substrates. This could help growers in case they cannot step up the seedlings or get them to the field as fast as they had once anticipated. One hundred percent sand had better germination with light than without. One hundred percent sand:light was consistently one of the better treatments. One hundred percent perlite and 100% vermiculite had highest germination numbers with no light. Seeds did show that germination occurred under both light conditions. In a regular propagation set up 100% sand:light would be suggested since most producers germinate seeds with light conditions rather than without. Using a rock based type of substrate is best to cut down on microbial and fungus growth which is said to hinder germination of seeds which was seen with the 50%:50% pine bark: peat moss. Fungal growth was seen on the 50%:50% pine bark: peat moss mix during every experiment. Adequate moisture of a rock based substrate would be recommended for germination. The 100% vermiculite treatment under both conditions showed to struggle with holding water therefore performing poorly as well.

Future work that needs to be considered is looking at pelletizing seeds and seed disease issues that might arise in the region. Also, how to obtain maximum leaf yield and maximize number of harvests per year. Mechanization of harvesting needs to be looked at to do as little damage to the plant between harvests. Finally, looking at the economic impact of a potential specialty crop such as *Stevia rebaudiana* compared to other crops grown in the south would be benefical.