GENETIC AND ENVIRONMENTAL EFFECTS ON GROWTH, RESIN AND
RUBBER PRODUCTION IN GUAYULE (PARTHENIUM ARGENTATUM, GRAY)

by

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Maren Elizabeth Veatch Blohm
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ABSTRACT

Guayule (Parthenium argentatum Gray) is a rubber producing plant native to the Chihuahuan Desert, which is currently being investigated as a source of hypoallergenic latex. Current efforts are focusing on increasing latex/rubber production in the plant by either manipulating the rubber biosynthetic pathway, altering agronomic practices to take advantage of environmental conditions that increase rubber synthesis, or both. Field and greenhouse studies were conducted to more fully understand the effect of genetic and environmental manipulation on rubber production in guayule. Three guayule breeding lines were transformed in order to increase the availability of the initiators of rubber synthesis. The tissue-culture-derived transgenic plants and their seed-generated progeny were grown in separate field experiments. Transformation with the genes for the initiators of rubber synthesis did not increase rubber concentration or yield. Height and width had high heritability estimates in the transgenic progeny and were the traits most correlated with rubber yield, while rubber concentration was poorly correlated with height and width. Greenhouse studies were conducted to understand why water stress and low night temperatures increase rubber concentration. Water stress increased the contribution of the stems to the total rubber in the plant and increased the bark to wood ratio of the stem. Most rubber is accumulated in the stems and these two effects of water stress contributed to the increased rubber concentration in water-stressed plants. Low night temperature reduced plant growth without a decrease in carbon exchange. Allocation of carbon fixation products to rubber synthesis rather than growth, contribute
to the high rubber production under low night temperatures. Contributions from both breeders and agronomists are needed to further improve guayule rubber/latex yield.
INTRODUCTION TO THE DISSERTATION

Guayule (*Parthenium argentatum* Gray) is a rubber producing plant native to the Chihuahuan Desert (Lloyd, 1911). An effort is currently underway to domesticate and commercialize guayule for the production of hypoallergenic latex (Thompson and Ray, 1989; Cornish et al., 2001). Rubber production has been increased both through breeding efforts (Ray et al., 1999; Dissanayake et al., 2004; Ray et al., 2005) and agronomic improvements (Foster and Coffelt, 2005), but improvements have been slow due to intermittent research and a very complicated reproductive system (Thompson and Ray, 1989; Ray et al., 2005). Researchers during the last two decades have learned much about the biochemical pathway that produces rubber (Backhaus, 1985; Archer and Audley, 1987; Cornish and Siler, 1995; Castillon and Cornish, 1999; Cornish, 2001), but many questions about the genetic and environmental factors that affect rubber production remain incompletely answered.

It has been demonstrated that rubber production in guayule is affected by genetic and environmental components (Thompson and Ray, 1989; Appleton and van Staden, 1991; Dierig et al., 2001; Coffelt et al., 2005). Of importance to breeders is how the rubber-producing potential of guayule can be improved through both traditional and transgenic methods, and if these traits will be passed reliably from generation to generation (Estilai et al., 1992; Ray et al., 1993; Dierig et al., 2001). Breeders need information on the heritability of traits associated with rubber concentration and yield (Ray et al., 1993; Dierig et al., 2001), so that selection can focus on the characteristics that have high genetic components (Estilai et al., 1992). Of equal importance to future
guayule growers are the environmental factors that also contribute to rubber production (Schloman et al., 1986a; Appleton and van Staden, 1991; Coffelt et al., 2005). Once the modes of action of these environmental effects are more fully understood, agronomic practices can be tailored to take advantage of and possibly manipulate favorable environmental conditions to maximize rubber production.

Rubber comprises less than ten percent of the plant biomass, leaving over ninety percent of the plant available for developing commercial applications, thereby increasing guayule’s potential economic value (Nakayama, 2005). One of the most abundant secondary compounds produced is resin, and uses for the resin have been and are the subject of research efforts (Bultman et al., 1991; Bultman et al., 1998; Nakayama et al., 2001; Nakayama, 2005). Therefore, it is also of great importance to understand genetic and environmental effects on resin production.

The research conducted for this dissertation includes four studies that focused on genetic and environmental effects on growth, and resin and rubber production in guayule. The two genetic studies evaluated the effects of transgene insertion into guayule both within and across generations. The two environmental studies focused on understanding why rubber concentration changes in response to water stress and low night temperatures. This dissertation includes these four studies, which have either been published or submitted for publication, and is organized in the following format: a general introduction, a literature review, a summary of the major methods, results, and conclusions from each study, and four appendices each describing the results of one of the studies.
APPENDIX A presents the evaluation of the first transgenic guayule field trials. Plants were transformed with the genes for the initiators of rubber synthesis, but no significant effects of the transgenes on growth or secondary compound production were found. These results indicate that other genes should be the focus of transgenic procedures to increase rubber production by manipulating the rubber synthesis pathway.

APPENDIX B examines the relationship of traits in the transgenic progeny to their transgenic parents. Weighted regression was used to calculate heritabilities for different traits related to growth and resin and rubber production. Progeny resin and rubber yield were highly correlated with parental height and width, which were the most heritable traits measured. We recommend that breeders increase resin and rubber yield indirectly by selecting for larger plants.

APPENDIX C examines how and why water stress increases rubber concentration in guayule. Distribution of secondary compounds was examined in guayule exposed to different levels of irrigation. Rubber concentration increased in water-stressed plants with a concomitant decrease in stem diameter and leaf to stem ratio. We propose that the increased rubber concentration is due to the higher contribution of stems to overall shoot rubber and a higher bark to wood ratio in the stems.

APPENDIX D evaluates the contributions of night temperature, carbon exchange, and plant age on rubber production during the winter. Secondary compound production and carbon exchange were measured in guayule grown under ambient winter and simulated summer night temperatures. Rubber concentration was highest when the plants were grown at the coldest night temperatures. This increase in rubber concentration is
thought to be due in part to minimal plant growth combined with levels of carbon exchange similar to that seen in warm night plants.
LITERATURE REVIEW

PLANT MORPHOLOGY AND HABITAT

Guayule (*Parthenium argentatum* Gray) is a rubber producing plant native to the Chihuahuan Desert of northeastern Mexico and southwestern Texas (Lloyd, 1911). The first collection was made by J.M. Bigelow and described by Asa Gray (Lloyd, 1911; Hammond and Polhamus, 1965). Guayule is a slow-growing perennial shrub of the composite family (West et al., 1991), which in the wild may live for as long as 40 or 50 years (McCallum, 1926). It usually grows symmetrically with many closely growing lateral branches (Lloyd, 1911). The leaves are a grayish green with a thick cuticle and are covered in trichomes (Lloyd, 1911; Gilliland et al., 1984). Leaf size and amount of lobing are dependent on water availability, with well watered plants having large, well lobed leaves (Lloyd, 1911). Leaves may stay on the plant for many months, even when they have dried, because the abscission layer is imperfectly formed (Lloyd, 1911). Flowering occurs in response to water (Lloyd, 1911) so that there are flowers at different stages of maturity throughout the plant (West et al., 1991). The flower heads produce achenes with seed that may be viable for years under good storage conditions (McCallum, 1926; National Academy of Sciences, 1977); however, the percentage of viable seed from each plant is generally low (Lloyd, 1911; Jorge and Ray, 2005). Guayule initially develops a taproot that may penetrate deep into the soil, but later develops an extensive lateral, fibrous root system, which is found very close to the soil surface to take advantage of infrequent precipitation (Lloyd, 1911; National Academy of Sciences, 1977).
The Chihuahuan Desert is a semiarid environment with an average annual rainfall ranging from 130 to 380 mm per year (McCallum, 1926; National Academy of Sciences, 1977; Nakayama, 1991), with the majority of the precipitation occurring in the late spring and early fall (Nakayama, 1991). The average high and low temperatures are 34 and 3 °C, respectively (Nakayama, 1991). Guayule may survive short exposure to temperatures as low as -15 °C, but with some leaf and root dieback (Mitchell, 1944; Nakayama, 1991). Guayule is found naturally on calcareous soils distributed between altitudes of 1,200 to 2,100 m above sea level, and grows best in well-drained, alkaline soil (Lloyd, 1911; Hammond and Polhamus, 1965; National Academy of Sciences, 1977).

HISTORY

The use of guayule rubber dates back to at least the 18\textsuperscript{th} century and earlier, when it was used by the Native Americans to form balls (Lloyd, 1911; Bonner, 1991). Around the turn of the 20\textsuperscript{th} century guayule was being used commercially in Mexico as an alternative to and a competitor of, rubber from the rubber tree (Hevea brasiliensis Müll. Arg.), which resulted in the widespread depletion of wild guayule stands (National Academy of Sciences, 1977). The first agronomic experiments occurred around this time in an attempt to domesticate guayule, to ensure a steady supply of the plant and halt the loss of wild plants (Lloyd, 1911; McCallum, 1926; Hammond and Polhamus, 1965).

The first intensive scientific research program was the Emergency Rubber Project (ERP) during World War II. The need for a native rubber source was made clear by the cut off of rubber supplies from Southeast Asia, which was the major supplier of rubber to the United States. In addition to research on guayule as a native rubber source, resources
were also put into the development of synthetic rubber. The major guayule research
effort came to an end shortly after the end of the war when natural rubber imports
resumed and synthetic rubber was developed (National Academy of Sciences, 1977).
Extensive research on guayule did not occur again for almost 30 years.

Interest in guayule as a rubber source was renewed during the oil crisis of the late
1970’s (Ray et al., 2005). Synthetic rubber is synthesized from petroleum products and
the rise in oil prices and the oil embargo threatened the stability of the synthetic rubber
market (Cornish, 2001). The future stability of the synthetic rubber market was also in
doubt because it is based on a non-renewable resource (Cornish, 2001). Although
synthetic rubber is suitable for many applications, it can not substitute for natural rubber
in many important applications (Davis, 1997, Cornish, 2001). Synthetic rubber has low
heat resistance and elasticity making it unsuitable for airplane tires and the side walls of
radial tires (Davis, 1997; Cornish, 2001). Synthetic lattices, especially the cheaper ones,
are unsuitable for many medical applications because of poor elasticity, high modulus,
high viral permeability, high incidence of tearing, and low resistance to puncture
(Handfield-Jones, 1998; Cornish, 2001; Murray et al., 2001).

The increased use of natural rubber latex from *Hevea* for medical purposes has
coincided with an increase in the incidence of Type I allergic responses, especially
among healthcare workers (Handfield-Jones, 1998; Cornish et al., 2001). Since synthetic
rubber latex cannot provide an adequate substitute for many medical applications
(Murray et al., 2001), there is a need and a potential market for hypoallergenic natural
rubber latex (Siler and Cornish, 1994; Cornish et al., 2001). Guayule latex has the same
properties that make *Hevea* latex suitable for medical applications, such as viral impermeability, break resistance, tensile strength, and elongation (Cornish and Lytle, 1999; Cornish, 2001). In addition, there is no cross reactivity between guayule and *Hevea* latex (Schloman et al., 1996; Schloman et al., 1997; Cornish et al., 2001).

Guayule latex contains 1.1 percent the amount of proteins found in *Hevea* latex, which reduces the risk of sensitization to guayule latex (Cornish et al., 2005). The current research focus is the cultivation of guayule in the arid Southwest of the United States as a source of hypoallergenic latex (Thompson and Ray, 1989). Guayule is also being considered as a rubber reserve (Cornish and Siler, 1996; Mooibroek and Cornish, 2000) in the event that *Hevea* rubber supplies from Southeast Asia, which provides 90% of the world’s natural rubber (Cornish, 2001), are threatened by political upheaval or disease, such as the leaf blight, which prevents its cultivation in South America (Davis, 1997; Onokpise, 2004).

In conjunction with research on increasing latex and rubber yield, researchers also are investigating ways to use guayule coproducts (Thames and Wagner, 1991; Nakayama, 2005). Uncompounded guayule rubber has higher elasticity and malleability than *Hevea* rubber at low temperatures (Cornish and Wood, 2002), which may make it more suitable for use in extreme cold. Latex/rubber usually comprise only three to ten percent of the plant dry weight, leaving the other 90 to 97 percent of the plant available for other commercial applications (Ray et al., 1999; Cornish et al., 2000; Nakayama et al., 2001; Nakayama, 2005). Guayule resin has shown great potential as an antitermitic (Bultman et al., 1998; Gutierrez et al., 1999; Nakayama et al., 2001) and an antifungal
compound (Bultman et al., 1991; Maatooq et al., 1996). The low molecular weight rubber has been tested as a non-volatile crosslinking agent in coatings (Thames et al., 1994; Gupta et al., 2001; Gupta et al., 2004). Guayulin A has shown promise as a biocidal agent (Watkins and Fronczek, 1985), and argentatin A is currently being investigated as an anticancer agent (Para-Delgado et al., 2005).

SECONDARY COMPOUND PRODUCTION

Both resin and rubber are formed by branches of the terpenoid biosynthesis pathway (Cornish, 2001). Resin, a mixture of trans-isoprenoid derivatives (Thompson and Ray, 1989), is a composite of acetone extractable compounds that are products of the terpenoid pathway that contributes to plant growth and development (Oh et al., 2000). Radio-labeling tests have shown that there is a constant supply of photosynthate going into resin production (Kelly and van Staden, 1993) and that isopentenyl pyrophosphate (IPP) is incorporated into the resin (Macrae et al., 1986). As expected, because they are also derived from IPP, the initiators of rubber biosynthesis are also precursors to compounds found in the resin (Ohnuma et al., 1994; Cornish, 2001). The resin is composed of a variety of compounds, such as guayulins, argentatins, plant hormones, and chlorophyll (Oh et al., 2000). One component of particular interest is guayulin A, a sesquiterpene cinnamic acid ester, which was found to cause contact dermatitis in guinea pigs (Rodriguez et al., 1981; Schloman et al., 1983; Watkins and Fronczek, 1985). There are three other known guayulins, but they occur at much lower levels than guayulin A and do not illicit the same sensitive reaction (Martinez et al., 1986; Sidhu et al., 1995).
The resin is an important component of guayule, making up anywhere from six to eleven percent of the plant biomass (Veatch et al., 2005). Resin is often found at much higher levels than rubber throughout the plant, and in different concentrations in different parts of the plant (Banigan et al., 1982; Sidhu et al., 1995; Teetor et al., 2003). The resin is stored in the leaves and in the resin canals of the stems and the roots (Gilliland and van Staden, 1983). The concentration of the guayulins and the other components of the resin vary by season (Schloman et al., 1986b); however, the overall resin concentration is much less affected by temperature (Jasso-Cantu et al., 1997) or water stress than rubber concentration (Hunter and Kelley, 1946; Allen et al., 1987).

In contrast, rubber concentration varies seasonally (Ji et al., 1993) and is affected by both temperature and water availability (Appleton and van Staden, 1991). Photosynthates are shuttled into rubber mainly during the winter when the plant is not actively growing (Benzioni and Mills, 1991; Kelly and van Staden, 1991; Kelly and van Staden, 1993). Rubber is a $\text{cis-1-4}$-polyisoprene, which is a polymer of IPP (Tanaka et al., 1996; Cornish and Siler, 1995). Acetate is a direct precursor to IPP through the mevalonate pathway (Backhaus, 1985; Macrae et al., 1986; Cornish, 2001), and the IPP units are added to the growing rubber molecule by rubber transferase (Backhaus, 1985; Cornish and Backhaus, 1990). Although IPP is the building block of the rubber molecule (Cornish and Siler, 1995), efficient production of the rubber molecule requires the presence of $\text{Mg}^{2+}$ as a cofactor for the rubber transferase (Siler and Cornish, 1993; Kang et al., 2000b; Oh et al., 2000) and an allylic pyrophosphate to initiate the rubber molecule (Cornish and Siler, 1995; Castillón and Cornish, 1999; Cornish, 2001).
In vitro, many different allylic pyrophosphates have been used to initiate rubber synthesis, including geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) (Archer and Audley, 1987; Cornish and Siler, 1995; Castillón and Cornish, 1999). The incorporation rate of IPP into rubber increases with the increased length of the initiator molecule, which makes FPP and GGPP the most effective initiators in vitro (Archer and Audley, 1987; Castillón and Cornish, 1999; Cornish, 2001). However, there is a delicate balance between incorporation rate and rubber molecular weight. If the concentration of the initiator is too high the chain transfer reaction is enhanced leading to a lower rubber molecular weight. Conversely, when initiator concentrations are not too high, molecular weight will be positively correlated with IPP concentration (Cornish, 2001). The most likely initiator in vivo is FPP, which not only has a high binding affinity for the rubber transferase (Kang et al., 2000a), but is also the only initiator located in the cytoplasm where rubber synthesis takes place (Backhaus, 1985; Cornish, 2001).

Over the years attempts have been made to isolate and clone the genes related to rubber synthesis for use in transgenic applications (Cornish and Siler, 1996). Although proteins have been located that enhance rubber synthesis in vitro and may be part of the rubber transferase (Backhaus et al., 1991; Siler and Cornish, 1994; Oh et al., 1999), attempts to confirm, sequence, and clone the rubber transferase gene(s) have been unsuccessful (Kim et al., 2004). Despite the lack of success in cloning the rubber transferase gene(s), other genes in the rubber synthesis pathway have been successfully isolated in bacteria and inserted into guayule. These genes include farnesyl
pyrophosphate synthase (FPPS) (Koyama et al., 1993), geranylgeranyl pyrophosphate synthase (GGPPS) (Ohnuma et al., 1994), and hexa-heptaprenyl pyrophosphate synthase (H-HPPS), which produces an altered form of GGPP (Ohnuma et al., 1996). Guayule plants transformed with these three genes have already been grown and evaluated in field trials in Arizona (Veatch et al., 2005). The 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene (HMGR) has also been cloned and recently inserted into guayule, and is currently being prepared for field testing (unpublished data).

Unlike *Hevea*, which stores its rubber within tube-like laticifers (Cornish, 2001), guayule stores the majority of its rubber in the bark parenchyma cells (Backhaus and Walsh, 1983; Gilliland et al., 1984). When the plant is young, rubber is accumulated in cells near the resin canals, but as the plant matures rubber is stored in the bark parenchyma (Backhaus and Walsh, 1983), and in much smaller amounts in other parts of the plant, such as the wood and leaves (Banigan et al., 1982; Gilliland and van Staden, 1983; Kuruvadi et al., 1997b). Within bark parenchyma cells, rubber is located mainly in the cytoplasm, and may eventually fill most of the cytoplasmic space in older stems (Backhaus and Walsh, 1983; Gilliland and van Staden, 1983; Gilliland et al., 1984).

**REPRODUCTION AND BREEDING OF GUAYULE**

Guayule, with a haploid chromosome number of 18, has a ploidy series ranging from diploid to tetraploid or more (Bergner, 1944; Thompson and Ray, 1989; de Rodriguez et al., 1993; Kuruvadi et al. 1997a). In both wild and cultivated settings, the majority of the plants are tetraploid, which are larger and produce more rubber than diploid plants (Kuruvadi et al., 1997a). Tetraploid guayule reproduces predominantly
through facultative apomixis (Ray et al., 1990; Ray et al., 2005). Apomixis is the formation of a viable embryo without fertilization; however, in guayule pollination is required for endosperm development (Esau, 1944; Thompson and Ray, 1989; Ray et al., 1990). The percent of apomixis in guayule varies by line and season, resulting in occasional sexual reproduction (Thompson and Ray, 1989; Keys et al., 2002). This occasional sexual reproduction, with outcrossing promoted by sporophytic incompatibility (Gerstel, 1950), generates and maintains genetic diversity within guayule populations (Naqvi, 1985; Ray et al., 1990; de Rodriguez et al., 1993; Kuruvadi et al. 1997c).

Over the years this variation has been exploited by plant breeders to improve the yield characteristics of guayule. New lines have been released within the last two decades that have outperformed the traditional varieties in California (Estilai, 1991; Estilai and Ray, 1991), Arizona (Ray et al., 1999), and Australia (Dissanayake et al., 2004), with additional field trials currently being conducted to evaluate new breeding lines (D.T. Ray, personal communication, 2005). There has also been some success in selecting guayule with increased resistance to *Verticillium* wilt (Ray et al., 1995).

