The Potential for Sunflower as a Rubber-Producing Crop for the United States.

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Abstract

There are strategic as well as economic incentives to develop a renewable, United States-based supply of natural rubber. Currently, nearly all commercial natural rubber comes from a single species, the Brazilian rubber tree (*Hevea brasiliensis*) and the US is almost completely dependent on imports from distant countries. Global natural rubber production will be best served in the future by multiple natural rubber crops. However, for most rubber crops, the ability to produce rubber that is economically competitive with rubber from *Hevea brasiliensis* is essential, because the largest increasing demand for rubber is to make tires as China and India industrialize.

Leaves of sunflower **(***Helianthus annuus*) produce rubber and researchers have postulated there is genetic potential for increasing the rubber content of cultivated *H. annuus*. Sunflower is a good candidate as a potential rubber source because this plant species already makes rubber which means it can compartmentalize this secondary product; it is adapted for agronomic production; it produces high biomass per acre; and sunflower agronomy is well understood and would need only minor adjustment as a rubber crop.

H. annuus currently produces little rubber of low molecular weight and also is notoriously recalcitrant to genetic transformation. In this paper, we describe progress in the development of tissue culture, transformation and regeneration of *H annuus*, and genetic engineering of sunflower for increased rubber quality and production.

Introduction

Almost all commercial natural rubber comes from a single plant species, the Brazilian rubber tree (*Hevea brasiliensis*) and the United States is completely dependent on imports from distant countries (Davis 1996). Primarily due to its molecular structure and high molecular weight (>1 million g/mol), natural rubber has high performance properties that cannot be matched by synthetic rubber produced from petroleum. These properties include resilience, elasticity, abrasion and impact resistance, efficient heat dispersion, and malleability at cold temperatures. Despite the best efforts of the chemical industry, these properties have not been achieved cost-effectively by synthetic materials, or non-rubber natural polymers or

blends. Natural rubber is essential in many high performance applications, including tires, particularly for aircraft and large equipment, surgical gloves, and latex balloon devices.

Thus, there are strategic as well as economic incentives to develop a renewable, United States-based supply of natural rubber, as has been recognized and stated by the U.S. Congress in Public Laws 95-592 and 98-284. Guayule (*Parthenium argentatum*) is being commercially produced in the southwestern United States as a source of natural rubber latex suitable for medical products (Cornish and Williams, 2006) (and that meets the protein standards of an ASTM D 1076-06 Category 4 latex). However, in the same way that many crops are used to produced carbohydrates (starch) and hydrocarbons (oils), we believe that global natural rubber production will best be served in the future by multiple natural rubber crops (Mooibroek and Cornish, 2000). However, for most rubber crops, the ability to produce rubber economically competitive with rubber from *Hevea brasiliensis* is essential, because the largest increasing demand for rubber is to make tires as China and India industrialize.

At least 2,500 plant species are known to produce natural rubber (Bowers 1990) but few do this in either high enough yields or the high molecular weight needed for commercial viability (Swanson et al., 1979). Most of the rubber plants known are wild plants, and the domestication process would be extremely long and arduous. Ideally, a rubber crop would be an annual plant with a large biomass, with established agronomic practices that could be readily adapted to rubber production.

The sunflower genus *Helianthus* has 69 species and subspecies native to the U.S. Leaves of *H. annuus* produce a small amount of rubber, and, of the 53 species and subspecies of *H. annuus* analyzed in the past, 14 produce more than 0.93% rubber (Stipanovic et al., 1982; Seiler et al., 1991). Those researchers postulated there is a high genetic potential for increasing the rubber content of cultivated *H. annuus*. *H. annuus* also has the potential to produce valuable co-products including specialty carbohydrates and feed for livestock. *H. annuus* appears to be an excellent candidate as a potential source of cultivated rubber because: *H. annuus* (1) already makes rubber which means it can compartmentalize this secondary product and will likely do the same with larger endogenous amounts; (2) is adapted for agronomic production in many parts of the United States as an annual crop; (3) produces high biomass per hectare; (4) agronomy is well understood and would likely need only minor adjustment for it to be grown for rubber across the country; (5) is adapted for mechanized agriculture; and (6) is a close relative of *P. argentatum*, a plant in which the biochemical regulation of rubber yield and quality has been extensively studied. *H. annuus* and *P. argentatum* rubber together would be able to meet the U.S. rubber needs and allow for marketing of high quality manufactured rubber products in international trade.