Researchers have examined the heritability of various traits that contribute to rubber, resin, and overall biomass in order to improve breeding efficiency. Broad sense heritability estimates have been variable, with height, width, and fresh and dry weight having the highest heritabilities (Estilai et al., 1992; Dierig et al., 2001). Rubber and resin concentration usually have lower heritabilities, and are difficult to predict from year to year (Dierig et al., 1989a; Dierig et al., 1989b; Estilai et al., 1992; Sidhu et al., 1993;
Dierig et al, 2001). For all traits used for selection, after two years of growth, heritability was close to zero as environmental effects compounded and masked genetic variation (Dierig et al., 2001). Although rubber and resin concentration are poorly correlated with other morphological characteristics, rubber and resin yield are positively correlated with height, width, and fresh and dry weight (Dierig et al., 1989b; Ray et al., 1993; Sidhu et al., 1993; de Rodriguez et al., 2001).

In addition to more traditional improvement methods, researchers are also investigating the merits of transgenic technology for manipulating the rubber synthesis pathway. Successful transformation of guayule (Pan et al., 1996) followed many years of perfecting the tissue culture methods that could be used to proliferate the transgenic plants (Dastoor et al., 1981; Radin et al., 1982; Smith, 1983; Staba and Nygaard, 1983). One goal is to improve rubber production by increasing the availability of initiator molecules. The first field-tested transgenic guayule plants were transformed with genes that synthesize allylic pyrophosphates that are known to be initiators of rubber synthesis in vitro (Veatch et al., 2005). Another goal of transgenic technology is to increase the availability of IPP, since high levels of IPP result in high molecular weight rubber (Cornish, 2001) and may be a limiting factor in rubber synthesis (Veatch et al., 2005). This is being attempted by increasing the amount of the IPP precursor 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG) (Cornish, 2001), by inserting the HMG reductase (HMGR) gene. Guayule plants transformed with HMGR are currently being prepared for field testing. The most desirable target of transgenic technology is the rubber transferase, which has so far proved difficult to isolate and clone.
ENVIRONMENTAL EFFECTS ON GROWTH AND SECONDARY COMPOUNDS

In addition to genetic factors, which are the focus of selection, guayule growth and secondary compound production are also affected by environmental factors (Ray et al., 1993; Nakayama, 1991; Dierig et al., 2001; Coffelt et al., 2005). Resin production seems to be mainly a function of plant age and growth status and is produced year round (Kelly and van Staden, 1993; Foster et al., 2002). Rubber production in guayule is seasonal, and occurs mainly in the winter months when guayule is exposed to the lowest nighttime temperatures and the lowest precipitation (Fangmeier et al., 1985). There is evidence that rubber production in guayule is triggered either by cold night temperatures, water stress, or both (Schloman et al., 1986a; Appleton and van Staden, 1991; Ji et al., 1993; Cornish and Backhaus, 2003).

Water

In its native environment, guayule receives an average annual rainfall between 130 to 380 mm (Nakayama et al., 1991), with the majority of the precipitation occurring during the spring and summer (McCallum, 1926; Benzioni et al., 1989). Therefore guayule has a natural history of exposure to water stress, and can often survive years of very minimal precipitation by becoming dormant (National Academy of Sciences, 1977; Nakayama, 1991). Guayule responds to water stress with decreased growth (Mills et al., 1990; Nakayama et al., 1991), changes in leaf morphology (Benedict et al., 1947; Benzioni et al., 1989), decreased leaf area (Bucks et al., 1985a; Bucks et al., 1985b), osmotic adjustment in the leaves (Allen et al., 1987; Allen and Nakayama, 1988), and increased size of the resin canals (Addicott and Pankhurst, 1944). There is also evidence
that photosynthesis is able to adapt and maintain relatively normal rates under water stress (Allen and Nakayama, 1988). Transplants that had been exposed to water stress survived transplanting better and resumed growth more rapidly (Kelley et al., 1945; Traub et al., 1946).

Water may have an effect on resin production in guayule; although the effect of water on resin concentration is unclear. Some studies showed that well watered and water-stressed plants had the same resin concentration (Hunter and Kelley, 1946; Benedict et al., 1947; Allen et al., 1987). Other studies showed that resin concentration increased with increasing irrigation (Bucks et al., 1985b; Miyamoto and Bucks, 1985; Benzioni et al., 1989). Regardless of how water affects resin concentration, resin yield increases with increasing irrigation, and seems to be directly related to plant biomass (Bucks et al., 1985b; Ehrler et al., 1985).

McCallum (1926), one of the first scientists to cultivate guayule, observed that irrigated guayule plants had lower rubber concentrations than wild plants, and hypothesized that the reduced rubber concentration would seriously compromise rubber yields. More recent studies have shown that this is not the case, and that rubber yields increase with increasing irrigation even though rubber concentration decreases (Bucks et al., 1985b; Ehrler et al., 1985; Fangmeier et al., 1985; Miyamoto and Bucks, 1985; Mills et al., 1990). With minimal irrigation rubber concentration increases but yield decreases (Miyamoto et al., 1984; Bucks et al., 1985b; Allen et al., 1987; Nakayama et al., 1991). Rubber yield appears to be directly related to biomass, while rubber concentration is inversely related to biomass (Kelley et al., 1945; Ehrler et al., 1985; Fangmeier et al.,
1985; Miyamoto and Bucks, 1985; Mills et al., 1990). Many hypotheses exist regarding how water stress increases rubber concentration including increased number of rubber-containing parenchyma cells (Addicott and Pankhurst, 1944; Latigo et al., 1996), increased rubber transferase activity (Reddy and Das, 1988), and normal photosynthetic rate during stress (Allen and Nakayama, 1988) that results in increased availability of substrates for rubber synthesis (Benzioni and Mills, 1991).

**Temperature**

Guayule’s growing range is limited by its sensitivity to cold temperatures (Mitchell, 1944; Nakayama, 1991), and is therefore being tested as a crop in warm arid environments (Foster and Coffelt, 2005). Very little information is available about how resin is affected by temperature. Radio-labeling experiments have shown that resin production is not a seasonal phenomenon like rubber, but that fructose is incorporated into resin throughout the year (Kelly and van Staden, 1993). Under field conditions resin concentration and yield did not respond to temperature (Jasso-Cantu et al., 1997).

Most rubber deposition occurs during the winter when the plant is dormant, often accumulating one and a third times as much rubber than it accumulates the rest of the year (Tingey and Foote, 1947; Ji et al., 1993; Jasso-Cantu et al., 1997; Cornish and Backhaus, 2003), with rubber concentration increasing steadily throughout the winter (Ji et al., 1993). One hypothesis is that rubber synthesis is triggered by the low night temperatures of the winter months (Bonner, 1943; Appleton and van Staden, 1991; Cornish and Backhaus, 2003). Researchers have been able to increase rubber concentration experimentally by exposing guayule plants to low temperatures in growth
chambers and refrigerated boxes (Bonner, 1943; Benedict, 1950; Goss et al., 1984; Appleton and van Staden, 1991; Norton et al., 1991; Sundar and Reddy, 2000; Sundar and Reddy, 2001; Cornish and Backhaus, 2003). The minimum temperature for rubber induction is unknown, but is estimated to be anywhere from 7 to 20 °C, and may vary among lines (Appleton and van Staden, 1991; Ji et al., 1993; Sundar and Reddy, 2000; Cornish and Backhaus, 2003).

The exact model of rubber induction has not been fully elucidated, but includes a role for plant dormancy, the activity of rubber synthesis related enzymes, photosynthesis, and plant maturity. Kelly and van Staden (1993) found that sucrose is allocated to rubber synthesis in the winter when the plant is more dormant, but is allocated to growth throughout the rest of the year. Other researchers noted that once dormancy had been broken and the plants were actively growing, cold temperatures could no longer induce rubber synthesis (Appleton and van Staden, 1991). Both rubber transferase and HMGR activity increase from October through December in field grown plants (Ji et al., 1993), and rubber transferase activity and concentration increased in guayule plants grown under cold night conditions in a growth chamber (Sundar and Reddy, 2000; Sundar and Reddy, 2001; Cornish and Backhaus, 2003). Sundar and Reddy (2000) observed that guayule plants treated with cold night temperatures of 15 °C had a higher photosynthetic rate than the control plants, which they hypothesized was related to the high rubber concentrations found in the treated plants. Other research showed that the leaves are the main source of sugars for rubber synthesis (Gilliland et al., 1985; Kelly and van Staden, 1987; Reddy and Das, 1995). Finally, there is evidence that guayule plants need to be greater than 200
days old to exhibit increased rubber synthesis in response to cold night temperatures (Goss et al., 1984; Cornish and Backhaus, 2003).

Possible uses for rubber by the guayule plant have been hypothesized over the years, such as using rubber as an energy reserve, which has been disproved (Lloyd, 1932; Traub, 1946; Benedict, 1949), or for drought tolerance, which has not been extensively tested, but seems unlikely (McCallum, 1926). Although the reason(s) guayule produces rubber is currently unknown, it is apparent that a variety of factors affect its production. In order for guayule to become a viable and economically successful commercial crop, it is important to understand how these different factors affect rubber and resin production. Once the effects of genetic and environmental factors are more fully understood, it will be easier to develop the varieties and agronomic practices that result in the highest possible rubber and resin yields.
PRESENT STUDY

The research for this dissertation is presented as four manuscripts, each in a separate appendix. Included in each manuscript are brief introductions, methods, results, discussion, and conclusions. The following are a summary of the most important findings from each of the manuscripts.

GROWTH, RUBBER, AND RESIN EVALUATION OF TWO-YEAR-OLD TRANSGENIC GUAYULE (APPENDIX A).

Rubber yields have been improved through the years by traditional breeding methods, and further improvements are being attempted by manipulating the rubber synthesis pathway. We hypothesized that increasing the availability of the initiators of rubber synthesis would increase rubber production throughout the year. The purpose of the study was to evaluate the effect of the transgene initiators farnesyl pyrophosphate synthase, geranylgeranyl pyrophosphate synthase, and hexa-heptaprenyl pyrophosphate synthase on not only rubber production, but growth and resin production in field grown guayule. Plants of guayule lines AZ 101, AZ-2, and N6-5 were transformed with one of the three initiator genes or with only the selectable marker and grown in the field for two years. Growth was monitored throughout the study, and resin and rubber production were monitored at regular intervals during the second year of growth. Transformation with the empty vector had an adverse effect on AZ 101 growth, but there were no positive effects of any of the initiator transgenes on growth, resin production, or rubber production. We concluded that transgenic manipulation of the initiator molecules would not increase rubber production throughout the year. We suggest that further efforts focus
on genes more directly involved in rubber synthesis, such as the rubber transferase gene(s).

AN ALTERNATIVE METHOD FOR ESTIMATING HERITABILITY IN
FACULTATIVE APOMICTIC GUAYULE (APPENDIX B).

Guayule reproduces by facultative apomixis, which has made it difficult to improve resin and rubber production. Researchers have attempted to improve guayule by examining heritabilities and correlations among traits related to rubber production. The purpose of this study was to propose a new way of estimating heritability in guayule that more accurately accounts for the contributions of apomictic and sexual reproduction. Seed-generated transgenic progeny were grown for two years in the field, and their growth and secondary compound production measured at one and two years of growth. Weighted parent-progeny regressions were used to calculate heritabilities of the different traits measured. Heritabilities of traits were presented as a range between the narrow (assuming sexual reproduction) and broad (assuming apomictic reproduction) sense heritabilities, which are the upper and lower limits respectively. Due to the predominately apomictic nature of guayule reproduction, heritabilities are more likely to be close to the broad sense estimate. Rubber and resin yield in the progeny were highly correlated with height and width in the parents after two years of growth, while rubber concentration was poorly correlated with almost all of the other traits measured. Based on both estimates of heritability, height and width had the highest heritabilities, and we recommend that rubber and resin yields be increased indirectly in the progeny by selecting for increased height and width in the parents.
WATER-STRESS INDUCED CHANGES IN RESIN AND RUBBER
CONCENTRATION AND DISTRIBUTION IN GREENHOUSE GROWN GUAYULE
(APPENDIX C).

Although guayule is adapted to arid regions, rubber yield has been found to increase under irrigated conditions, but with a concomitant decrease in rubber concentration. Conversely water stressed conditions increased rubber concentration but reduced rubber yield. It has been hypothesized that rubber production is stimulated by water stress. The effect of water stress on resin concentration is unclear. The purpose of this study was to understand how resin concentration responds to water stress and why rubber concentration is increased by exposure to water stress. Guayule grown under well-watered or water-stress conditions were evaluated in both summer and winter experiments. In addition to growth and biomass production, resin and rubber concentration and distribution in shoots, leaves, and stems were evaluated. Water-stressed plants had decreased growth, reduced stem diameter, and reduced resin and rubber yield, but a higher contribution of the stem to overall biomass, and higher rubber concentrations in the whole shoot. In water-stressed plants the stems contributed significantly more of the overall resin and rubber in the plant than in the well-watered plants, and generally had a smaller stem diameter that resulted in a greater bark to wood ratio. Since most rubber deposition occurs in the parenchyma cells of the bark, we suggest that the increased rubber concentration in the water-stressed plants was due to the increased contribution of the stem to overall biomass and the higher bark to wood ratio in the stems compared to the well-watered plants.
THE INFLUENCE OF NIGHT TEMPERATURE AND CARBON EXCHANGE ON RUBBER PRODUCTION IN GUAYULE (APPENDIX D).

Rubber production in guayule occurs mainly during the winter and there is evidence that rubber production is induced by cold night temperatures. The purpose of this study was to understand how immature plants respond to night temperature, and the role of night temperature and carbon exchange in rubber production in mature plants. Guayule was grown in differentially heated containers, with some plants receiving simulated summer night temperatures and the others ambient winter night temperatures. Growth and carbon exchange were monitored throughout the winter, and resin and rubber production were measured after harvest. Rubber production increased in immature plants only in response to cold night temperatures below 10 °C. Rubber concentration and yield in mature plants were higher under ambient winter cold night temperatures all three years, with the largest increase occurring during the first year when night temperatures consistently fell below 10 °C. The mature cold night plants also had carbon exchange rates that were as high as or higher than the warm night plants. In two of the three years, cold night plants grew less than warm night plants. Our data indicate that the threshold temperature for high rubber induction is somewhere between 7 and 10 °C. Our data also indicate that reduced growth in conjunction with normal carbon exchange rates contributed to high rubber production during the winter by allocating carbon to rubber production when it is not being used for active growth.
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Heritability of height, width, resin, rubber, and latex in guayule (*Parthenium argentatum*). Ind. Crops Prod. 13:229-238.


APPENDIX A. GROWTH, RUBBER, AND RESIN EVALUATION OF TWO YEAR OLD TRANSGENIC GUAYULE
GROWTH, RUBBER, AND RESIN EVALUATION OF TWO-YEAR-OLD TRANSGENIC GUAYULE

Maren E. Veatch, Dennis T. Ray, Chris J.D. Mau, Katrina Cornish

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Abstract

Guayule (Parthenium argentatum Gray) is a desert shrub that is a source of hypoallergenic, high quality latex and rubber. Improvements in rubber content and yield have been made through conventional selection techniques. Further improvements are being attempted by transforming guayule with one of three genes encoding various allylic diphosphate synthases in the rubber biosynthesis pathway. The objective of this study was to evaluate the effect of these transgenes on growth, rubber and resin production, in field-grown guayule. Tissue culture generated transgenic plants of the lines AZ 101, AZ-2 and N6-5 were planted in two field plots in 2001 and 2002. In both plots, plant height and width were measured monthly. Branches from each plant were sampled every four months starting at one year of growth. Resin and rubber were quantified by gravimetric analysis after being sequentially extracted with acetone (resin) and cyclohexane (rubber). The 2001 plot was harvested at the end of two years of growth. Transformation had no significant effect on growth of AZ-2 and N6-5 in the two years of the 2001 planting and the first year of the 2002 planting. In the 2001 planting, transformation appeared to have a drastic effect on the height and width of transformed AZ 101 compared with its empty vector control; however, the field in this study was not randomized and lacked non-transformed controls. In the 2002 planting, which was randomized and contained both positive and negative controls, the AZ 101 transformants were significantly larger than the empty vector AZ 101 control, but were not significantly different from the non-transformed controls. In the 2001 planting, resin concentration increased throughout the year up to January 2003, but decreased by the time of harvest in March 2003. Rubber
concentration, on the other hand, was high in May 2002, but decreased throughout the summer, before steadily increasing during the winter months.

Key Words: Guayule; Transgenes; Farnesyl pyrophosphate synthase (FPP); Geranylgeranyl pyrophosphate synthase (GGPP); Hexa-heptaprenyl pyrophosphate synthase (H-HPP); Resin; Rubber
Guayule (Parthenium argentatum Gray) is a shrub native to the Chihuahuan Desert and is currently being investigated for cultivation in the arid southwestern United States as a source of high quality, hypoallergenic latex and rubber (Thompson and Ray, 1989). The bulk of guayule rubber synthesis occurs during the winter months (Ji et al., 1993). Breeding efforts have been aimed at increasing the rubber content and overall yield with the most recent efforts focused on single-plant selections among polyploid apomictic plants and interspecific hybridization with other Parthenium species (Thompson and Ray, 1989). Breeding for increased rubber yield has been difficult because the most commonly used varieties of guayule are polyploid and reproduce apomictically at variable percentages (Thompson and Ray, 1989; Ray et al., 1990; Keys et al., 2002).

An alternative approach to increase rubber production is to target the rubber biosynthesis pathway directly using recombinant DNA technology (Cornish, 2001). The obvious and most desirable target is the rubber transferase gene, which catalyzes the addition of isopentenyl moieties from isopentenyl pyrophosphate (IPP) units to the rubber molecule (Backhaus et al., 1991; Cornish, 2001). However, the rubber transferase gene(s) have not yet been cloned; and therefore, other steps in the rubber synthesis pathway have been targeted.

An indirect approach to increase rubber production is to increase the amount of allylic pyrophosphates, which are initiators of rubber biosynthesis, available to the rubber transferase. This follows from in vitro experiments showing that the rate of rubber biosynthesis is dependent upon the concentration of the initiator (Tanaka et al., 1995;
Tanaka et al., 1996; Tangpakdee and Tanaka, 1998; Castillon and Cornish, 1999; Cornish, 2001). However, allylic pyrophosphates are also precursors to numerous other compounds, such as chlorophyll and gibberellins that are essential for plant growth and development (Oh et al., 2000). Therefore, any transformation with genes for allylic pyrophosphate synthases could lead to multiple phenotypic effects and not just changes in rubber production.

In this report, we evaluate the effect of three different allylic pyrophosphate synthase transgenes placed into three guayule lines, on growth, resin and rubber production under field conditions.

**Materials and Methods**

*Generation of transgenic guayule lines*

Three guayule lines, AZ 101, AZ-2, and N6-5 were used in the transformation. AZ 101 is an interspecific hybrid, with low rubber content but high biomass production. AZ-2 was selected for high biomass, but also has low rubber concentration. N6-5 in contrast was selected for high rubber concentration, yet has low biomass (Thompson and Ray, 1989; Ray et al., 1999). Each breeding line was transformed with at least one of three different genes using the *Agrobacterium*-mediated transformation (Pan et al., 1996). The genes, farnesyl pyrophosphate synthase (FPP) (Koyama et al., 1993), geranylgeranyl pyrophosphate synthase (GGPP) (Ohnuma et al., 1994), and a mutated form of geranylgeranyl pyrophosphate synthase, hexa-heptaprenyl pyrophosphate synthase (H-HPP) (Ohnuma et al., 1996), are all responsible for producing initiator molecules for rubber biosynthesis in the isoprenoid pathway. Each gene, under control of a constitutive
promoter, was integrated into the guayule genome using a binary vector containing a neomycin phosphotransferase II marker gene (McBride and Summerfelt, 1990). The presence of the genes was confirmed by PCR (Katrina Cornish, personal communication). Positive controls were transformed with a binary vector containing the kanamycin selectable marker only. All transplants, for both field plantings were generated from tissue culture and were grown in a greenhouse at the University of Arizona for three months before being transplanted into the field. The APHIS permit number for transgenic field release was 00-214-05n.

**Field Study for 2001**

The 2001 field study was conducted at The University of Arizona Maricopa Agricultural Center, Maricopa, Arizona from May 2001 to March 2003. A total of 130 plants were planted in the field, representing 21 different transformation events (FPP: AZ 101 n = 5, AZ-2 n = 6, N6-5 n = 5; GGPP: N6-5 n = 16; H-HPP: AZ 101 n = 3, N6-5 n = 68) and six positive controls (AZ 101 n = 20, AZ-2 n = 2, N6-5 n = 5). All plants from the same transformation event were grouped together in the field without randomization. Transformants of the same variety were also grouped together within the field without randomization. The field was irrigated every 14 days from March to October and every 42 days from October to March.

Height and width were measured monthly starting in November 2001. Plant width was the average of two perpendicular width measurements per plant. In addition to height and width, secondary compound production was monitored throughout the second year of growth, from May 2002 through March 2003. One or two branches were
removed from each plant within the plot in May, September, and January, dried at 80 °C for two days and then ground in a coffee grinder. Resin and rubber concentration were determined using 0.5000 g samples by gravimetric analysis (Black et al., 1983) using acetone and cyclohexane, sequentially, to isolate resin and rubber, respectively. In March 2003, the above ground biomass was harvested, run through a chipper, and dried at 80 °C for two days before the secondary compounds were extracted as described previously. Fresh weight was recorded at the time of harvest, and dry weight was calculated by subtracting the percent moisture of the chipped fresh weight and dry weight of a sample dried at 100 °C for two days. Total resin and rubber yields per plant were calculated from the percent resin or rubber and the dry weight of the plant.

*Field Study for 2002*

The May 2002 field planting was planted adjacent to the May 2001 planting. The second field of transgenic plants, contained 195 plants from seven different transformation events (FPP: AZ 101 n = 37, AZ-2 n = 17, and N6-5 n = 17; GGPP: N6-5 n = 16; H-HPP: N6-5 n = 34), with two negative and two positive controls, and was set up as a randomized complete block with four replications. Non-transformed AZ-2 (n = 19) and AZ 101 (n = 20) were included as negative controls, as well as AZ 101 (n = 20) and N6-5 (n = 19) empty vector positive controls. All other lines were the same as in the 2001 planting, with the exception of AZ 101 transformed with H-HPP, which was not available. Growth was measured as described for the 2001 planting.
Data analysis

All data from a line that contained the same gene were analyzed together, as there were no differences between transformation events as determined using linear regression (i.e., all 13 H-HPP transformations of N6-5 were analyzed as one data set). The data were analyzed using multiple regression in the Fit Model platform of JMP (Sall et al., 2001). Comparisons among transformants and among genes were done using Tukey’s HSD. Comparisons between a transformant and its controls were done using orthogonal contrast. A P-value = 0.05 was used for testing significance.

Results

Growth

In the 2001 planting, all plants actively grew between April and October, with no active growth from October through March (Fig. A1). Due to the similar growth trends among all three lines, only the data set for AZ 101 is shown. No significant differences were observed in either height or width between N6-5 FPP, N6-5 GGPP, and N6-5 H-HPP and their corresponding empty vector control. No significant differences in growth were present between the AZ-2 FPP and AZ-2 empty vector control (data not shown). On the other hand, AZ 101 FPP and AZ 101 H-HPP were significantly taller and wider than the empty vector control (Figs. A1 and A2). The differences in width were significant from November 2001 through September 2002. By final harvest no significant difference existed in width between AZ 101 FPP and AZ 101 empty vector control (Fig. A2). Not only were there differences between AZ 101 FPP, AZ 101 H-HPP and AZ 101 empty vector control, but also all of the N6-5 and AZ-2 transformants were
significantly shorter than AZ 101 FPP and AZ 101 H-HPP (data not shown). Differences in width between transformants were much more variable than differences in height, but no significant differences were observed among transformants at final harvest (data not shown).

Through the first year of growth, plants of the 2002 planting showed trends similar to the plants of the 2001 planting, with the plants actively growing from April to October (Fig. A3). As seen in the 2001 planting, no significant growth differences were observed among any of the N6-5 or AZ-2 transformants and their empty vector controls (data not shown). AZ 101 FPP was significantly larger than the empty vector control as seen in the 2001 planting data, but was not significantly larger than the non-transformed AZ 101 (Fig. A3). The subsequent growth data from this field plot will be analyzed at a later date, as the field is not scheduled for harvest until March 2004.

**Biomass Yield**

Fresh weight of the plants harvested from the 2001 planting ranged from 0.2 to 8.5 kg, with a mean weight of 2.3 kg. However, no significant differences occurred between transformants and their empty vector controls or among transformants for all lines. Dry weight ranged from 0.1 to 3.9 kg, with a mean weight of 1.0 kg, with no significant differences between any transformants and their empty vector controls. The only significant difference among transformants was between transformants of different breeding lines: N6-5 H-HPP and AZ 101 H-HPP, with mean dry weights of 0.9 and 2.3 kg, respectively.
Resin Production

A significant increase in resin concentration was present for each gene and empty vector control in the plants from May to January, but no significant change in resin concentration, due to any one gene, between January and March (Table A1). In general, plants transformed with FPP had the highest resin concentration at all sample points and plants with either FPP or GGPP had significantly higher resin concentration than the empty vector control in both January and March (Table A1). The N6-5 GGPP and N6-5 H-HPP were not different from the N6-5 empty vector control at any of the sample points. However, N6-5 FPP had a higher resin concentration than N6-5 empty vector control at the final harvest (Table A2). No differences were observed between AZ-2 FPP and the AZ-2 empty vector control at any of the sample points (Table A2). Both AZ 101 FPP and AZ 101 H-HPP, in contrast, had significantly higher resin concentration than the AZ 101 empty vector control throughout the year (Table A2). Varieties that had been transformed with the same gene were compared as well. For those plants carrying only the empty vector, AZ-2 had the highest percent resin during all four months, almost doubling the resin concentration between May and March (Table A2). For those plants transformed with FPP, N6-5 FPP had the lowest resin concentration in May and March, with AZ 101 FPP having a significantly higher resin concentration than N6-5 FPP in those two months (Table A2). The effect of GGPP could not be compared among lines because the only successful transformation was into N6-5, but an increase in resin concentration occurred from approximately 4% in May to around 6% in March (Table A2). A consistent difference existed between the N6-5 and AZ 101 plants transformed
with H-HPP, which was significantly higher in AZ 101 H-HPP at all sample dates with the exception of January (Table A2). AZ 101 H-HPP was the only transformant to have a significant increase in resin concentration between January and March (Table A2).

When resin yield was calculated on a per plant basis, AZ 101 FPP and AZ 101 H-HPP produced significantly more resin than the AZ 101 empty vector control (Fig.A4). Neither the AZ-2 nor the N6-5 transformants had greater resin yield than their empty vector controls (Fig. A4). Among transformants of different lines, both AZ 101 FPP and AZ 101 H-HPP had greater resin yields than N6-5 FPP, N6-5 GGPP and N6-5 H-HPP, whereas only AZ 101 H-HPP had greater resin yield than AZ-2 FPP (Fig. A4).

**Rubber Production**

The change in rubber concentration throughout the year was very similar for all genes, with rubber concentration being lowest in September and highest in March (Table A3). For all genes and the empty vector, with the exception of the plants transformed by GGPP, there was a significant increase in rubber concentration between January and March 2003 (Table A3). In January, plants transformed with GGPP had a significantly higher rubber concentration than plants transformed with FPP (Table A3). No transformant had a significantly different rubber concentration than its empty vector control (Table A4). However, some differences were present between transformants of different lines. N6-5 GGPP had a higher rubber concentration in January than both AZ 101 FPP and AZ-2 FPP (Table A4), which is consistent with the results found when comparing the overall effect of FPP and GGPP (Table A3). Most of the N6-5 H-HPP plants had higher rubber concentration than AZ 101 FPP and AZ-2 FPP in January and
than AZ-2 FPP in March (Table A4). N6-5 FPP also had a significantly higher rubber concentration than AZ 101 FPP and AZ-2 FPP in March (Table A4). When rubber yield was calculated on a per plant basis no differences were observed between any of the transformants and their controls. Also, no differences were present in rubber yield among any of the transformants, which ranged from 3.7 to 106.3 g per plant.

Discussion

The purpose of this study was to evaluate the effect of FPP, GGPP and H-HPP on growth and secondary compound production in guayule. The ultimate goal of the transformations was to increase rubber yield in the transgenic plants by increasing the availability of the initiators for rubber synthesis. These three genes are also precursors to many other compounds in the complex terpenoid pathway. Growth and resin production were measured to determine whether these genes had any effect on other parts of the terpenoid pathway.

In the AZ-2 and N6-5 transformants, there appeared to be no effect of the genes on growth. Any differences that were exhibited among the transformants of these two varieties was probably a varietal effect and not due to the presence of the genes. There appeared to be some effect of FPP and H-HPP on growth in AZ 101. The exceptionally large plants from these transformations indicated a positive effect on growth. However, the results were not conclusive without a non-transformed AZ 101 check. In the 2002 planting, this was rectified and no unusual boost to growth was found when AZ 101 FPP was compared with the non-transformed AZ 101. It may be that during the transformation process the empty vector inserted into an area in the genome that negatively affected
growth in AZ 101. Between the two sequential yearly field plantings, there were five separate empty vector transformations of AZ 101 (four in 2001 and one in 2002) that all exhibited the same reduction in growth, and were not significantly different from each other. Therefore, it is possible that a decrease in growth is not due to where the plasmid inserted, but an antagonistic effect of what was carried on the plasmid, which was cancelled out by the genes carried by AZ 101 FPP and AZ 101 H-HPP.

Interestingly, there appeared to be an effect of transformation on resin production from May to January, but not rubber production. Both of the AZ 101 transformants, AZ 101 FPP and AZ 101 H-HPP, had high resin yields per plant. This could be accounted for in two of different ways. The first is higher overall biomass in the plant. The transformants were large, but statistically there was no difference in biomass compared with the empty vector control. However, this may have been due to the small number of plants within each transformation, such that a very small or very large plant within any transformation would have a very large effect on the variability, obscuring any significant biological differences. The other possible explanation for the relatively high resin yields is a higher resin concentration within the transformants. Both AZ 101 FPP and AZ 101 H-HPP had much higher resin concentration than not only their empty vector control but also all of the other transformants as well (Table A2). This high resin concentration could be an indication that FPP and H-HPP are positively affecting resin production in AZ 101, but no definitive conclusion can be made until analysis of the data from the 2002 planting, which also had a negative control.
Little or no effect of the transgenes is apparent on rubber production (Tables A3 and A4). The only difference was that N6-5 FPP had a higher rubber concentration than AZ-2 FPP and AZ 101 FPP (Table A4). The higher rubber concentration in N6-5 FPP could be a line effect due to their relatively small size compared with AZ-2 FPP and AZ 101 FPP, whose parent lines were selected for size (Thompson and Ray, 1989; Ray et al., 1999). Size differences could also affect rubber yield by diluting rubber in the larger plants that would be expressed by a lower rubber concentration, but similar rubber yields as smaller plants with a higher rubber concentration.

Although the rubber concentration and rubber yield data indicate that there was no effect of these three genes on rubber production, other possible alternative explanations for this lack of difference can be considered. High initiator concentrations, as are probably produced in the transgenics due to their enhanced endogenous prenyl transferase activity (data not shown), would enhance the production of new rubber molecules, but an overall increase in the amount of rubber would only occur at nonlimiting IPP concentrations (Cornish, 2001; Cornish and Scott, 2004). The low affinity of the guayule rubber transferase for IPP ensures that rubber can only be made when IPP is not in demand by other metabolic processes. Thus, enhancing the production of IPP in guayule may have a stronger effect on rubber production. Alternatively, the initiators may have rapidly been metabolized by the resin biosynthetic pathway. The remarkably strong affinity of the rubber transferase for the initiators suggests that bypassing rubber biosynthesis is unlikely and that substrate limitations by IPP are the most likely cause of the unaltered rubber levels. Due to apomictic reproduction, altering
guayule according to traditional breeding techniques has been difficult. Transgenic technology may give breeders the tools they need to increase rubber synthesis significantly. Although the initial round of transformations increased resin content without impacting rubber levels, it did show that transformation is possible and should be a practical approach once the rubber transferase gene is cloned.
References


Table A1. Resin content of transgenic guayule by gene during the second year of growth (% resin ± standard error).

<table>
<thead>
<tr>
<th></th>
<th>May-02</th>
<th>Sep-02</th>
<th>Jan-03&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mar-03&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Vector Control (n = 27)</td>
<td>4.53 ± 0.10 b&lt;sup&gt;2&lt;/sup&gt; B&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5.28 ± 0.15 abB</td>
<td>6.46 ± 0.14 cA</td>
<td>6.06 ± 0.13 cA</td>
</tr>
<tr>
<td>FPP&lt;sup&gt;4&lt;/sup&gt; (n = 16)</td>
<td>5.26 ± 0.28 aB</td>
<td>5.69 ± 0.15 aB</td>
<td>7.20 ± 0.19 abA</td>
<td>7.42 ± 0.21 aA</td>
</tr>
<tr>
<td>GGPP&lt;sup&gt;5&lt;/sup&gt; (n = 16)</td>
<td>4.37 ± 0.16 bB</td>
<td>5.66 ± 0.16 bB</td>
<td>6.59 ± 0.10 aA</td>
<td>6.16 ± 0.10 aA</td>
</tr>
<tr>
<td>H-HPP&lt;sup&gt;6&lt;/sup&gt; (n = 71)</td>
<td>4.73 ± 0.09 bC</td>
<td>5.59 ± 0.08 bD</td>
<td>6.13 ± 0.09 bcB</td>
<td>5.91 ± 0.11 bA</td>
</tr>
</tbody>
</table>

<sup>1</sup>Data log<sub>10</sub> transformed for analysis.

<sup>2</sup>Numbers with the same lower case letter within a column are not significantly different according to Tukey’s HSD with <i>P</i> < 0.05.

<sup>3</sup>Numbers with the same upper case letter within a row are not significantly different according to Tukey’s HSD <i>P</i> < 0.05.

<sup>4</sup>Farnesyl pyrophosphate synthase.

<sup>5</sup>Geranylgeranyl pyrophosphate synthase.

<sup>6</sup>Hexa-heptaprenyl pyrophosphate synthase.
Table A2. Resin content of transgenic guayule by line and gene during second year of growth (% resin ± standard error).

<table>
<thead>
<tr>
<th>Line</th>
<th>May-02</th>
<th>Sep-02</th>
<th>Jan-03¹</th>
<th>Mar-03¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Vector Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ 101 (n = 20)</td>
<td>4.44 ± 0.11 c²</td>
<td>5.09 ± 0.14 d</td>
<td>6.20 ± 0.12 cd</td>
<td>5.85 ± 0.07 de</td>
</tr>
<tr>
<td>G7-11 (n = 2)</td>
<td>4.80 ± 0.29 bc</td>
<td>7.01 ± 0.31 ab</td>
<td>8.08 ± 0.60 ab</td>
<td>8.02 ± 0.41 ab</td>
</tr>
<tr>
<td>N6-5 (n = 5)</td>
<td>4.77 ± 0.29 c</td>
<td>5.33 ± 0.24 cd</td>
<td>6.84 ± 0.17 bc</td>
<td>6.11 ± 0.12 cde</td>
</tr>
<tr>
<td>FPP³</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ 101 (n = 5)</td>
<td>6.33 ± 0.43 ab</td>
<td>6.22 ± 0.30 bc</td>
<td>7.70 ± 0.52 ab</td>
<td>8.31 ± 0.34 a</td>
</tr>
<tr>
<td>G7-11 (n = 6)</td>
<td>5.02 ± 0.35 c</td>
<td>5.42 ± 0.11 cd</td>
<td>7.09 ± 0.08 ab</td>
<td>7.12 ± 0.28 b</td>
</tr>
<tr>
<td>N6-5 (n = 5)</td>
<td>4.47 ± 0.34 c</td>
<td>5.48 ± 0.25 cd</td>
<td>6.84 ± 0.21 bc</td>
<td>6.91 ± 0.08 bc</td>
</tr>
<tr>
<td>GGPP⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N6-5 (n = 16)</td>
<td>4.37 ± 0.16 c</td>
<td>5.66 ± 0.16 cd</td>
<td>6.59 ± 0.10 bc</td>
<td>6.16 ± 0.10 cde</td>
</tr>
<tr>
<td>H-HPP⁵</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ 101 (n = 3)</td>
<td>7.74 ± 0.50 a</td>
<td>7.70 ± 0.54 a</td>
<td>8.81 ± 0.59 a</td>
<td>9.63 ± 0.88 a</td>
</tr>
<tr>
<td>N6-5 (n = 68)</td>
<td>4.59 ± 0.05 c</td>
<td>5.50 ± 0.06 cd</td>
<td>6.01 ± 0.05 d</td>
<td>5.74 ± 0.05 e</td>
</tr>
</tbody>
</table>

¹ Data log₁₀ transformed for analysis.

² Numbers with the same letter within a column are not significantly different according to Tukey’s HSD P < 0.05.

³ Farnesyl pyrophosphate synthase.

⁴ Geranylgeranyl pyrophosphate synthase.

⁵ Hexa-heptaprenyl pyrophosphate synthase.
Table A3. Rubber content of transgenic guayule by gene during the second year of growth (% rubber ± standard error).

<table>
<thead>
<tr>
<th>May-02</th>
<th>Sep-02&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Jan-03</th>
<th>Mar-03</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Empty Vector Control (n = 27)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.19 ± 0.18 C&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.58 ± 0.07 B</td>
<td>2.66 ± 0.10 A</td>
<td>3.86 ± 0.09 b&lt;sup&gt;3&lt;/sup&gt;A</td>
</tr>
<tr>
<td><strong>FPP&lt;sup&gt;4&lt;/sup&gt; (n = 16)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.86 ± 0.23 B</td>
<td>1.30 ± 0.09 B</td>
<td>2.11 ± 0.14 A</td>
<td>3.36 ± 0.22 abA</td>
</tr>
<tr>
<td><strong>GGPP&lt;sup&gt;5&lt;/sup&gt; (n = 16)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00 ± 0.31 C</td>
<td>1.47 ± 0.07 B</td>
<td>3.23 ± 0.19 A</td>
<td>4.06 ± 0.10 abAB</td>
</tr>
<tr>
<td><strong>H-HPP&lt;sup&gt;6&lt;/sup&gt; (n = 71)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.31 ± 0.10 C</td>
<td>1.61 ± 0.05 B</td>
<td>3.12 ± 0.07 A</td>
<td>3.86 ± 0.06 aAB</td>
</tr>
</tbody>
</table>

<sup>1</sup> Data natural log transformed for data analysis.

<sup>2</sup> Numbers with the same upper case letter within a row are not significantly different according to Tukey’s HSD P < 0.05.

<sup>3</sup> Numbers with the same lower case letter within a column are not significantly different according to Tukey’s HSD P < 0.05.

<sup>4</sup> Farnesyl pyrophosphate synthase.

<sup>5</sup> Geranylgeranyl pyrophosphate synthase.

<sup>6</sup> Hexa-heptaprenyl pyrophosphate synthase.
Table A4. Rubber content of transgenic guayule by line and gene during the second year of growth (% rubber ± standard error).

<table>
<thead>
<tr>
<th>Line</th>
<th>May-02</th>
<th>Sep-02&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Jan-03</th>
<th>Mar-03</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Vector Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ 101 (n = 20)</td>
<td>2.24 ± 0.22</td>
<td>1.64 ± 0.08 a&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.68 ± 0.10 bc</td>
<td>3.90 ± 0.10 ab</td>
</tr>
<tr>
<td>G7-11 (n = 2)</td>
<td>0.99 ± 0.05</td>
<td>1.23 ± 0.10 ab</td>
<td>1.83 ± 0.06 cd</td>
<td>3.31 ± 0.39 a-d</td>
</tr>
<tr>
<td>N6-5 (n = 5)</td>
<td>2.49 ± 0.19</td>
<td>1.50 ± 0.21 ab</td>
<td>2.90 ± 0.31 abc</td>
<td>3.89 ± 0.14 abc</td>
</tr>
<tr>
<td>FPP&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ 101 (n = 5)</td>
<td>2.71 ± 0.42</td>
<td>1.27 ± 0.48 ab</td>
<td>2.02 ± 0.20 cd</td>
<td>3.10 ± 0.25 cd</td>
</tr>
<tr>
<td>G7-11 (n = 6)</td>
<td>1.22 ± 0.08</td>
<td>1.12 ± 0.15 b</td>
<td>1.69 ± 0.09 d</td>
<td>2.70 ± 0.14 d</td>
</tr>
<tr>
<td>N6-5 (n = 5)</td>
<td>1.77 ± 0.37</td>
<td>1.56 ± 0.30 ab</td>
<td>2.70 ± 0.24 a-d</td>
<td>4.42 ± 0.26 a</td>
</tr>
<tr>
<td>GGPP&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N6-5 (n = 16)</td>
<td>2.00 ± 0.31</td>
<td>1.47 ± 0.07 ab</td>
<td>3.23 ± 0.19 ab</td>
<td>4.06 ± 0.10 ab</td>
</tr>
<tr>
<td>H-HPP&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ 101 (n = 3)</td>
<td>3.61 ± 0.46</td>
<td>1.16 ± 0.20 ab</td>
<td>2.07 ± 0.07 cd</td>
<td>3.21 ± 0.12 bcd</td>
</tr>
<tr>
<td>N6-5 (n = 68)</td>
<td>2.26 ± 0.09</td>
<td>1.63 ± 0.05 a</td>
<td>3.16 ± 0.07 a</td>
<td>3.89 ± 0.06 ab</td>
</tr>
</tbody>
</table>

<sup>1</sup> Data natural log transformed for data analysis.