However, *H. annuus* currently produces little rubber and what it does produce is of low molecular weight. *H. annuus* also is notoriously recalcitrant to genetic transformation. In this paper, we describe our progress in the development of tissue culture, transformation and regeneration of *H annuus*, and how we are applying these methods to the metabolic engineering of sunflower for increased rubber quality and production, in a manner analogous to our efforts with *P. argentatum*.

Methods and Materials

Colorado field study. Agronomic characteristics and latex production were determine in a selection of modern *H. annuus* cultivars during a field performance test at Fruita during 2001, 2002, and 2003. The experiments were randomized complete blocks with four

replications. Plot size was 3.04 m wide (four, 0.76 m beds) x 15.2 m long beds. Fertilizers, herbicides, insecticides, and other inputs were applied as needed each year for best management practices and to obtain high economic yields. Plots were furrow irrigated as needed each year to prevent plants from experiencing water stress and to obtain high biomass yields. Planting occurred on 15 May 2001, 20 May 2002, and 22 May 2003 at 54,362 seeds/ha (22,000 seeds/acre). Harvest occurred on 20 Sept 2001, 6 Sept. 2002, and 28 Aug. 2003. Aerial phytomass was determined by harvesting the two center rows of each 4-row plot with a commercial forage chopper. The forage mass was blown into a weigh truck that traveled alongside the forage chopper as plots were harvested. A forage subsample was obtained from each plot for moisture determination. Subsamples were weighed immediately after harvest and oven-dried at 65°C until constant weights were obtained. Aerial phytomass was calculated on a dry matter basis. Additional subsamples were sealed in plastic and shipped overnight in coolers to Albany, California for latex extraction, purification, and analysis.

In 2001, plants were deheaded to test whether preventing assimilates from being converted into seed oil would increase latex yield. Flower buds were manually removed once reproductive growth could be visually identified on plants. Ten consecutive plants were deheaded. When physiological mature, the plants were harvested at ground level and partitioned into leaves, stems, and head. Petioles were partitioned as stems. Plant material was oven-dried at 65ºC to a constant weight and weighed.

Latex Extraction and Purification. All leaves were excised from the plants and ground in a one gallon Waring blender set on low for 1 minute in 0.2% NH_4OH, 0.1% Na_2SO_3, pH 10 (AAO) 1:1 w:v. The homogenate was filtered through four layers of grade 60 cheese cloth. The filtrate was adjusted to pH 10 with NH_4OH and then to 200 mM EDTA. The homogenate was centrifuged for 25 min at 5850 x g in a bucket rotor. The floated latex layer was scooped off into 5 ml of AA0 and purified by repeated creaming in 0.1% ammonium alginate as described for *P. argentatum* latex (Cornish and Brichta, 2002). Creaming was repeated twice beyond when the subnatent became colorless. In some experiments, the plants were subdivided into leaves, stems, and flowers, and the flowers further subdivided into pappus and disc. Each plant part was homogenized and analyzed for latex content in a similar manner to the leaves.

Latex Quantification. 35 ml aliquots of homogenate were centrifuged for 25 min at 5,870 x g in a bucket rotor. 2 ml of glacial acetic acid was pipetted onto the floated latex layer and the homogenate was recentrifuged. Coagulated latex was lifted off the homogenate onto tared weigh paper with a metal spatula, or collected by vacuum filtration onto tared 0.25 µm cellulose acetate/cellulose nitrate filters, dried overnight at 37ºC, and weighed.

Molecular Weight Analysis. Purified latex samples dissolved in tetrahydrofuran were analyzed on a HPLC/MALLS system with a HP1047 refractive index detector (Agilent Technologies, Palo Alto, California), a multiple angle laser light scattering detector containing 18 light scattering detectors with a 632.8 nm wavelength laser (Dawn DSP Laser Photometer, Wyatt Technologies, Santa Barbara, CA), a Phenogel 5 µm Linear/ Mixed Guard Column (Phenomenex, Torrence, CA) and a PLgel 10 µm Mixed-B size exclusion column (Agilent Technologies, Palo Alto, CA) maintained at 35°C (Dayal and Mehta, 1994). Latex rubber polymers were eluted within 17 min by THF (1.0 ml/min). Astra software (Wyatt Technologies, Santa Barbara, CA) was used to calculate molecular weight (M_w).