<sup>2</sup> Numbers with the same letter within a column are not significantly different according to Tukey’s HSD P < 0.05.

<sup>3</sup> Farnesyl pyrophosphate synthase.

<sup>4</sup> Geranylgeranyl pyrophosphate synthase.

<sup>5</sup> Hexa-heptaprenyl pyrophosphate synthase.
List of Figures

Figure A1. Mean height of transgenic guayule (AZ 101) from November 2001 through March 2003. The transgenes included are farnesyl pyrophosphate synthase (FPP) n = 5, hexa-heptaprenyl pyrophosphate synthase (H-HPP) n = 3, and an empty vector control (C) n = 20. Vertical bar represents ± standard error.

Figure A2. Mean width of transgenic guayule (AZ 101) from November 2001 through March 2003. The transgenes included are farnesyl pyrophosphate synthase (FPP) n = 5, hexa-heptaprenyl pyrophosphate synthase (H-HPP) n = 3, and an empty vector control (C) n = 20. Vertical bar represents ± standard error.

Figure A3. Mean height of transgenic guayule (AZ 101) from July 2002 through August 2003. The transgenes included are farnesyl pyrophosphate synthase (FPP) n = 37, and empty vector control (C) n = 16, and non-transformed AZ 101 n = 20. Vertical bar represents ± standard error.

Figure A4. Mean resin yield (g) per plant of transgenic guayule. Three lines, AZ 101, AZ-2 and N6-5, were transformed with farnesyl pyrophosphate synthase (FPP) n = 16, geranylgeranyl pyrophosphate synthase (GGPP) n = 16, hexa-heptaprenyl pyrophosphate synthase (H-HPP) n = 71 or an empty vector control (C) n = 27. Resin yield was log₁₀ transformed for analysis. Vertical bar represents ± standard error.
Figure A1.
Figure A2.
Figure A3.
Figure A4.
APPENDIX B. AN ALTERNATIVE METHOD FOR ESTIMATING HERITABILITY IN FACULTATIVE APOMIC TIC GUAYULE
AN ALTERNATIVE METHOD FOR ESTIMATING HERITABILITY IN
FACULTATIVE APOMICTIC GUAYULE

Maren E. Veatch-Blohm, Dennis T. Ray, Valerie H. Teetor, Bruce Walsh
Abstract

Guayule (Parthenium argentatum Gray) has been difficult to improve through classical plant breeding because of its facultative apomictic reproductive system. Attempts have been made to increase rubber concentration and yield by examining traits correlated with rubber production and their heritabilities. In this paper we propose a new way of estimating heritability in guayule that more accurately accounts for the contributions of apomictic and sexual reproduction. At two years of growth there was a significant relationship between the parents and the progeny for all traits measured except rubber and guayulin B concentrations. Due to the facultative apomictic nature of guayule reproduction, heritabilities are more accurately presented as a range of values between the narrow and broad sense heritabilities. Since guayule is more apomictic than sexual most heritabilities will be closer to the broad sense values. To increase resin and rubber yield in the progeny, selection should focus on height and width in the parents because height and width are highly correlated with rubber yield with the highest heritabilities (0.65 to 1.00 and 0.97 to 1.0) of the traits measured.

Key words: Parthenium argentatum, rubber, resin, guayulin, apomixis, heritability
Guayule (*Parthenium argentatum* Gray) is a rubber producing plant, which has been used on a large scale mainly in times of emergency (Ray et al., 2005). Research has been conducted during three major periods, all associated with a crisis. The first period of extensive research, the Emergency Rubber Project, was in response to the natural rubber supplies from Southeast Asia being cut off during World War II (National Academy of Sciences, 1977). The next round of research was stimulated by the oil crisis of the late 1970s and early 1980s (Ray et al., 2005). The current round of research is in response to the increasing incidence of type I allergy to *Hevea brasiliensis* Muell. Arg. latex and the need for a hypoallergenic natural rubber latex substitute (Cornish et al., 2001). Due to these intermittent research efforts, improvement of guayule has been slow (Thompson and Ray, 1989; Dierig et al., 2001; Ray et al., 2005).

Even though guayule breeding research has been intermittent there has been significant progress. In 1999, Ray et al. released six new germplasm selections, which were selected for uniformity, increased growth, and increased rubber yield. These and other new lines performed better than traditional varieties in field trials in California (Estilai, 1991), Arizona (Ray et al., 1999), and Australia (Dissanayake et al., 2004). Mass selection has also been used to increase tolerance to *Verticillium* wilt both in the greenhouse and in the field (Ray et al., 1995).

Multiple strategies have been used over the years to improve guayule. One approach is to generate interspecific hybrids with close relatives in order to combine desirable characteristics such as cold tolerance and increased biomass with the rubber producing potential of guayule (Naqvi et al., 1984; Hashemi et al., 1989). Sexual
diploids and the few naturally occurring sexual polyploids of guayule have also been
considered for inclusion in breeding programs (Esau, 1944; Gardner, 1946; Kuruvadi et
al., 1997a). The most successful guayule breeding methods have used mass, family,
and/or single plant selections (Thompson and Ray, 1989; Ray et al., 1995; Ray et al.,
1999; Ray et al., 2005). A more recent tool for improvement of guayule is transformation
(Pan et al., 1996), to insert genes from the rubber biosynthetic pathway into the plant in
an attempt to increase rubber concentration and rubber yield (Veatch et al., 2005).

The economics of guayule production will improve significantly if higher
yielding lines can be developed using reliable and rapid methods of selecting plants with
the best possible traits that will be passed faithfully to their progeny. One method is to
examine the linear correlation between different morphological traits and either rubber
content or yield. Correlations between traits aid in selection by using non-destructive
measurements, such as plant height or width. For example, rubber yield has been found
to be positively correlated with fresh weight, dry weight, height, and width (Dierig et al.,
1989b; Thompson and Ray, 1989; Ray et al., 1993; De Rodríguez et al., 2001). Rubber
concentration generally has a low positive or even a negative correlation with the traits
that are positively correlated with rubber yield (Dierig et al., 1989b; Ray et al., 1993).
Rubber concentration is also poorly correlated with rubber molecular weight and resin
concentration (Sidhu et al., 1993).

Breeding programs also need to select for characteristics with high heritabilities,
indicating low environmental effects. Heritability estimates of the traits associated with
rubber concentration and rubber yield have been variable. In a study by Dierig et al.
(2001), where clones were compared to open-pollinated progeny, height, resin concentration, and rubber concentration had high heritability at two years of growth, but by the third year heritability was almost zero as environmental effects compounded. In another study using path coefficient analysis, all traits measured at one year were found to be highly heritable in California but much less in Arizona (Estilai et al., 1992). In a third study, when parents and progeny from single plant selections were compared at two and three years of age, there were no significant regressions between parents and progeny, which is indicative of low heritabilities (Ray et al., 1993). Additionally, resin and rubber concentration have been difficult to predict from year to year, possibly because of large environmental effects (Dierig et al., 1989a; Coffelt et al., 2005).

Improvement of guayule is further complicated because of its complex genetic and reproductive system (Thompson and Ray, 1989, Ray et al., 2005). Guayule has a haploid chromosome number of 18, with a natural ploidy series ranging from diploid to tetraploid or higher (Bergner, 1944; Powers, 1945; Thompson and Ray, 1989; De Rodríguez et al., 1993). In native populations approximately 95% of the accessions surveyed are polyploid with the majority being tetraploid (Kuruvadi et al., 1997a). Chromosome numbers are also variable because the plants withstand a fair amount of aneuploidy (Powers, 1945; Thompson and Ray, 1989; Ray et al., 1990). This natural variation in chromosome number makes it difficult to predict gene expression, since different ploidy levels may exhibit different gene dosage effects (Thompson and Ray, 1989). The chromosomes of guayule are also very small and difficult to distinguish from each other (Thompson and Ray, 1989), which has made it difficult to map genes to
specific chromosomes (Kim et al., 2004). There are also few genetic markers available for breeding (Estilai and Ray, 1991). The differences in ploidy level and the resulting gene dosage effects, no genetic map, and lack of genetic markers make selecting for increased rubber concentration more difficult.

Diploid guayule plants reproduce sexually, but tetraploid guayule reproduces predominantly by facultative apomixis (Esau, 1944; Ray et al, 1990). Apomixis, embryo development without fertilization, ranges from 70 to 100% by line and season (Thompson and Ray, 1989; Keys et al., 2002), which complicates the development of breeding and selection methods (Thompson and Ray, 1989). Guayule also takes two to three years to reach harvestable size resulting in longer generation times for a breeding program (Thompson and Ray, 1989).

Most of the guayule germplasm, upon which the present breeding program is based, comes from only a few plants (Thompson and Ray, 1989). Despite this apparently narrow genetic base, there appears to be a large amount of variation in cultivated guayule (Ray et al., 2005), not only between lines but also within lines (Naqvi, 1985; Dierig et al., 1989a; Ray et al., 1990). There is also great deal of unexploited variation available in native populations (De Rodríguez et al., 1993; Kuruvadi et al., 1997b). Guayule plants that occasionally reproduce sexually are self-incompatible (Ray et al., 1993) with a sporophytic system of self-incompatibility (Gerstel, 1950). This system of enforced outcrossing promotes variation within cultivated and native populations.

This variation could be a valuable tool in guayule improvement; however, methods that determine the genetic contribution to this variation and subsequent response
to selection need to take into account guayule’s unusual mode of reproduction. In this paper we propose a new way of estimating heritability in guayule that more accurately accounts for the contributions of apomictic and sexual reproduction.

**Materials and Methods**

Open pollinated seed were collected from the tetraploid parent plants (n = 21) during the first year and a half of growth and used to generate progeny plants (n = 92). Both the parents and the progeny were established in a greenhouse for three months prior to transplantation in the field, so that they were approximately the same morphological age at the time of transplantation. The progeny were grown at the University of Arizona Maricopa Agricultural Center, Maricopa, Arizona from May 2003 through March 2005 in the same field where the parents had grown from May 2001 through March 2003. Irrigation of both the parents and progeny was on the same schedule of every 14 days from March to October and every 42 days from October to March. The parent field was not randomized (Veatch et al., 2005), but the progeny field was set up in a completely randomized design.

The number of progeny germinated from the respective parents ranged from one to sixteen. The parents were tissue culture generated transformed plants of guayule lines AZ 101, AZ-2, and N6-5. There were eight AZ 101 parents with an average family size of 6.8. There were four AZ-2 parents with an average family size of 6.5. There were nine N6-5 parents with an average family size of 1.5.

Height and width were measured at one and two years of growth. Width measurements were taken in two directions, which were perpendicular to each other, and
the width measurements averaged. After one year of growth resin, rubber, and guayulins A and B were extracted from two branches harvested from each plant. The branches were dried for two days at 60 °C to a constant weight, and ground in a coffee grinder. Resin and rubber concentrations were determined from 0.5 gram samples by a modification of the gravimetric method of Black et al. (1983) using acetone and cyclohexane to extract resin and rubber, respectively. Guayulins A and B were isolated from the resin fraction, which had been dissolved in absolute ethyl alcohol, injected into an HPLC with an acetonitrile/water gradient, with retention times of 24 and 25 minutes for guayulin A and guayulin B respectively. Guayulin A and B concentrations were calculated by the formula (Area Count (AC) x Response Factor x mL ethyl alcohol)/ (mg resin x 10^7), with a response factor of 1.234/1000 AC for guayulin A, and 0.867/1000 AC for guayulin B. The above ground biomass was harvested in March at the end of the second year of growth, the fresh weight measured, and the plants run through a chipper. The chipped samples were dried and secondary compounds extracted as described above. Resin and rubber yields were calculated using their respective concentrations and the dry weight of each plant.

Due to the unequal number of progeny available for each parent, weighted regressions were conducted to examine the relationship between parents and progeny using the Fit Y by X platform of JMP 4.0 statistical software, academic version (Sall et al., 2001). Multiple regressions were performed using the Fit Model platform (Sall et al., 2001) in order to check for significant effects of line and line by parental trait interaction and to examine differences between parent and progeny means. Linear correlations of
traits within the progeny and also among the traits of the progeny and the parents were calculated using the Multivariate platform (Sall et al., 2001). Means, standard deviations, and ranges were generated in the Distribution platform (Sall et al., 2001). Narrow sense heritabilities were calculated by multiplying the regression coefficient by two, and broad sense heritabilities were calculated as being equal to the regression coefficient. A $P$-value < 0.05 was considered significant throughout.

**Results**

At one year the parents and the progeny had significantly different means for only guayulin A and B concentrations (Table B1). At harvest, at the end of the second year, there were significant differences for resin concentration, fresh and dry weight, and resin and rubber yield (Table B1). There was a significant correlation between almost every parental trait and its corresponding progeny trait except rubber concentration and rubber yield and guayulin B concentration (Table B1). There was also a significant relationship between the parents and the progeny for many of the traits (Table B2). The weighted regression coefficients were significant for three of the six traits measured at year one, and for eight of the ten traits measured at year two (Table B2). Heritabilities were higher at year two than at year one, and height and width had the highest heritabilities (Table B2). There were only a few significant differences between the transformants, and were mainly due to a line effect rather than a transgene effect. Line had a significant effect on height and width at year one and guayulin A concentration both years (data not shown).

At two years the progeny resin and rubber yield were positively correlated with plant growth and biomass production (Table B3). Resin concentration was positively
correlated with height and rubber concentration, and rubber concentration was also positively correlated with rubber yield and guayulin A and B concentration (Table B3). Parent-progeny correlations for all traits showed a similar trend. Progeny resin and rubber yield were positively correlated with parental growth and biomass (Table B4). Progeny resin concentration was positively correlated with parental growth, resin concentration and yield, and guayulin B concentration, but progeny rubber concentration had no significant correlation with any of the traits measured (Table B4). Progeny guayulin A concentration was negatively correlated with every parental trait except rubber concentration and guayulin A and B concentrations (Table B4). On the other hand, progeny guayulin B concentration was not correlated with any of the traits measured (Table B4).

**Discussion**

Due to the facultative apomictic nature of guayule reproduction (Esau, 1944; Ray et al., 1990; Keys et al., 2002), heritability can be difficult to calculate using traditional methods. Generally, when progeny values are regressed on one parent, narrow sense heritability can be calculated by multiplying the regression coefficient by two (Falconer and Mackay, 1996). In a completely outcrossed species, narrow sense heritability, which factors in only the additive effects of genes, is the preferred method to calculate heritability (Nyquist, 1991). On the other hand, broad sense heritability, which includes not only the additive effects of genes but also the interactions among genes, is the most appropriate way to measure heritability in an apomictic species since entire genotypes, and not just individual genes, are transmitted to the next generation (Nyquist, 1991;
Stratton, 1991; Lynch and Walsh, 1998; Di Renzo et al., 2003). In the case of an apomictic species the broad sense heritability would be equal to the regression coefficient of the parent-progeny regression, which is the same as the regression of the progeny on the midparent value, since the known parent is the only parent (Nyquist, 1991).

Due to the variable amounts of apomixis displayed by different guayule varieties and at different seasons of the year (Ray et al., 1993; Keys et al., 2002), heritability of traits in guayule is more accurately presented as a range of possible values falling somewhere between the narrow and the broad sense heritabilities, which are the high and low estimates, respectively. In general, guayule is more apomictic than sexual (Thompson and Ray, 1989; Keys et al., 2002); therefore, heritability is likely to be closer to the broad sense heritability estimate.

There are distinct differences in heritability estimates among the different traits. Height and width appear to be the most heritable traits, heritability of fresh and dry weight, resin and rubber yield, and resin concentration are low to moderate, and heritability of rubber concentration is very low (Table B2). Rubber yield is a function of rubber concentration and plant biomass, and our data shows a greater effect of biomass than rubber concentration on rubber yield (Tables B1 and B2). Research has shown that there is a substantial effect of the environment on rubber production (Dierig et al., 2001; Coffelt et al., 2005). It may be that traits with low heritabilities, such as resin and rubber concentration (Table B2), are very sensitive to even slight environmental differences, such that the environmental variance masks the genetic variance of the population.
Despite the rather low heritabilities estimated for resin and rubber yield in this environment (Table B2), there were still significant correlations of parent height and width with progeny rubber and resin yields (Table B4). Since height and width appear to be the most heritable characteristics based on regression (Table B2), resin and rubber yields can be increased indirectly by selecting for height and width as has been suggested by other researchers (Dierig et al., 1989a; Ray et al. 1993; De Rodríguez et al., 2001). Another effect of selecting for increased height and width in the parents, is a probable decrease in the progeny of guayulin A concentration, a potential cause of contact dermatitis (Schloman et al., 1983), indicated by the negative correlation of these traits (Table B4).

Here we present a new way of examining and estimating heritability in guayule, which accounts for variable rates of apomixis. Breeders can use this method to get a more realistic estimate of the magnitude of response to selection, and tailor selection techniques accordingly.
References


Gardner, E.J. 1946. Sexual plants with high chromosome number from an individual plant selection in a natural population of guayule and mariola. Genetics 31:117-123.


Powers, L. 1945. Fertilization without reduction in guayule (Parthenium argentatum Gray) and a hypothesis as to the evolution of apomixis and polyploidy. Genetics 30:323-346.


Table B1. Means ± standard error (SE) and ranges, and Pearson’s product correlation coefficient of traits measured on parent and progeny plants of transgenic guayule at one and two years of growth.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Parent (n = 21)</th>
<th>Progeny (n = 92)</th>
<th>Parent-Progeny Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Range)</td>
<td>(Range)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Year One</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>54 ± 4</td>
<td>53 ± 2</td>
<td>0.27*</td>
</tr>
<tr>
<td></td>
<td>(24 – 81)</td>
<td>(17 – 74)</td>
<td></td>
</tr>
<tr>
<td>Width (cm)</td>
<td>54 ± 4</td>
<td>56 ± 2</td>
<td>0.22*</td>
</tr>
<tr>
<td></td>
<td>(25 – 90)</td>
<td>(14 – 84)</td>
<td></td>
</tr>
<tr>
<td>Resin Concentration (%)</td>
<td>5.5 ± 0.3</td>
<td>5.6 ± 0.1</td>
<td>0.34*</td>
</tr>
<tr>
<td></td>
<td>(3.8 – 8.6)</td>
<td>(4.1 – 7.7)</td>
<td></td>
</tr>
<tr>
<td>Rubber Concentration (%)</td>
<td>2.3 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>(0.9 – 4.3)</td>
<td>(0.7 – 3.3)</td>
<td></td>
</tr>
<tr>
<td>Guayulin A Concentration (%)</td>
<td>0.91 ± 0.39*</td>
<td>0.21 ± 0.03</td>
<td>0.29*</td>
</tr>
<tr>
<td></td>
<td>(0.04 – 7.34)</td>
<td>(0 – 1.10)</td>
<td></td>
</tr>
<tr>
<td>Guayulin B Concentration (%)</td>
<td>0.19 ± 0.05*</td>
<td>0.06 ± 0.01</td>
<td>-0.05</td>
</tr>
<tr>
<td></td>
<td>(0 – 0.91)</td>
<td>(0 – 0.29)</td>
<td></td>
</tr>
<tr>
<td>Year Two</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>79 ± 4</td>
<td>78 ± 3</td>
<td>0.43*</td>
</tr>
<tr>
<td></td>
<td>(56 – 114)</td>
<td>(18 – 120)</td>
<td></td>
</tr>
<tr>
<td>Width (cm)</td>
<td>92 ± 4</td>
<td>91 ± 3</td>
<td>0.49*</td>
</tr>
<tr>
<td></td>
<td>(58 – 132)</td>
<td>(15 – 133)</td>
<td></td>
</tr>
<tr>
<td>Resin Concentration (%)</td>
<td>7.2 ± 0.4*</td>
<td>6.5 ± 0.1</td>
<td>0.55*</td>
</tr>
<tr>
<td></td>
<td>(5.1 – 11.1)</td>
<td>(4.7 – 8.1)</td>
<td></td>
</tr>
<tr>
<td>Rubber Concentration (%)</td>
<td>3.4 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>(2.3 – 4.8)</td>
<td>(3.0 – 5.3)</td>
<td></td>
</tr>
<tr>
<td>Guayulin A Concentration (%)</td>
<td>0.38 ± 0.09</td>
<td>0.43 ± 0.05</td>
<td>0.21*</td>
</tr>
<tr>
<td></td>
<td>(0.03 – 1.55)</td>
<td>(0 – 3.67)</td>
<td></td>
</tr>
<tr>
<td>Guayulin B Concentration (%)</td>
<td>0.06 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>(0 – 0.19)</td>
<td>(0 – 0.44)</td>
<td></td>
</tr>
<tr>
<td>Fresh Weight (kg plant(^{-1}))</td>
<td>3.2 ± 0.4*</td>
<td>1.9 ± 0.2</td>
<td>0.29*</td>
</tr>
<tr>
<td></td>
<td>(0.9 – 8.5)</td>
<td>(0.03 – 6.8)</td>
<td></td>
</tr>
<tr>
<td>Dry Weight (kg plant(^{-1}))</td>
<td>1.5 ± 0.2*</td>
<td>0.8 ± 0.1</td>
<td>0.31*</td>
</tr>
<tr>
<td></td>
<td>(0.4 – 3.8)</td>
<td>(0.01 – 2.7)</td>
<td></td>
</tr>
<tr>
<td>Resin Yield (g plant(^{-1}))</td>
<td>114 ± 19*</td>
<td>55 ± 4</td>
<td>0.37*</td>
</tr>
<tr>
<td></td>
<td>(26 – 325)</td>
<td>(0.7 – 159)</td>
<td></td>
</tr>
<tr>
<td>Rubber Yield (g plant(^{-1}))</td>
<td>48 ± 5*</td>
<td>26 ± 2</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>(19 – 106)</td>
<td>(0.2 – 75)</td>
<td></td>
</tr>
</tbody>
</table>
Parent and progeny means significantly different at $P < 0.05$ based on ANOVA, and Pearson’s product correlation coefficient significant at $P < 0.05$. 
Table B2. Regression coefficients \((b)\) ± standard error (SE) and range of heritability values of parent and progeny plants of transgenic guayule at one and two years of growth.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Regression ± SE</th>
<th>Range of Heritabilities&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Year One</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>0.21 ± 0.08&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.21 – 0.42</td>
</tr>
<tr>
<td>Width (cm)</td>
<td>0.17 ± 0.08&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.17 – 0.34</td>
</tr>
<tr>
<td>Resin Concentration (%)</td>
<td>0.15 ± 0.04&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.15 – 0.30</td>
</tr>
<tr>
<td>Rubber Concentration (%)</td>
<td>-0.01 ± 0.05</td>
<td>0 – 0&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Guayulin A Concentration (%)</td>
<td>0.08 ± 0.07</td>
<td>0.08 – 0.16</td>
</tr>
<tr>
<td>Guayulin B Concentration (%)</td>
<td>-0.02 ± 0.03</td>
<td>0 – 0&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh Weight (kg plant&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.21 ± 0.07&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.21 – 0.42</td>
</tr>
<tr>
<td>Dry Weight (kg plant&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.21 ± 0.07&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.21 – 0.42</td>
</tr>
<tr>
<td>Resin Yield (g plant&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.17 ± 0.05&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.17 – 0.34</td>
</tr>
<tr>
<td>Rubber Yield (g plant&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.14 ± 0.07&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.14 – 0.28</td>
</tr>
<tr>
<td><strong>Year Two</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>0.58 ± 0.13</td>
<td>0.58 – 1.00</td>
</tr>
<tr>
<td>Width (cm)</td>
<td>0.86 ± 0.16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.86 – 1.00</td>
</tr>
<tr>
<td>Resin Concentration (%)</td>
<td>0.26 ± 0.04&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.26 – 0.52</td>
</tr>
<tr>
<td>Rubber Concentration (%)</td>
<td>0.07 ± 0.16</td>
<td>0.07 – 0.14</td>
</tr>
<tr>
<td>Guayulin A Concentration (%)</td>
<td>0.38 ± 0.19&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.38 – 0.76</td>
</tr>
<tr>
<td>Guayulin B Concentration (%)</td>
<td>0.11 ± 0.19</td>
<td>0.11 – 0.22</td>
</tr>
</tbody>
</table>

<sup>1</sup> The lower estimate is the broad sense heritability and was assumed to equal the regression coefficient. The upper estimate is the narrow sense heritability and was assumed to equal to two times the regression coefficient.

<sup>2</sup> Regression coefficient significant at \(P < 0.05\).

<sup>3</sup> Both the upper and lower estimates of heritability are zero because the regression coefficient was negative.
Table B3. Correlation of rubber and resin concentration (%) and rubber and resin yield to various morphological traits at two years of growth of progeny plants of transgenic guayule.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Resin yield</th>
<th>Rubber yield</th>
<th>Resin (%)</th>
<th>Rubber (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>0.77*</td>
<td>0.74*</td>
<td>0.23*</td>
<td>0.08</td>
</tr>
<tr>
<td>Width</td>
<td>0.88*</td>
<td>0.84*</td>
<td>0.16</td>
<td>0.03</td>
</tr>
<tr>
<td>Fresh Weight</td>
<td>0.98*</td>
<td>0.95*</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Dry Weight</td>
<td>0.99*</td>
<td>0.96*</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Resin Yield</td>
<td>1.00</td>
<td>0.97*</td>
<td>0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>Rubber Yield</td>
<td>0.97*</td>
<td>1.00</td>
<td>0.10</td>
<td>0.25*</td>
</tr>
<tr>
<td>Resin (%)</td>
<td>0.15</td>
<td>0.12</td>
<td>1.00</td>
<td>0.33*</td>
</tr>
<tr>
<td>Rubber (%)</td>
<td>0.10</td>
<td>0.25*</td>
<td>0.33*</td>
<td>1.00</td>
</tr>
<tr>
<td>Guayulin A (%)</td>
<td>-0.24*</td>
<td>-0.18</td>
<td>-0.13</td>
<td>0.40*</td>
</tr>
<tr>
<td>Guayulin B (%)</td>
<td>-0.04</td>
<td>0</td>
<td>0.19</td>
<td>0.25*</td>
</tr>
</tbody>
</table>

1 Pearson’s product correlation coefficient significant at $P < 0.05$. 
Table B4. Parent-progeny correlation of all traits in transgenic guayule measured at two years of growth.

<table>
<thead>
<tr>
<th>Parent Measurement</th>
<th>Height</th>
<th>Width</th>
<th>Fresh Weight</th>
<th>Dry Weight</th>
<th>Resin Yield</th>
<th>Rubber Yield</th>
<th>Resin (%)</th>
<th>Rubber (%)</th>
<th>Guayulin A (%)</th>
<th>Guayulin B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>0.43*</td>
<td>0.44*</td>
<td>0.38*</td>
<td>0.40*</td>
<td>0.45*</td>
<td>0.39*</td>
<td>0.38*</td>
<td>0.01</td>
<td>-0.27*</td>
<td>-0.11</td>
</tr>
<tr>
<td>Width</td>
<td>0.50*</td>
<td>0.49*</td>
<td>0.39*</td>
<td>0.41*</td>
<td>0.44*</td>
<td>0.38*</td>
<td>0.29*</td>
<td>-0.06</td>
<td>-0.35*</td>
<td>-0.14</td>
</tr>
<tr>
<td>Fresh Weight</td>
<td>0.31*</td>
<td>0.32*</td>
<td>0.29*</td>
<td>0.30*</td>
<td>0.31*</td>
<td>0.24*</td>
<td>0.09</td>
<td>-0.15</td>
<td>-0.25*</td>
<td>-0.19</td>
</tr>
<tr>
<td>Dry Weight</td>
<td>0.32*</td>
<td>0.33*</td>
<td>0.29*</td>
<td>0.31*</td>
<td>0.33*</td>
<td>0.26*</td>
<td>0.15</td>
<td>-0.12</td>
<td>-0.24*</td>
<td>-0.16</td>
</tr>
<tr>
<td>Resin Yield</td>
<td>0.36*</td>
<td>0.37*</td>
<td>0.32*</td>
<td>0.33*</td>
<td>0.37*</td>
<td>0.30*</td>
<td>0.29*</td>
<td>-0.07</td>
<td>-0.27*</td>
<td>-0.14</td>
</tr>
<tr>
<td>Rubber Yield</td>
<td>0.24*</td>
<td>0.25*</td>
<td>0.22*</td>
<td>0.23*</td>
<td>0.26*</td>
<td>0.19</td>
<td>0.17</td>
<td>-0.10</td>
<td>-0.21*</td>
<td>-0.15</td>
</tr>
<tr>
<td>Resin (%)</td>
<td>0.34*</td>
<td>0.33*</td>
<td>0.25*</td>
<td>0.27*</td>
<td>0.33*</td>
<td>0.30*</td>
<td>0.55*</td>
<td>-0.13</td>
<td>-0.22*</td>
<td>0.06</td>
</tr>
<tr>
<td>Rubber (%)</td>
<td>-0.55*</td>
<td>-0.53*</td>
<td>-0.46*</td>
<td>-0.46*</td>
<td>-0.45*</td>
<td>-0.42*</td>
<td>-0.10</td>
<td>0.05</td>
<td>0.39*</td>
<td>0.19</td>
</tr>
<tr>
<td>Guayulin A (%)</td>
<td>-0.27*</td>
<td>-0.21*</td>
<td>-0.20</td>
<td>-0.20</td>
<td>-0.18*</td>
<td>-0.15</td>
<td>-0.16</td>
<td>0.12</td>
<td>0.21*</td>
<td>0.02</td>
</tr>
<tr>
<td>Guayulin B (%)</td>
<td>0.05</td>
<td>0.08</td>
<td>0.04</td>
<td>0.05</td>
<td>0.11</td>
<td>0.12</td>
<td>0.21*</td>
<td>0.14</td>
<td>0.05</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Pearson’s product correlation coefficient significant at $P < 0.05$. 

10 Pearson’s product correlation coefficient significant at $P < 0.05$. 


APPENDIX C. WATER-STRESS INDUCED CHANGES IN RESIN AND RUBBER CONCENTRATION AND DISTRIBUTION IN GREENHOUSE GROWN GUAYULE
WATER-STRESS INDUCED CHANGES IN RESIN AND RUBBER
CONCENTRATION AND DISTRIBUTION IN GREENHOUSE GROWN
GUAYULE

Maren E. Veatch-Blohm, Dennis T. Ray, William B. McCloskey
Abstract

Guayule (Parthenium argentatum Gray) is naturally subjected to periods of water stress in its native habitat. Under cultivation it has been shown that rubber yields increase with increasing irrigation (due to increased biomass production), but rubber concentration per plant decreases. The effect of irrigation on resin concentration is unclear. The purpose of this study was to understand how resin concentration is affected by water stress, and why water stress increases rubber concentration. Greenhouse grown guayule plants were subjected to water stress in four experiments of three months each. Two experiments were in the summer, the active growth period, and two experiments in the winter, during the normally dormant period. The water-stressed plants were irrigated when the average soil water potential reached either -0.6 (first summer experiment) or -0.3 (all subsequent experiments) MPa. Water stress effects were monitored by measuring growth, carbon exchange, biomass, and resin and rubber production. Resin and rubber were extracted from dried, ground plant samples with the leaves and stems analyzed separately. Water-stressed plants had lower carbon exchange, growth, and leaf-to-stem ratio than well-watered plants. Resin and rubber yield were lower in water-stressed plants due to decreased growth. Resin concentration did not respond consistently to water stress. Rubber concentration was generally higher in the water-stressed plants as a result of decreased leaf biomass and decreased stem diameter. Rubber is deposited mainly in the bark, and the water-stressed plants had a greater bark to wood ratio than the well-watered plants, which contributed to their observed higher rubber concentration.
Guayule (*Parthenium argentatum* Gray) is a native of the Chihuahuan desert with a natural history of exposure to water stress (Nakayama et al., 1991). In its native habitat, guayule receives between 17 and 38 cm of precipitation annually, occurring mainly during the summer months (McCallum, 1926; Benzioni et al., 1989). Guayule is currently being investigated as a source of hypoallergenic latex (Siler and Cornish, 1994, Cornish et al., 2001) and as a potential source of natural rubber in the event that rubber supplies from *Hevea brasiliensis* Müll. Arg. are unable to meet natural rubber demands (Mooibroek and Cornish, 2000).

Latex is a liquid suspension of rubber, which is a *cis*-1-4-polyisoprene (Cornish and Siler, 1995). Resin, a *trans*-isoprenoid, is a composite of acetone extractable compounds that contribute to plant growth and development (Thompson and Ray, 1989; Oh et al., 2000). Resin comprises six to eleven percent of the mature plant biomass (Teetor et al., 2003; Veatch et al., 2005), and is currently being used in coproduct development as an antitermitic and an antifungal compound (Maatooq et al., 1996; Nakayama et al., 2001; Nakayama, 2005).

Guayule is typically grown from transplants in a two year cycle, with active growth occurring between April and October, with the bulk of new growth completed at the end of the second summer when the plant is 17 to 18 months old. Guayule is a highly branched shrub, which at maturity ranges from 0.3 to 1.0 m in height, and 0.6 to 1.2 m in width (Foster et al., 2002; Foster et al., 2005; Veatch et al., 2005). Resin is produced throughout the year, and is usually highest during periods of active growth (Schloman et al., 1986). Guayule produces the majority of its rubber during the winter months, during
a time in which the plant appears to be dormant and it encounters the lowest temperatures and amount of rainfall (Fangmeier et al., 1985; Ji et al., 1993). There is evidence that rubber production in guayule is triggered by either cold temperatures or water stress or both (Schloman et al., 1986; Appleton and van Staden, 1991).

Water requirements for guayule have been studied extensively during every major period of guayule research (Ray et al., 2005), from the Emergency Rubber Project (Addicott and Pankhurst, 1944; Kelley et al., 1945; Hunter and Kelley, 1946; Benedict et al., 1947) to studies in the 1980s (Bucks et al., 1985a, b, c; Miyamoto and Bucks, 1985). The relationship of resin concentration to the amount of irrigation is unclear. Some field studies have shown that the amount of irrigation does not affect resin concentration (Benedict et al., 1947; Allen et al., 1987), while others have shown that resin concentration increases with increasing irrigation (Bucks et al., 1985b; Miyamoto and Bucks, 1985; Benzioni et al., 1989). However, resin yield is directly related to plant biomass and increases with increasing irrigation (Bucks et al., 1985b, Ehrler et al., 1985). Field studies have shown that as the application of irrigation water increases, rubber concentration within the plant decreases, but rubber yields increase (Fangmeier et al., 1985; Miyamoto and Bucks, 1985; Mills et al., 1990). On the other hand, rubber concentration within the plant increases as the plants are subjected to water stress, but with a rather large loss in rubber yield (Nakayama and Bucks, 1984; Ehrler et al., 1985). The decrease in rubber yield in water-stressed plants is attributed to reduced biomass production (Ehrler et al., 1985; Nakayama et al., 1991).
Water stress initiates a number of changes within the plant, many of which contribute to guayule’s drought tolerance. Water-stressed plants have less leaf lobing, smaller leaves, and smaller leaf area than well-watered plants (Benedict et al., 1947; Bucks et al., 1985 a, b; Benzioni et al., 1989). Carbon exchange (often reported as photosynthesis) measurements taken about two hours before solar noon on whole plants, showed no difference in carbon exchange rate between well-watered and water-stressed plants (Allen et al., 1987; Allen and Nakayama, 1988). Other researchers have shown that photosynthates, such as sucrose, are allocated into growth in actively growing plants compared with dormant plants, and that water stress reduces photosynthetic translocation in the phloem (Benzioni and Mills, 1991; Kelley and van Staden, 1993). Guayule also undergoes osmotic adjustment in response to water stress, which some researchers believe is related to the rapid recovery of water-stressed leaves in response to even small amounts of added water (Ehrler and Nakayama, 1984; Nakayama and Bucks, 1984; Bucks et al., 1985a; Ehrler et al., 1985; Allen and Nakayama, 1988).

Previous research has focused on water stress under field conditions. Examining water stress under greenhouse conditions allows us to more easily examine the response of individual plants to different irrigation treatments. The purpose of this study was to understand how resin concentration is affected by water stress, and why water stress increases rubber concentration. To answer these questions we investigated growth, carbon exchange, and resin and rubber production and distribution in the leaves and stems of both well-watered and water-stressed plants.
Materials and Methods

Experiments were conducted in an evaporatively cooled, temperature controlled greenhouse at the Campus Agricultural Center of the University of Arizona, Tucson, Arizona. The experiment was conducted four times for three months at a time. Two experiments ran from June through August in two consecutive summers (2003 and 2004). Two experiments ran from December through February in two consecutive winters (2003-2004 and 2004-2005). Separate one-year-old plants were used in all four experiments. For the summer 2003 experiment, plants were produced via tissue culture and included non-transgenic lines AZ 101 and AZ-2, and three transgenic lines. Two of the transgenic lines were derived from AZ 101, one carrying the farnesyl pyrophosphate (FPP) synthase gene (EC 2.5.10) and the other acting as an empty vector control (carrying only kanamycin resistance), and the third transgenic line was derived from AZ-2 and carried the FPP synthase gene. For the winter 2003-2004 experiment the plants were also produced via tissue culture and included a non-transgenic AZ-2 line, and transgenics of AZ 101 and N6-5 both carrying only kanamycin resistance. For the summer 2004 experiment, all of the plants used were seed derived and were non-transgenic lines AZ 101 and AZ-2. For the winter 2004-2005 experiment only seed derived non-transgenic AZ 101 plants were used in the experiment. Lines used were based on plant availability.

The experiments were set up as a randomized complete block with four replications except in the winter 2003-2004 experiment, which had five replications. Two to five plants per line were assigned randomly to each treatment within each
replication. The plants were grown in pots for a year in the greenhouse with irrigation every day during the summer and irrigation every two days during the winter. The plants were transplanted into the experimental pots, containing a sandy loam soil, at least one month prior to the initiation of the irrigation treatments. The plants within a row were placed 36 cm apart, which is the same planting distance used under field conditions; however, due to space limitations within the greenhouse there were only 57 cm between rows of plants compared to 102 cm under field conditions. During the experiments the plants were grown in pots that were 0.61 to 0.76 m tall, with a total pot volume of 18 and 30 L respectively. The pots were taller than conventional greenhouse pots to provide a deeper root zone, such that water stress symptoms took longer to develop (Earl, 2003). The 0.76 m pots were only available for use in the first experiment (summer 2003).

Irrigation frequency was determined by soil water potential, measured by a combination of predawn leaf water potential measurements, using a pressure chamber, and soil water potential measurements, using Watermark Soil Matric Potential Blocks (Campbell Scientific, Inc., Logan, UT). The well-watered treatment was irrigated daily for 10 min with a drip irrigation system that delivered approximately 500 ml of water to the soil. Summer 2003 water-stressed plants were watered for 10 min when the soil water potential was approximately -0.6 MPa, about halfway to the permanent wilting point. Although the water-stressed plants survived, their growth in summer 2003 was so reduced that in all subsequent experiments the water-stressed treatments were irrigated when the soil water potential was approximately -0.3 MPa. The average irrigation frequency of the water-stressed plants was every five days summer 2003, every three
days summer 2004, every seven days winter 2003-2004, and every eight days winter 2004-2005. For all treatments, the plants were fertilized with a one-half strength solution of Peter’s 20:20:20 with micronutrients (20 percent nitrogen: 2.1 percent nitrate and 17.9 percent urea; 20 percent phosphorous: P₂O₅; 20 percent potassium: K₂O; 0.05 percent each of chelated copper, chelated manganese, chelated iron; 0.02 percent chelated boron; 0.10 percent chelated iron; and 0.0009 percent molybdenum) each seven irrigations.

High and low temperatures were recorded and the average daily high and low temperatures were 32 ± 1.2 and 23 ± 1.7 ºC, respectively summer 2003, 28 ± 1.9 and 16 ± 2.0 ºC winter 2003-2004, 32 ± 3.2 and 22 ± 1.9 ºC summer 2004, and 29 ± 3.6 and 17 ± 1.9 ºC winter 2004-2005.

Plant height, width, and stem diameter were recorded at the start of irrigation treatments and growth was measured as changes from these initial measurements. Width was an average of two measurements taken perpendicular to each other. Changes in height and width were measured for all experiments except winter 2003-2004, when only height was measured. Changes in stem diameter were measured during the summer 2004 and winter 2004-2005 experiments only. Changes in leaf osmotic potential were measured with a Wescor Vapor Pressure Osmometer (Wescor, Logan, UT) on leaves sampled from the top of the plant the day after irrigation for both treatments. Photosynthesis was estimated by measuring carbon exchange for single leaves at the top of the plant, with a LI-6200 (Li-Cor Biosciences, Lincoln, NE). Due to instrument availability the majority of the carbon exchange measurements were taken during summer 2003 and winter 2003-2004. Summer 2003 carbon exchange measurements
were taken the day before and the day after irrigation of the water-stressed treatment. Winter 2003-2004, irrigation of the water-stressed plants occurred at different times for the different replications, which dried out at different rates; therefore, carbon exchange measurements were taken twice a week, without reference to irrigation, but were usually within three to four days after irrigation of the water-stressed plants. The carbon exchange measurements were taken within one hour of solar noon on sunny days using an air flow rate of 185 to 190 µmol s⁻¹, ambient CO₂ between 380 and 410 ppm, an ambient average PAR of 1300 µmol m⁻² s⁻¹, and ambient humidity, which ranged from 25 to 40 percent relative humidity during the summer and 11 to 30 percent relative humidity during the winter. The relative humidity under field conditions was similar throughout the majority of the experiment, with a slightly higher relative humidity in the greenhouse on very dry days and slightly lower humidity in the greenhouse on extremely humid days. Diurnal carbon exchange measurements were also taken once a month in summer 2003 every two hours from 0800 to 1600 h approximately two days after irrigation of the water-stressed plants and once during winter 2003-2004 every two hours from 0700 to 1700 h approximately four days after irrigation of the water-stressed plants.

Three months after the start of the experiment, when the plants were 15 months old, the aboveground biomass was harvested at the soil surface. In addition to shoot biomass the root ball was also harvested for all experiments except summer 2003. A subset of plants was defoliated; leaf area and leaf weight measured, and the proportion of leaf fresh weight of the overall shoot fresh weight calculated. The subset included one plant per line per replication, except winter 2003, which included defoliated plants from
only three of the five replications. Plant samples were dried for two days at 80 ºC, weighed and ground in a coffee grinder, which was wiped clean between samples. Resin and rubber were extracted sequentially from the dried, ground samples with acetone and cyclohexane respectively using the common extraction method, which is a variation of the gravimetric analysis method developed by Black et al. (1983). A 0.5 g sample of the ground plant material was homogenized in 20 ml of acetone for 30 seconds with a Polytron homogenizer (Kinematica Inc., Newark, NJ) at a speed of 27,000 rotations per minute (rpm) to extract resin. The homogenizer was rinsed with an additional 10 ml of acetone and the sample was centrifuged at 3500 rpm for 12 minutes. The supernatant was poured through filter paper into a preweighed aluminum dish. The procedure was repeated twice more, with the supernatant from all three extractions being poured into the same dish. The acetone in the aluminum dish evaporated overnight and any residual liquid was removed in a vacuum oven for 30 minutes at 60 ºC. The acetone also evaporated from the sample residue overnight, from which rubber was extracted with cyclohexane using the procedure described above for acetone. Resin and rubber concentrations were calculated with the following formula \(((\text{final pan weight } – \text{ initial pan weight})/\text{sample weight}) \times 100\). For all experiments except summer 2003, resin and rubber were also extracted from the roots, and separately from the stems and leaves of the defoliated plants. Resin and rubber yield per plant were calculated by multiplying the plant dry weight by the resin and rubber concentration of each plant.

All data were analyzed using multiple regression in the fit model platform of the JMP 4.0 statistical software, academic version (Sall et al., 2001), for a randomized
complete block with four replications (five replications for winter 2003-2004), with line, season, irrigation, and their interactions as explanatory variables. Fresh weight, dry weight, leaf area, resin and rubber concentration, and resin and rubber yield were log$_{10}$ transformed for analysis. Specific comparisons were done using orthogonal contrast (Sall et al., 2001). A $P$-value of $< 0.05$ was considered significant throughout.

**Results**

No significant differences between lines and no significant interactions between any of the lines and irrigation treatments or seasons were found; however, there were significant differences between seasons and between irrigation treatments and significant interactions between season and irrigation treatment. Therefore, all data presented are for irrigation treatments within season. The water-stressed plants had significantly smaller changes in height and width than the well-watered plants in all experiments (Table C1). The water-stressed plants also had a significantly smaller change in stem diameter than the well-watered plants in summer 2004 and winter 2004-2005 (Table C1). Both fresh and dry weights were significantly reduced in the water-stressed plants (Table C2). The water-stressed plants also had reduced leaf area (Table C2) and a significantly smaller proportion of leaf fresh weight to the overall shoot fresh weight (Table C2) than the well-watered plants.

The osmotic potential of the leaves of both well-watered and water-stressed plants decreased over much of the experimental period, and was significantly lower in the water-stressed plants compared to the well-watered plants in both the summer and the winter (Fig. C1). There were also significant differences between treatments during
midday carbon exchange measurements. The day before irrigation, summer 2003, the water-stressed plants had much lower carbon exchange compared to the well-watered plants (Fig. C2). Carbon exchange rates of the water-stressed plants increased significantly the day after irrigation, but the water-stressed plants still had significantly lower carbon exchange than the well-watered plants (Fig. C2). Carbon exchange was also significantly reduced in the water-stressed plants winter 2003-2004, but reduction in carbon exchange took longer to develop than it did in the summer (Fig. C3). No significant differences in carbon exchange were found between treatments in December, but carbon exchange was significantly lower in the water-stressed plants than the well-watered plants in January and February (Fig. C3). The diurnal carbon exchange measurements summer 2003 were significantly higher in the well-watered plants compared to the water-stressed plants only at solar noon and 1400 h, with no significant differences between treatments observed at other times during the day (Fig. C4). In contrast, during the winter 2003-2004 diurnal measurements, the water-stressed plants had significantly lower carbon exchange than the well-watered plants at all time points except 0700 and 1700 h (Fig. C5).

Resin concentration in the shoots (leaves and stems) was significantly higher in the well-watered plants in two of the four experiments; however, resin concentration was not consistently different between treatments in either the summer or the winter (Table C3). Resin yield was significantly higher in the well-watered plants in all experiments (Table C3). Resin concentration in the roots was significantly higher in the water-stressed plants in the summer and significantly higher in the well-watered plants in the
winter (Table C4). Leaf resin concentration was significantly higher in the well-watered plants in the winter, but no significant differences between treatments were found in the summer (Table C4). Stem resin concentration was higher in the water-stressed plants in the summer, but not significantly different between treatments in the winter (Table C4). The proportion of the total shoot resin was significantly higher in the stems than in the leaves in both treatments, with the proportion of total shoot resin significantly higher in the leaves and stems of the well-watered and the water-stressed plants respectively (Table C5).

Rubber concentration was higher in the water-stressed plants than the well-watered plants in whole shoots (leaves and stems) in three of the four experiments, both summer experiments and winter 2003-2004 (Table C3). The well-watered plants had significantly higher rubber concentration in the shoots in the winter, but this was due to the very high rubber concentration in the well-watered plants winter 2004-2005 (Table C3). Rubber yield was significantly higher in the well-watered plants in all experiments (Table C3). No treatment had consistently higher rubber concentration in the roots, and leaf rubber concentration was not significantly different between treatments (Table C4). Stem rubber concentration was significantly greater in the water-stressed plants in the summer and in the well-watered plants in the winter (Table C4). The high rubber concentration in the stems of well-watered plants in the winter was due to the high rubber concentration in the well-watered plants compared to the water-stressed plants winter 2004-2005 (Table C4). In contrast stem rubber concentration was not significantly different between treatments winter 2003-2004 (Table 4). The proportion of total plant
rubber was significantly greater in the stems than in the leaves, and the proportion of total plant rubber in the stems was significantly greater in the water-stressed plants in both the summer and the winter (Table C5).

**Discussion**

The water-stressed plants, even at the reduced stress level used after summer 2003, had reduced growth compared to the well-watered plants (Table C1). Reduced growth under the greenhouse conditions used in this study was expected, as it is a common response to water stress in the field (Nakayama and Bucks, 1984; Ehrler et al., 1985). Reduced leaf area observed in this study (Table C2) could help delay the most severe effects of water stress by reducing transpirational demand on the plant (Bucks et al., 1985a). However, the reduced leaf area had the negative effect of reducing available photosynthetic area (Table C2). The reduced photosynthetic area in combination with reduced carbon exchange on an individual leaf basis (Figs. C2 and C3) contribute to a smaller photosynthate pool available for growth. The water-stressed leaves also had lower osmotic potential than the well-watered leaves (Fig. C1), which may have protected the plant from many of the damaging effects of water stress (Allen et al., 1987). However, the low leaf osmotic potential after irrigation may have reduced the ability of the water-stressed plants to recover full carbon exchange capacity after irrigation, since many of the solutes used for osmotic adjustment, such as soluble sugars, reduce photosynthesis by feedback inhibition (Basu et al., 1998). In contrast, plants from the well-watered treatment had a larger photosynthetic area (Table C2), higher carbon exchange measurements (Figs. C2 and C3), and higher osmotic potential (Fig. C1) than
the water-stressed plants. Therefore, these plants could maintain steady growth. In actively growing plants, such as the well-watered plants, photosynthates are allocated to growth and not rubber production (Kelley and van Staden, 1987; Kelley and van Staden, 1993).

In summer 2003, resin concentration was higher in the well-watered plants and rubber concentration was higher in the water-stressed plants (Table C3). The well-watered plants had a much higher leaf-to-stem ratio than the water-stressed plants (Table C2). Teetor et al. (2003) found that green leaves and green stems contribute between 35 and 45 percent of the total resin in the shoot, with the highest concentrations found in green stems. In the same study, rubber concentration was highest in the stems and lowest in the leaves. We hypothesized that the higher shoot resin concentration in the well-watered treatment was due to the high contribution of leaves to the total resin within the shoot. In contrast, shoot rubber concentration in the well-watered plants would be lower because the leaves, which have very little rubber, would have a diluting effect on the high concentration of rubber in the stems.

To test this hypothesis, resin and rubber were extracted separately from the leaves and stems of the defoliated plants from all subsequent experiments. We hypothesized that if the relative amounts of leaf and stem were the major contributing factor to the differences in resin and rubber concentration between treatments, then the resin and rubber concentrations in different plant parts would not be different between the treatments. Winter 2003-2004, the hypothesis held true for rubber concentration in the leaves and stems, but the well-watered leaves had higher resin concentration than the
water-stressed leaves even though resin concentration within the stems was not significantly different between treatments (Table C4). The contribution of the leaves to the total shoot resin was significantly higher in the well-watered plants than the water-stressed plants (Table C5); although, the well-watered plants did not have significantly higher shoot resin concentrations than the water-stressed plants (Table C3). Winter 2004-2005 resin concentration in the leaves and the stems and rubber concentration in the stems was significantly higher in the well-watered plants (Table C4); however, the proportion of total shoot resin and rubber from the leaves and stems was not significantly different between treatments (Table C5). Summer 2004 resin and rubber concentration in the leaves was not significantly different between treatments, but the stems of the water-stressed plants had significantly higher resin and rubber concentrations than the well-watered plants (Table C4). The higher resin concentration in the stems of the water-stressed plants may be due to the smaller mean diameter of the stems (8.1 mm) compared to the stems of the well-watered plants (11.0 mm) (Table C1), as small stems are where the highest resin concentrations are found (Teetor et al., 2003). The number of resin canals is also increased in water-stressed plants (Addicott and Pankhurst, 1944), which could contribute to the higher resin concentration in the stems. Despite the lower resin concentration in the stems of the well-watered plants, the leaves contributed a significantly higher proportion of the total shoot resin in the well-watered plants than the leaves in the water-stressed plants (Table C5), such that resin concentration in the shoots was not significantly different between treatments (Table C3).
The increased rubber concentration in the water-stressed plants in all but winter 2004-2005 has two probable contributing factors. First, rubber deposition occurs mainly in the bark parenchyma of guayule stems and to a much lesser extent in other parts of the plant (Gilliland et al., 1984). The greater contribution of stems to total shoot biomass, with a smaller contribution of leaves (Table C2), could increase the overall rubber concentration in the water-stressed plants. This hypothesis is supported by examining the proportion of the total shoot rubber contributed by the stems in each treatment. The treatment with the highest proportion of the total shoot rubber from the stems also had the highest rubber concentration in the shoot (Tables C3 and C5).

Second, the significant differences in rubber concentration between the well-watered and water-stressed plants, especially summer 2004, were contributed to by differences in stem diameter between treatments. Smaller stems tend to have higher rubber concentrations than larger stems (Dierig et al., 1989) and the greatest number of parenchyma cells (Latigo et al., 1996). Summer 2004 the water-stressed plants had a smaller change in stem diameter (Table C1) and smaller mean stem diameter (8.1 mm) than the well-watered plants (11.0 mm), resulting in a greater bark to wood ratio. Lloyd (1911) and Mitchell et al. (1945) found that as stem diameter increased, the bark to wood ratio decreased. Very little rubber is accumulated in the woody portion of the stems (Gilliland et al., 1984), with one estimate of 82 percent of the stem rubber in the bark and only 18 percent of the stem rubber in the wood (Kuruvadi et al., 1997). Therefore, plants with a greater bark to wood ratio would have higher rubber concentrations within the stem, and would contribute more to the overall rubber concentration within the plant.
Winter 2004-2005 the mean stem diameter of the well-watered plants (11.9 mm) at harvest was significantly greater than the water-stressed plants (11.1 mm), but rubber concentration in the stem was not significantly different between treatments (Table C4). With a difference in mean stem diameter between treatments of only 0.8 mm, the water-stressed plants may not have had a large enough increase in the bark to wood ratio to increase the stem rubber concentration in the water-stressed plants compared to the well-watered plants. Despite the large difference in mean stem diameter between the well-watered (13.2) and the water-stressed plants (10.7) winter 2004-2005, rubber concentration within the shoots and the stems were not what we had anticipated based on the results of the three previous experiments (Tables C3 and C4). These unanticipated findings are thought to be the result of two factors. First, there were only a small number of plants available for this experiment such that any extreme value in either treatment could strongly affect the results. Second, in contrast to the water-stressed plants in the other experiments, the water-stressed plants in this experiment had a much more rapid and severe response to water stress, from which they did not significantly recover until a couple of weeks before harvest.

The plants grown during the winter had lower resin and rubber concentrations than the plants grown during the summer (Tables C3 and C4). The decrease in resin concentration was probably related to the lower growth rates in the winter (Table C1), since resin is generally higher in actively growing plants (Schloman et al., 1986). The soil water potential in these experiments did not decrease as rapidly in the winter as they did in the summer, with almost twice as much time between irrigations. This was due to the
lower daily temperatures in the winter, so that the winter grown plants were not exposed to severe water stress as often as the summer grown plants. Winter is the time when most rubber deposition occurs in guayule (Ji et al., 1993), and water stress decreases translocation of rubber precursors to the stem (Benzioni and Mills, 1991), with the decrease in carbon exchange in the water-stressed leaves further reducing the available pool of rubber precursors. The water-stressed plants may not have had the opportunity to accumulate substantial quantities rubber before water stress altered the leaf to stem ratio and reduced changes in stem diameter. Also the low night temperatures during the winter experiments were warmer than the temperatures thought to trigger high rubber production, which may have been responsible for the low rubber concentrations observed in both winter experiments.
References


Irrigated guayule- production and water use relationships. Agric. Water Manage. 10:95-102.


Table C1. Mean plant height, width, and stem diameter at the start of irrigation treatments, and mean change in height, width, and stem diameter ± standard error in well-watered and water-stressed guayule in the summer (2003 and 2004) and the winter (2003-2004 and 2004-2005).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Height (cm)</th>
<th>Width (cm)</th>
<th>Stem Diameter (mm) (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Summer</td>
<td>Winter</td>
<td></td>
</tr>
<tr>
<td>Initial mean(^2)</td>
<td>32.6 ± 0.28</td>
<td>31.9 ± 1.0</td>
<td>10.3 ± 0.15</td>
</tr>
<tr>
<td>Mean change well-watered (n = 102)</td>
<td>9.2 ± 0.43 a(^3)</td>
<td>4.8 ± 0.61 a</td>
<td>3.0 ± 0.22 a</td>
</tr>
<tr>
<td>Mean change water-stressed (n = 101)</td>
<td>3.0 ± 0.40 b</td>
<td>0.3 ± 0.48 b</td>
<td>0.4 ± 0.2 b</td>
</tr>
</tbody>
</table>

\(^1\) Change in stem diameter was measured only during the summer 2004 and the winter 2004-2005 experiments on 20 plants and 10 plants in each treatment for each year, respectively.

\(^2\) Mean plant height, width, and stem diameter at the start of irrigation treatments.

\(^3\) Numbers in the same column, within the same season, with the same letter are not significantly different according to orthogonal contrast \(P < 0.05\).
Table C2. Mean shoot (leaves and stems) fresh and dry weight, proportion of leaf fresh weight to shoot fresh weight, and leaf area ± standard error of well-watered and water-stressed guayule in the summer (2003 and 2004) and the winter (2003-2004 and 2004-2005).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh weight (g)$^1$</th>
<th>Dry weight (g)$^1$</th>
<th>Proportion of leaf weight (g kg$^{-1})^2$</th>
<th>Leaf area (cm$^2)^{2,3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered (n = 102)</td>
<td>274 ± 16.6 a$^4$</td>
<td>113 ± 5.5 a</td>
<td>45 ± 1.6 a</td>
<td>4768 ± 971 a</td>
</tr>
<tr>
<td>Water-stressed (n = 101)</td>
<td>105 ± 3.0 b</td>
<td>60 ± 2.3 b</td>
<td>33 ± 2.1 b</td>
<td>1203 ± 98 b</td>
</tr>
<tr>
<td>Winter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered (n = 71)</td>
<td>228 ± 6.2 a</td>
<td>72 ± 4.1 a</td>
<td>52 ± 1.6 a</td>
<td>3657 ± 187.1 a</td>
</tr>
<tr>
<td>Water-stressed (n = 71)</td>
<td>106 ± 2.3 b</td>
<td>45 ± 1.7 b</td>
<td>36 ± 1.7 b</td>
<td>1516 ± 55.5 b</td>
</tr>
</tbody>
</table>

$^1$ Fresh and dry weight data were log$_{10}$ transformed for analysis.

$^2$ Proportion of leaf weight and leaf area were measured on one plant per line, per treatment, per replication, except for winter 2003-2004 when the plants were taken from three of the five replications.

$^3$ Leaf area data were log$_{10}$ transformed for analysis.

$^4$ Numbers in the same column, within the same season, with the same letter are not significantly different according to orthogonal contrast $P < 0.05$. 
Table C3. Mean shoot (leaves and stems) resin and rubber concentrations and yields ± standard error of well-watered and water-stressed guayule in the summer (2003 and 2004) and the winter (2003-2004 and 2004-2005).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Resin (g kg⁻¹) ¹</th>
<th>Resin (g) ¹</th>
<th>Rubber (g kg⁻¹) ¹</th>
<th>Rubber (g) ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Summer</td>
<td>Winter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 102)</td>
<td>8.6 ± 0.14 a²,³</td>
<td>4.4 ± 0.12 a⁴</td>
<td>1.9 ± 0.04 b</td>
<td>1.1 ± 0.08 a</td>
</tr>
<tr>
<td>Water-stressed</td>
<td>8.4 ± 0.12 a</td>
<td>4.1 ± 0.10 a</td>
<td>2.4 ± 0.05 a</td>
<td>0.7 ± 0.03 b</td>
</tr>
<tr>
<td>(n = 101)</td>
<td>5.0 ± 0.21 b</td>
<td>2.0 ± 0.11 b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Resin and rubber concentrations and yields were log₁₀ transformed for analysis.

² Numbers in the same column, within the same season, with the same letter are not significantly different according to orthogonal contrast \( P < 0.05 \).

³ Resin concentration was not significantly different between treatments over the two years, but was significantly different between treatments summer 2003, with a mean of 8.7 and 8.1 g kg⁻¹ in the well-watered and water-stressed plants, respectively.

⁴ Resin concentration was not significantly different between treatments over the two years, but was significantly different between treatments winter 2004-2005, with a mean of 5.8 and 5.0 g kg⁻¹ in the well-watered and water-stressed plants, respectively.

⁵ Rubber concentration was not significantly different between treatments over the two years, but was significantly different between treatments winter 2003-2004, with a mean of 1.5 and 1.7 g kg⁻¹ in the well-watered and water-stressed plants, respectively, and was
significantly different between treatments winter 2004-2005, with a mean of 1.7 and 1.4 g kg$^{-1}$ in the well-watered and water-stressed plants, respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root</th>
<th></th>
<th>Leaf</th>
<th></th>
<th>Stem</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resin</td>
<td>Rubber</td>
<td>Resin</td>
<td>Rubber</td>
<td>Resin</td>
<td>Rubber</td>
</tr>
<tr>
<td></td>
<td>(g kg⁻¹)</td>
<td>(g kg⁻¹)</td>
<td>(g kg⁻¹)</td>
<td>(g kg⁻¹)</td>
<td>(g kg⁻¹)</td>
<td>(g kg⁻¹)</td>
</tr>
<tr>
<td><strong>Summer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>5.9 ±</td>
<td>0.9 ±</td>
<td>7.5 ±</td>
<td>1.6 ±</td>
<td>8.7 ±</td>
<td>2.5 ±</td>
</tr>
<tr>
<td>(n = 40)</td>
<td>0.11 b²</td>
<td>0.05 a</td>
<td>0.31 a</td>
<td>0.05 a</td>
<td>0.31 b</td>
<td>0.18 b</td>
</tr>
<tr>
<td>Water-stressed</td>
<td>6.6 ±</td>
<td>1.0 ±</td>
<td>7.9 ±</td>
<td>1.9 ±</td>
<td>10.2 ±</td>
<td>4.2 ±</td>
</tr>
<tr>
<td>(n = 40)</td>
<td>0.13 a</td>
<td>0.05 a</td>
<td>0.24 a</td>
<td>0.11 a</td>
<td>0.25 a</td>
<td>0.28 a</td>
</tr>
<tr>
<td><strong>Winter</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Well-watered</td>
<td>3.6 ±</td>
<td>0.8 ±</td>
<td>4.2 ±</td>
<td>1.4 ±</td>
<td>4.7 ±</td>
<td>1.7 ±</td>
</tr>
<tr>
<td>(n = 69)</td>
<td>0.24 a³</td>
<td>0.04 a⁴</td>
<td>0.11 a</td>
<td>0.04 a</td>
<td>0.26 a⁵</td>
<td>0.14 a⁶</td>
</tr>
<tr>
<td>Water-stressed</td>
<td>2.9 ±</td>
<td>0.9 ±</td>
<td>3.9 ±</td>
<td>1.5 ±</td>
<td>4.3 ±</td>
<td>1.4 ±</td>
</tr>
<tr>
<td>(n = 71)</td>
<td>0.10 b</td>
<td>0.06 a</td>
<td>0.13 b</td>
<td>0.05 a</td>
<td>0.22 a</td>
<td>0.09 b</td>
</tr>
</tbody>
</table>

¹ Leaf and stem resin and rubber concentrations were measured on one plant per line, per treatment, per replication, except winter 2003-2004 when the plants were taken from three of the five replications.

² Numbers in the same column, within the same season, with the same letter are not significantly different according to orthogonal contrast $P < 0.05$.

³ Root resin concentration was significantly different between treatments over the two years, but was not significantly different between treatments winter 2003-2004, with a mean of 2.5 and 2.6 g kg⁻¹ in the well-watered and water-stressed plants, respectively.
4 Root rubber concentration was not significantly different between treatments over the two years, but was significantly different between treatments winter 2003-2004, with a mean of 0.8 and 1.0 g kg\(^{-1}\) in the well-watered and water-stressed plants, respectively.

5 Stem resin concentration was not significantly different between treatments over the two years, but was significantly different between treatments winter 2004-2005, with a mean of 6.3 and 5.5 g kg\(^{-1}\) in the well-watered and water-stressed plants, respectively.

6 Stem rubber concentration was significantly different between treatments over the two years, but was not significantly different between treatments winter 2003-2004, with a mean of 1.4 g kg\(^{-1}\) for each treatment.
Table C5. Mean proportion of total shoot resin and rubber yields ± standard error from leaves and stems of well-watered and water-stressed guayule in the summer (2004) and the winter (2003-2004 and 2004-2005).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Resin Leaf (g kg(^{-1}))(^1)</th>
<th>Resin Stem (g kg(^{-1}))(^1)</th>
<th>Rubber Leaf (g kg(^{-1}))(^1)</th>
<th>Rubber Stem (g kg(^{-1}))(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Summer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered (n = 8)</td>
<td>48 ± 1.0 a(^2)B(^3)</td>
<td>52 ± 1.0 bA</td>
<td>41 ± 1.2 aB</td>
<td>59 ± 1.2 bA</td>
</tr>
<tr>
<td>Water-stressed (n = 8)</td>
<td>42 ± 1.3 bB</td>
<td>58 ± 1.3 aA</td>
<td>31 ± 2.1 bB</td>
<td>69 ± 2.1 aA</td>
</tr>
<tr>
<td><strong>Winter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered (n = 25)</td>
<td>47 ± 1.3 aB(^4)</td>
<td>53 ± 1.3 bA(^4)</td>
<td>46 ± 2.0 aB(^4)</td>
<td>54 ± 2.0 bA(^4)</td>
</tr>
<tr>
<td>Water-stressed (n = 26)</td>
<td>35 ± 1.5 bB</td>
<td>65 ± 1.5 aA</td>
<td>39 ± 1.7 bA</td>
<td>61 ± 1.7 aA</td>
</tr>
</tbody>
</table>

\(^1\) The proportion of the total shoot resin and rubber yield from leaves and stems were measured on one plant per line, per treatment, per replication, except winter 2003-2004 when the plants were taken from three of the five replications.

\(^2\) Numbers in the same column, within the same season, with the same lowercase letter are not significantly different according to orthogonal contrast \(P < 0.05\).

\(^3\) Numbers within the same row and compound with the same uppercase letter are not significantly different according to orthogonal contrast \(P < 0.05\).

\(^4\) The proportion of leaf and stem resin and rubber to total shoot resin and rubber were significantly different between treatments over the two years, but were not significantly different between treatments winter 2004-2005, with a mean leaf resin proportion of 45 and 41 g kg\(^{-1}\), a mean stem resin proportion of 55 and 59 g kg\(^{-1}\), a mean leaf rubber
proportion of 41 and 44 g kg$^{-1}$, and a mean stem rubber proportion of 59 and 56 g kg$^{-1}$ in the well-watered and water-stressed plants, respectively.
List of Figures

Figure C1. Mean leaf osmotic potential (MPa) of well-watered (W) and water-stressed (D) guayule in the summer and winter the day after irrigation. The osmotic potential was measured on 19 plants in each irrigation treatment summer 2003, on 8 plants in each irrigation treatment summer 2004, on 9 plants in each irrigation treatment winter 2003-2004, and on 4 in each irrigation treatment winter 2004-2005. The vertical bar indicates ± standard error.

Figure C2. Mean midday carbon exchange rate (µmol CO₂ m⁻² s⁻¹) of well-watered (W) and water-stressed (D) guayule the day before and the day after irrigation during June, July, and August 2003. Carbon exchange measurements were recorded within an hour of solar noon. Measurements were taken on 19 plants in each irrigation treatment. The vertical bar indicates ± standard error.

Figure C3. Mean midday carbon exchange rate (µmol CO₂ m⁻² s⁻¹) of well-watered (W) and water-stressed (D) guayule December 2003, and January and February 2004 an average of three to four days after irrigation of the water-stressed plants. Carbon exchange measurements were recorded within an hour of solar noon. Measurements were taken on 12 plants in each irrigation treatment. The vertical bar indicates ± standard error.
Figure C4. Mean diurnal carbon exchange rates (µmol CO₂ m⁻² s⁻¹) of well-watered (W) and water-stressed (D) guayule July 2003. Diurnal measurements were taken two days after irrigation of the water-stressed plants. Carbon exchange measurements were recorded every two hours beginning at 0800 and ending at 1600 h. Measurements were taken on 10 plants in each irrigation treatment. The vertical bar indicates ± standard error.

Figure C5. Mean diurnal carbon exchange rates (µmol CO₂ m⁻² s⁻¹) of well-watered (W) and water-stressed (D) guayule February 2004. Diurnal measurements were taken an average of four days after irrigation of the water-stressed plants. Carbon exchange measurements were recorded every two hours beginning at 0700 and ending at 1700 h. Measurements were taken on 8 plants in each irrigation treatment. The vertical bar indicates ± standard error.
Figure C1.

Leaf Osmotic Potential (MPa)

-3 -2.5 -2 -1.5 -1 -0.5 0

1 week 6 weeks 12 weeks

-3 -2.5 -2 -1.5 -1 -0.5 0

Summer D Summer W
Winter D Winter W
Figure C2.

![Graph showing carbon exchange over months](image)

The graph illustrates the carbon exchange (µmol CO₂ m⁻² sec⁻¹) from June to August. It compares data for D Before, D After, W Before, and W After conditions.
Figure C3.

- Carbon Exchange (µmol CO₂ m⁻² sec⁻¹)

- December, January, February
Figure C4.
Figure C5.
APPENDIX D. THE INFLUENCE OF NIGHT TEMPERATURE ON CARBON EXCHANGE AND RUBBER PRODUCTION IN GUAYULE
THE INFLUENCE OF NIGHT TEMPERATURE ON CARBON EXCHANGE
AND RUBBER PRODUCTION IN GUAYULE

Maren E. Veatch-Blohm, Dennis T. Ray, Aleida Gehrels
Abstract

Guayule (Parthenium argentatum Gray), a native of the Chihuahuan desert, produces the majority of its rubber during the winter months. Increased rubber production is thought to be induced by cold night temperatures; however, the factors involved in rubber induction are not completely understood. The purpose of this study was to answer three questions about rubber production in the winter: (1) how do immature plants (<180 d) respond to cold night temperatures?; (2) how is rubber production in mature plants (∼1 year) related to night temperature?; and (3) what is the relationship of carbon exchange to rubber production under cold night temperatures? Plants were grown in differentially heated enclosures over three consecutive winters, with the warm night plants receiving simulated summer night temperatures and the cold night plants receiving ambient winter night temperatures. Plant responses to different night temperatures were monitored by measuring growth, carbon exchange, fresh and dry weight, and resin and rubber production. Immature plants exposed to cold nights had higher rubber concentration in the first year, which had the lowest night temperatures. For the mature plants, dry weight was not significantly different between treatments, but rubber concentration and yield were significantly greater in the cold night plants. Plants in both treatments had similar carbon exchange rates; therefore, the similarity in dry weight between treatments was most likely due to increased growth in the warm night plants and increased rubber deposition in the cold night plants. Rubber concentration was significantly related to night temperature and appears to be stimulated most by temperatures below 10 °C. Reduced growth and high carbon exchange rates contribute to increased rubber
production during the winter by allocating carbon into rubber synthesis when it is not being used for active growth.

Key words: Guayule, Night Temperature, Rubber Induction, Carbon Exchange
Guayule (Parthenium argentatum Gray), is native to the Chihuahuan desert and grows almost exclusively in warm, arid environments (Foster and Coffelt, 2005), with cold temperatures appearing to be the range-limiting factor (Mitchell, 1944; Hashemi et al., 1988). Guayule can withstand exposure to temperatures below freezing for only a few hours without die off of the branches, roots, or both (Mitchell, 1944); however, despite guayule’s sensitivity to cold temperatures, rubber synthesis is thought to be triggered by moderate cold night temperatures (Bonner, 1943; Goss et al., 1984; Appleton and van Staden, 1991; Ji et al., 1993; Cornish and Backhaus, 2003).

Most of the rubber deposition in guayule occurs during the winter months when the plant is not actively growing (Ji et al., 1993; Jasso-Cantú et al., 1997; Cornish and Backhaus, 2003), accumulating 1 x to 1.3 x as much rubber as it does throughout the rest of the year (Tingey and Foote, 1947). Rubber concentration increases steadily throughout the winter (Ji et al., 1993), and rubber concentration has been increased experimentally by exposing guayule to cold night temperatures in refrigerated boxes or growth chambers (Bonner, 1943; Benedict, 1950; Goss et al., 1984; Appleton and van Staden, 1991; Sundar and Reddy, 2000; Sundar and Reddy, 2001; Cornish and Backhaus, 2003). Rubber induction is not a continuous process (Cornish and Backhaus, 2003), and once bud break has occurred rubber synthesis cannot be reinduced by cold temperatures (Appleton and van Staden, 1991), implying that plant growth takes precedence over rubber production.

Although it is apparent that rubber production in guayule responds to cold night temperatures, there are questions about the interaction between night temperature and
rubber synthesis that remain unanswered. For instance, there have been conflicting reports as to the minimum temperature required for rubber induction. Estimates have varied widely from 15 °C to 20 °C (Sundar and Reddy, 2000; Sundar and Reddy, 2001; Cornish and Backhaus, 2003) to as low as 7 °C (Bonner, 1943; Goss et al., 1984; Appleton and van Staden, 1991; Ji et al., 1993).

The amount of rubber produced during the winter is related to the size of the shrub (Tingey and Foote, 1947; Jasso-Cantú et al., 1997), which is most often related to plant age (Veatch et al., 2005), with more mature stems having more rubber than immature stems (Gilliland et al., 1985). It is unclear whether rubber production will respond to cold temperatures in immature plants (< 180 d), which lack much of the bark parenchyma laid down during secondary growth of more mature plants (closer to one year) (Backhaus and Walsh, 1983; Gilliland et al., 1984, Cornish and Backhaus, 2003). Therefore, plant maturity may be critical for rubber induction since the bark parenchyma is where the majority of the rubber in guayule is produced and stored (Gilliland et al., 1984). Rubber concentration has been experimentally increased in young plants, but only after four or more months of exposure to cold temperatures, so that the plants were older than 180 d at the end of the experiment (Goss et al., 1984; Cornish and Backhaus, 2003). However, rubber synthesis has been induced in guayule tissue culture callus after only 28 d of 4 °C nights and 25 °C days (Norton et al., 1991). Therefore, it is difficult to determine whether increased rubber concentration in young guayule plants is a direct response to cold temperatures or a consequence of plant maturity.
Of particular interest are the physiological processes triggered by cold night temperatures that may be responsible for increasing rubber concentration during the winter. One hypothesis is that cold night temperatures increase the level and/or activity of enzymes involved in rubber synthesis. For example, Ji et al. (1993) observed that the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, which provides a precursor to the isoprene building blocks of rubber, increased in field plants from October through December during the time when rubber concentration was increasing. In addition, rubber transferase activity increased throughout the winter in field grown guayule (Ji et al., 1993; Cornish and Backhaus, 2003), and its activity was higher in plants that were exposed to cold night temperatures in a growth chamber (Sundar and Reddy, 2001; Cornish and Backhaus, 2003). Exposure to cold night temperatures also increased the level of a number of unidentified proteins, which may or may not be involved in rubber synthesis (Sundar et al., 2003).

Another process that may be important in rubber synthesis is carbon exchange (commonly expressed as photosynthesis). Even though guayule has chloroplasts in its stems, the leaves are the primary source of sugars from carbon exchange (Gilliland et al., 1985), and rubber concentration during the winter is drastically reduced in plants that have been defoliated (Kelly and van Staden, 1987; Reddy and Das, 1995). In radiolabelling experiments, sucrose was the main translocated product in guayule, and a precursor to acetate, which is a direct precursor to rubber (Kelly and van Staden, 1987). Further labeling experiments showed that during the winter sucrose is incorporated into rubber, but during the summer it is incorporated into growth, whereas fructose
incorporation into the resin occurs throughout the year (Kelly and van Staden, 1993). Therefore, resources are only incorporated into rubber when growth is not a priority (Kelly and van Staden, 1991). Only one study has examined the response of carbon exchange to cold night temperatures. After an initial decrease in carbon exchange in the cold night treated plants, carbon exchange increased to a level higher than the warm night control plants (Sundar and Reddy, 2000). Guayule also responds to cold temperatures by increasing antioxidative metabolism, which protects the photosynthetic enzymes involved in carbon exchange (Sundar and Reddy, 2000; Sundar et al., 2004).

The purpose of this study was threefold to answer the following questions. First, how do immature plants (<180 d) respond to cold night temperatures? Second, how is rubber production in mature plants (≈ one year) related to night temperature? Third, what is the relationship of carbon exchange to rubber production under cold night temperatures? The results were integrated into the current model of rubber induction during the winter.

**Materials and Methods**

The experiments were conducted in an unheated greenhouse at the Campus Agricultural Center of the University of Arizona, Tucson, AZ. Guayule plants were grown three consecutive winters (December 3, 2002 – March 19, 2003; December 1, 2003 – March 5, 2004; and November 29, 2004 – March 3, 2005). Different lines were used each year based on plant availability. In 2002-2003 there were both mature and immature guayule plants. The mature plants were four tissue culture derived transgenics of the guayule line N6-5, containing kanamycin resistance as a selectable marker.
(McBride and Summerfelt, 1990) in combination with either farnesyl pyrophosphate synthase (FPPS) (Koyama et al., 1993), geranylgeranyl pyrophosphate synthase (GGPPS) (Ohnuma et al., 1994), hexa-heptaprenyl pyrophosphate synthase (H-HPPS) (Ohnuma et al., 1996), or kanamycin resistance alone. The immature plants were seed derived plants of guayule lines 11591 and AZ-6. In 2003-2004 there were also mature and immature plants. The mature plants were all tissue culture derived and included the following guayule lines: transgenic N6-5 containing either H-HPPS with kanamycin resistance or kanamycin resistance alone, transgenic AZ 101 containing only kanamycin resistance, and non-transgenic AZ-2. The immature plants were seed derived plants of guayule lines 11591 and AZ-6. In 2004-2005 only mature seed derived plants of guayule lines AZ 101, N6-5, 11591, and AZ-6 were used.

The experiment was set up as a split plot with four replications except during 2004-2005, which had three replications, with night temperature as the main plot and line as the sub-plot. Within the unheated greenhouse, plants were placed at random into smaller greenhouse type enclosures with differential heating. Air temperature within each container was monitored by a Sentry High/Low Memory Thermometer, and soil temperature was measured with a Digital Dial Thermometer (VWR, West Chester, PA). Plants subjected to the cold night treatment had no supplemental heating and were exposed to the ambient night temperatures within the greenhouse (Table D1), which were on average one or two degrees warmer than the outside temperatures (data not shown). The plants grown with warm night temperatures were exposed to simulated summer night temperatures between 21 and 24 °C (Table D1) with a Titan TCM16 1500 W ceramic
heater (Holmes Products, Fontana, CA) connected to a vented dryer duct to distribute heat evenly throughout the enclosure. All enclosures were uncovered during the day so that both treatments were exposed to similar daytime temperatures (Table D1), while all enclosures were covered at night when the different temperature treatments were applied. Between 0700 and 0800 h the covers were removed, night high and low air temperatures and current soil temperatures recorded, heaters turned off, and the thermometers reset. Between 1700 and 1800 h the enclosures were covered, the heaters turned on, daily high and low air temperatures recorded, and the thermometers reset. The plants were irrigated twice a week, with one of the irrigations containing half strength Peter’s 20:20:20 plus micronutrients fertilizer, and supplemental irrigation applied as needed.

Height and width were measured at the start of the temperature treatments and growth was measured as the change from this initial measurement. Changes in height were measured monthly all three years, and changes in width were measured monthly in 2004-2005. Biomass (fresh weight) of the shoots was measured when the plants were harvested in March by cutting the shoots off at the soil line. A subset of plants, which included one plant per line in each treatment in each replication in 2002-2003, in two replications in 2003-2004 and in one replication in 2004-2005, was defoliated and leaf area measured. Plant material was dried in an oven at 80 °C for two days until the plant material reached a constant weight and the dry weight was recorded. The dried material was then ground in a coffee grinder and resin and rubber extracted with acetone and cyclohexane, respectively, from 0.5 g samples using a variation of the gravimetric method of Black et al. (1983) (Valerie Teetor, personal communication, 2002). Resin
and rubber were extracted separately from the stems of the defoliated plants in 2003-2004 and 2004-2005. Resin and rubber yields were calculated by multiplying their concentrations within the plant by the plant’s dry weight.

Carbon exchange measurements were taken all three years from individual leaves near the top of the plant. Most measurements were taken for mature plants, due to the small size of the leaves of the immature plants. In 2002-2003 and 2004-2005, measurements were taken with a CIRAS-2 (PP Systems, Amesbury, MA) within an hour of solar noon on sunny days using a light source with a PAR of 1000 µmol m\(^{-2}\) s\(^{-1}\), a CO\(_2\) concentration of 370 ppm, an air flow rate of 191 µmol s\(^{-1}\), and ambient humidity. Due to instrument availability measurements were taken mainly in January and February. In 2003-2004 midday carbon exchange measurements were taken weekly December through February with a LI-6200 (Li-Cor Biosciences, Lincoln, NE), using an ambient PAR of 950 to 1200 µmol m\(^{-2}\) s\(^{-1}\), an ambient CO\(_2\) concentration of 380 to 410 ppm, an air flow rate of 185 to 190 µmol s\(^{-1}\), and ambient humidity. Diurnal measurements were taken once in January 2003 from 0700 to 0900, 1100 to 1300, and 1500 to 1700 h, and once in February 2004 every two hours for 24 hours from 0700 through 0500 h.

Statistical analyses were done using JMP 4.0 statistical software, academic version (Sall et al., 2001). Treatments were analyzed using multiple regressions in the Fit Model platform, with night temperature as the main plot and line as the subplot. Multiple comparisons were done using Tukey’s HSD, and specific comparisons were done using orthogonal contrast. To examine the relationship of resin and rubber concentration to night temperature, we ran a regression of mean resin and rubber concentration of each
treatment within each replication against the mean low night temperature of each
treatment within each replication, combining all years. The following data were
transformed in order to normalize the data for analysis. In 2002-2003 carbon exchange,
change in height of mature plants and shoot rubber concentration of mature and immature
plants were log_{10} transformed. In 2003-2004 shoot resin yield of mature plants was
square root transformed. In 2004-2005 fresh and dry weight, shoot resin and rubber
concentration, and shoot resin and rubber yield were log_{10} transformed. A P-value <0.05
was considered significant throughout.

Results

Immature Plants

Lines responded the same within treatments (data not shown), thus, unless
otherwise noted only the effects of temperature are shown and discussed. There were
significant differences in growth between treatments, as measured by changes in height,
in 2002-2003 but not in 2003-2004, and dry weight was not significantly different
between treatments either year (Table D2). Rubber concentration was significantly
greater in the cold night plants in 2002-2003, but not in 2003-2004 (Table D2). Resin
concentration was not significantly related to night temperatures (Fig. D1A); however,
rubber concentration was negatively related to night temperature and was highest when
the night temperature was below 10 °C (Fig. D1B).
Mature Plants

Growth

Lines responded the same within treatments (data not shown), thus, unless otherwise noted only the effects of temperature are shown and discussed. Warm night treated plants 2002-2003 had significantly more new growth, as measured by change in height, than cold night treated plants (Table D3). In 2003-2004 and 2004-2005 there was no difference in new growth as measured by change in height (Table D3), but in 2004-2005 there was a significantly greater change in width in the warm night plants (Table D2), especially in line AZ6 (data not shown). Stem diameter was not significantly different between the treatments in either 2003-2004 or 2004-2005 (data not shown).

At harvest, in 2002-2003, warm night plants had significantly greater fresh weight than cold night plants, with the opposite result in 2003-2004 (Table D3). Fresh weight was variable between the warm and cold night plants in 2004-2005, masking any significant differences in fresh weight between treatments (Table D3). Despite the inconsistent response of fresh weight to the different treatments over the three winters, there was never any difference in dry weight between cold and warm night plants (Table D3). Leaf area was also not significantly different between cold and warm night plants (data not shown).

Secondary compound production

Resin concentration and yield in the shoot, which is composed of the leaves and stems, were not significantly different between treatments all three years (Table D4). Rubber concentration and yield in the shoots were significantly higher in the cold night
treated plants all three years (Table D4). In 2004-2005 rubber concentration in the cold AZ6 plants was significantly higher than all other lines in both treatments (data not shown). There was no significant relationship between resin concentration and night temperature (Fig. D2A). Rubber concentration had a negative relationship with night temperature, increasing most at average minimum night temperatures below 10 °C (Fig. D2B). In 2003-2004 rubber concentration, but not resin concentration, in the stems was significantly higher in the cold night than the warm night plants (Table D4). In 2004-2005 stem measurements were taken on a subset of plants from only one replication, because it was the only replication that had not lost any of the lines due to *Verticillium* wilt. Although we were unable to statistically show a difference between the treatments, it appears that rubber concentration was higher in the stems of the cold night plants (Table D4).

*Carbon Exchange*

Carbon exchange data from mature and immature plants are presented together since there was no significant effect of age on carbon exchange (data not shown). Mean midday carbon exchange was significantly higher in the cold night plants in 2002-2003, and not significantly different between treatments in 2003-2004 and 2004-2005 (Fig. D3). During diurnal measurements carbon exchange was highest during the midday measurement, declined significantly by 1500 h, was at its lowest just after sunset, and hovered around zero for the remainder of the night (data not shown).
Discussion

There is evidence that guayule plants need to be at least 180 days old before rubber synthesis can be induced by cold nights (Cornish and Backhaus, 2003). It has been reported that immature plants need exposure to cold nights for at least four to six months before rubber concentration is substantially increased (Goss et al., 1984; Cornish and Backhaus, 2003). We observed increased rubber concentration in the immature cold night plants only in 2002-2003 (Table D2). In 2002-2003 the immature plants were exposed to 107 d of cold nights compared to only 96 d in 2003-2004. There was also an almost 6 °C difference in average low night temperature between these two winters (Table D1). Our data support the hypothesis that immature plants are not as responsive to cold night temperatures as mature plants and may need either an extra few days of cold nights, low night temperatures close to or below 10 °C (Fig. D1B), or both in order to increase rubber production during the winter.

It is apparent that rubber production is affected by cold night temperatures, yet most of the evidence has been either circumstantial or from growth chamber studies with widely varying estimates of the temperatures required for increased rubber production ranging from 7 to 20 °C, (Bonner, 1943, Benedict, 1950; Goss et al., 1984; Appleton and van Staden, 1991; Ji et al., 1993; Sundar and Reddy, 2000; Sundar and Reddy, 2001; Cornish and Backhaus, 2003). From our data it appears that night temperatures between 10 and 15 °C result in a slight increase in rubber concentration within the plant, but that temperatures below 10 °C and probably closer to 7 °C may be the threshold where rubber
production increases in a more exponential rather than linear fashion (Table D4, Fig. D2B).

As we examined the data, we evaluated various reasons for the increased rubber concentration observed in the cold night plants. In a separate experiment we found that water stress caused differences in rubber concentration due to differences in the relative contribution of leaves and stems to the total rubber within the shoots and differences in stem diameter (Veatch-Blohm et al., in press). In the present study rubber contribution from the leaves and the stems, and the stem diameter were not significantly different between treatments (data not shown).

Since increased rubber concentration did not seem to be related to differences in rubber distribution in the plant or differences in stem diameter, we examined the contributions of growth and carbon exchange on rubber concentration. Only one previous study has examined the role of carbon exchange, which showed an initial decrease in the cold night plants, but eventually greater carbon exchange in the cold night plants compared to the untreated control. However, in this experiment, which ran for only 60 days, only the cold night plants were transferred to a growth chamber for the night temperature treatment (Sundar and Reddy, 2000).

Cold night treated plants maintained carbon exchange rates higher than or as high as the warm night plants in 2002-2003 and 2004-2005, respectively (Fig. D3), but there was significantly less new growth (height or width) in the cold night plants compared to the warm night plants (Table D3). One of the main products of carbon fixation is sucrose, and radio-labeling tests show that sucrose goes into rubber in the winter, but
contributes to growth during the summer (Gilliland et al., 1985; Kelly and van Staden, 1993). Increased rubber concentration in the cold night plants could be due to sugars produced during carbon fixation going into rubber synthesis since they were not being used for new growth. Dry weight was also not significantly different between treatments (Table D3), with the cold night plants having higher rubber concentrations and yields (Table D4). It appears that carbon was incorporated into biomass in both treatments, but the difference was in what form the biomass was accumulated, new growth or rubber production.

Our data show that normal carbon exchange combined with low growth rates does play a significant role in increased rubber concentration in cold night plants; however, a number of studies have shown that rubber transferase activity increases as temperatures decrease (Sundar and Reddy, 2000; Sundar and Reddy, 2001; Cornish and Backhaus, 2003). Our data from 2003-2004 provide circumstantial evidence that rubber transferase also plays a role in rubber synthesis in response to cold night temperatures. In 2003-2004, not only were there no differences in carbon exchange between treatments (Fig. D3), but there were also no significant differences in growth (Table D3). The higher rubber concentration seen in the cold night plants could not have been merely the result of carbon being diverted into rubber synthesis instead of growth; otherwise the shoots of the warm night plants should have had the same rubber concentration as the cold night plants (Table D4). In fact we found that there was an even greater difference in rubber concentration between treatments within the stems (Table D4), where the majority of rubber is laid down (Dierig et al., 1989), which would also be an area with high rubber
transferase activity. Therefore a model for rubber synthesis during the winter should include the contributions of growth, carbon exchange, and rubber transferase activity.

Based on the data from 2002-2003 and 2003-2004 we expected that the plants in 2004-2005, which had temperatures between the first two years, would have rubber concentrations between the first two years (Table D4). This was not the case and this discrepancy seems to be related to die off from *Verticillium* wilt, which affected the warm night plants more than the cold night plants. A consequence of the few plants in the warm night treatment was that any extreme value factored more into the mean than it did in the cold night treatments. Early guayule research found that plants exposed to warm temperatures do not necessarily have low rubber concentration, and may have increased rubber concentration because of other known or unknown environmental factors (Benedict, 1950). Another explanation was that there was less of a difference between high and low temperatures in 2004-2005 compared to the other two years (data not shown). Appleton and van Staden (1991) found that the day-night temperature differential needs to be around 20 °C in order for rubber concentration to increase. There were a number of days especially in January and February of 2005 that were very cloudy and rainy with a day-night temperature differential in the cold night plants of less than 10 °C (data not shown).

In conclusion we found that rubber induction and synthesis during the winter is a complex process affected by multiple factors. Rubber synthesis is greatly affected by temperatures below 10 °C. Rubber transferase activity is increased at these temperatures (Sundar and Reddy, 2000; Sundar and Reddy 2001; Cornish and Backhaus, 2003), while
carbon exchange is relatively unaffected. Reduced growth, which is the general trend in cold exposed plants (Bonner, 1943; Kelly and van Staden, 1993), in combination with high carbon exchange rates, allow more photosynthates to be shuttled into rubber synthesis than is normally shuttled into rubber synthesis during active growth (Kelly and van Staden, 1993).

**Acknowledgements:** We would like to thank David Blohm, Tyler Shumway, Alisha Shumway, Marcal Jorge, and Bill Liesenbein for their assistance in the set up of the experiments.
References


Table D1. Mean daytime high, nighttime low and average, and morning soil temperatures (°C) ± standard error in warm and cold night treated guayule plants.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean High Temp (Day)</th>
<th>Mean Low Temp (Night)</th>
<th>Mean Temp (Night)</th>
<th>Mean Soil Temp (Morning)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm</td>
<td>26.9 ± 0.15</td>
<td>14.6 ± 0.15</td>
<td>21.0 ± 0.13</td>
<td>17.0 ± 0.15</td>
</tr>
<tr>
<td>Cold</td>
<td>24.7 ± 0.22</td>
<td>7.7 ± 0.15</td>
<td>13.5 ± 0.15</td>
<td>9.4 ± 0.14</td>
</tr>
<tr>
<td></td>
<td><strong>2002 – 2003</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n = 107)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warm</td>
<td>28.8 ± 0.17</td>
<td>18.2 ± 0.16</td>
<td>24.0 ± 0.13</td>
<td>22.7 ± 0.13</td>
</tr>
<tr>
<td>Cold</td>
<td>26.9 ± 0.14</td>
<td>13.0 ± 0.13</td>
<td>17.4 ± 0.10</td>
<td>16.0 ± 0.08</td>
</tr>
<tr>
<td></td>
<td><strong>2003 – 2004</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n = 96)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warm</td>
<td>25.9 ± 0.17</td>
<td>17.6 ± 0.19</td>
<td>23.7 ± 0.16</td>
<td>21.3 ± 0.16</td>
</tr>
<tr>
<td>Cold</td>
<td>23.1 ± 0.17</td>
<td>10.4 ± 0.19</td>
<td>14.4 ± 0.16</td>
<td>12.3 ± 0.20</td>
</tr>
<tr>
<td></td>
<td><strong>2004 – 2005</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n = 95)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table D2. Mean change in height, mean fresh and dry weight, and mean shoot resin and rubber concentration (%) ± standard error of immature (<180 d) warm and cold night treated guayule.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change in Height (cm)$^1$</th>
<th>Fresh Weight (g plant$^{-1}$)$^2$</th>
<th>Dry Weight (g plant$^{-1}$)$^2$</th>
<th>Resin (%)</th>
<th>Rubber (%)$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm (n = 27)</td>
<td>9.3 ± 0.97 a$^4$</td>
<td>12 ± 1.9 a</td>
<td>2 ± 0.3 a</td>
<td>3.5 ± 0.14 a</td>
<td>1.2 ± 0.03 b</td>
</tr>
<tr>
<td>Cold (n = 29)</td>
<td>6.6 ± 1.44 b</td>
<td>6 ± 1.0 b</td>
<td>1 ± 0.2 a</td>
<td>3.9 ± 0.19 a</td>
<td>1.5 ± 0.11 a</td>
</tr>
<tr>
<td>Warm (n = 39)</td>
<td>12.2 ± 1.14 a</td>
<td>77 ± 6.6 a</td>
<td>13 ± 1.3 a</td>
<td>3.9 ± 0.10 a</td>
<td>1.2 ± 0.03 a</td>
</tr>
<tr>
<td>Cold (n = 39)</td>
<td>10.2 ± 0.83 a</td>
<td>85 ± 7.5 a</td>
<td>17 ± 1.7 a</td>
<td>3.9 ± 0.09 a</td>
<td>1.3 ± 0.03 a</td>
</tr>
</tbody>
</table>

1 Change in height in 2002-2003 was log$_{10}$ transformed for analysis.

2 Fresh and dry weight were log$_{10}$ transformed for analysis.

3 Rubber concentration (%) in 2002-2003 was log$_{10}$ transformed for analysis.

4 Numbers in the same column, within the same year, with the same letter are not significantly different according to orthogonal contrast $P < 0.05$. 
Table D3. Mean change in height and width, and mean fresh and dry weight ± standard error of mature (≈ one year) warm and cold night treated guayule.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change in Height (cm)</th>
<th>Change in Width (cm)</th>
<th>Fresh Weight (g plant⁻¹)¹</th>
<th>Dry Weight (g plant⁻¹)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002 – 2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warm (n = 79)</td>
<td>4.0 ± 0.60 a²</td>
<td>NA</td>
<td>65 ± 1.4 a</td>
<td>15 ± 0.4 a</td>
</tr>
<tr>
<td>Cold (n = 80)</td>
<td>2.2 ± 0.43 b</td>
<td>NA</td>
<td>61 ± 1.4 b</td>
<td>15 ± 0.4 a</td>
</tr>
<tr>
<td>2003 – 2004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warm (n = 56)</td>
<td>3.8 ± 0.67 a</td>
<td>NA</td>
<td>156 ± 7.4 b</td>
<td>44 ± 1.5 a</td>
</tr>
<tr>
<td>Cold (n = 56)</td>
<td>3.6 ± 0.67 a</td>
<td>NA</td>
<td>177 ± 9.2 a</td>
<td>48 ± 2.0 a</td>
</tr>
<tr>
<td>2004 – 2005</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warm (n = 21)</td>
<td>1.4 ± 1.77 a</td>
<td>8.8 ± 1.63 a</td>
<td>46 ± 4.8 a</td>
<td>17 ± 1.6 a</td>
</tr>
<tr>
<td>Cold (n = 43)</td>
<td>-2.6 ± 0.75 a</td>
<td>1.3 ± 0.57 b</td>
<td>67 ± 6.8 a</td>
<td>27 ± 2.5 a</td>
</tr>
</tbody>
</table>

¹ Fresh and dry weight were log₁₀ transformed for analysis.

² Numbers in the same column, within the same year, with the same letter are not significantly different according to orthogonal contrast $P < 0.05$. 
Table D4. Mean shoot resin and rubber concentration (%), shoot resin and rubber yield, and stem resin and rubber concentration (%) ± standard error of mature (∼ one year) warm and cold night treated guayule.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot (^1,2)</th>
<th>Stem (^3,4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resin (%)</td>
<td>Resin yield (mg plant(^{-1}))</td>
</tr>
<tr>
<td>Warm</td>
<td>4.2 ± 0.03 a</td>
<td>644 ± 16.5 a b</td>
</tr>
<tr>
<td>(n = 79)</td>
<td>4.2 ± 0.05 a</td>
<td>651 ± 19.7 a</td>
</tr>
<tr>
<td>Cold</td>
<td>4.2 ± 0.03 a</td>
<td>644 ± 16.5 a b</td>
</tr>
<tr>
<td>(n = 80)</td>
<td>4.2 ± 0.05 a</td>
<td>651 ± 19.7 a</td>
</tr>
</tbody>
</table>

2003 – 2004

| Warm      | 3.7 ± 0.08 a  | 1663 ± 64.9 b | 1.6 ± 0.04 b | 682 ± 26.0 b | 3.5 ± 0.20 a | 1.3 ± 0.09 b |
| (n = 56)  | 3.8 ± 0.07 a  | 1856 ± 104.4 a | 1.7 ± 0.04 a | 787 ± 29.5 a | 3.7 ± 0.17 a | 1.7 ± 0.13 a |
| Cold      | 3.8 ± 0.07 a  | 1856 ± 104.4 a | 1.7 ± 0.04 a | 787 ± 29.5 a | 3.7 ± 0.17 a | 1.7 ± 0.13 a |
| (n = 56)  | 3.8 ± 0.07 a  | 1856 ± 104.4 a | 1.7 ± 0.04 a | 787 ± 29.5 a | 3.7 ± 0.17 a | 1.7 ± 0.13 a |

2004 – 2005

| Warm      | 4.9 ± 0.19 a  | 829 ± 80.7 a b | 1.4 ± 0.08 b | 234 ± 23.2 b | 4.1 ± 0.51 a | 1.8 ± 0.60 a |
| (n = 21)  | 4.8 ± 0.11 a  | 1321 ± 76.8 a  | 1.5 ± 0.08 a | 380 ± 41.7 a | 4.0 ± 0.47 a | 2.4 ± 0.92 a |
| Cold      | 4.8 ± 0.11 a  | 1321 ± 76.8 a  | 1.5 ± 0.08 a | 380 ± 41.7 a | 4.0 ± 0.47 a | 2.4 ± 0.92 a |
| (n = 43)  | 4.8 ± 0.11 a  | 1321 ± 76.8 a  | 1.5 ± 0.08 a | 380 ± 41.7 a | 4.0 ± 0.47 a | 2.4 ± 0.92 a |

\(^1\) Shoots are composed of the leaves and stems.

\(^2\) Shoot resin concentration (%) in 2004-2005 was log\(_{10}\) transformed, shoot resin yield in 2003-2004 was square root transformed and in 2004-2005 was log\(_{10}\) transformed, and shoot rubber concentration (%) and yield in 2002-2003 was log\(_{10}\) transformed for analysis.
Resin and rubber concentration in the stems were measured on a subset of defoliated plants. In 2003-2004: \( n = 14 \) for both treatments. In 2004-2005: \( n = 4 \) for both treatments.

Stems are defoliated shoots.

Numbers in the same column, within the same year, with the same letter are not significantly different according to orthogonal contrast \( P < 0.05 \).

Orthogonal contrast could not be performed on the stem data in 2004-2005 because stem data was collected on the only replication that included all lines in both treatments.
List of Figures

Figure D1. *P*-value and regression in immature plants (<180d) of mean resin and rubber concentration (%) from each treatment of each replication on mean temperature of each treatment of each replication including 2002-2003 and 2003-2004. A- Regression of mean resin concentration (%) on mean temperature. B- Regression of mean rubber concentration (%) on mean temperature.

Figure D2. *P*-value and regression in mature plants (≈1 year) of mean resin and rubber concentration (%) from each treatment of each replication on mean temperature of each treatment of each replication including 2002-2003, 2003-2004, and 2004-2005. A- Regression of mean resin concentration (%) on mean temperature. B- Regression of mean rubber concentration (%) on mean temperature.

Figure D3. Mean midday carbon exchange of cold and warm night treated guayule plants. Carbon exchange measurements were taken within an hour of solar noon on 28, 21, or 12 plants within each treatment in 2003, 2004 and 2005 respectively. Vertical bars represent ± standard error.
Figure D1.

A

\[ y = -0.003x + 3.82 \]

\[ p=0.900 \]

B

\[ y = -0.023x + 1.60 \]

\[ p=0.020 \]
Figure D2.

A

\[ y = -0.017x + 4.46 \]

B

\[ y = -0.054x + 2.46 \]

\[ p=0.526 \]

\[ p=0.002 \]
Figure D3.

![Graph showing CO₂ emissions over time]

- **µmol CO₂ m⁻² s⁻¹**
- **Cold**
- **Warm**