Tissue Culture, Transformation and Regeneration. Tissue culture and regeneration protocols were tested using a wide range of explants from several different sunflower

cultivars in attempts to determine the least recalcitrant system. Also, we concentrated on developing a system that could be used with mature tissues due to the difficulties presented by use of immature seed – it is difficult, on a laboratory scale, to obtain many seed at exactly the same developmental stage from sunflower, and it also is difficult to determine at exactly what developmental stage a seed is at any particular time in greenhouse plants because seed develops in series across the flowering disc and not in synchrony. Promising regeneration systems and cultivars were tested for their transformation ability using constructs containing the GUS reporter gene before being transformed with genes of biochemical interest.

Agrobacterium-mediated transformations were performed with constructs containing the GUS reporter gene. A herbicide-resistant selectable marker was used initially to verify and fine tune protocols, but ultimately a kanamycin-resistant selectable marker was used in a series of vectors containing the target genes involved in rubber biosynthesis. Transformed tissue from mature seed was regenerated using a sequence of semisolid agar medium in Petri dishes for shoot production followed by rooting on a sterilized peat pellet in a liquid medium in ventilated Magenta boxes. Plantlets were grown in a tissue culture growth chamber and when well rooted, were transferred into pots with soil and moved into a BL2-P+ greenhouse.

Results

Table 1. Average dry matter production of sunflower grown at Fruita, Colorado.

Year	No. of cultivars evaluated	Dry matter yield kg/ha
2001	17	9,650
2002	15	11,484
2003	18	10,565

Sunflower biomass yields were reasonably consistent over three years (Table 1), as was the partitioning of dry matter among the different parts of the plant (Table 2) in two different years. On average, we can estimate that in 2002 and 2003, the leaf yields were 2,148 and 1,764 kg/ha, respectively.

Table 2. Average dry matter partitioning of sunflower cultivars grown at Fruita, Colorado

Year	No. of cultivars	Plant weight (g)	Leaf weight (%)	Stem weight (%)	Head weight (%)
2002	15	236.8	18.7	36.1	45.2
2003	18	220.7	16.7	39.7	43.6

The latex was found almost entirely in leaves of young and mature sunflowers. No latex was found in mature stems or in the pappus of the flowers. Observations suggested that a small amount of latex might be present in the flower discs but the presence of a large number of oil bodies made it difficult to accurately or reproducibly assess latex content in this tissue.

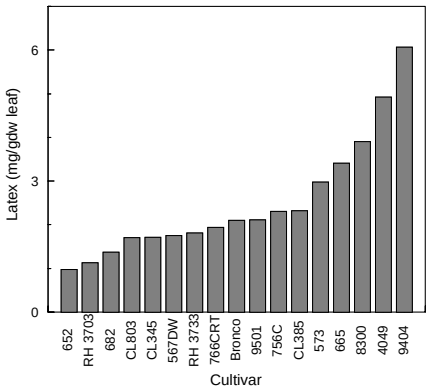

Figure 1. Leaf latex content in different cultivars of *H. annuus* grown in Fruita, Colorado, in 2001.

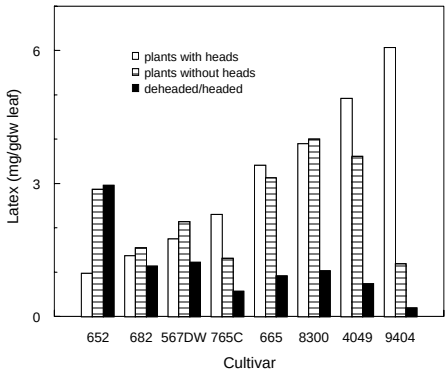

Figure 2. Effect of deheading on leaf latex content in a selection of *H. annuus* cultivars grown in Fruita, Colorado, in 2001.

When the latex was quantified in leaves of a selection of modern cultivars a considerable range was observed (Fig. 1) but even the highest value was less than 1% on a dry weight basis – far below a commercially-viable level of 5-10%. Also, we observed that deheading sunflowers did not enhance latex production in leaves through assimilate redistribution, and although the effect varied among cultivars (Fig. 2), in most cases deheaded plants had substantially lower levels of leaf latex.

The molecular weight of latex rubber from cultivars 593 and 4049 was found to be similar (Fig. 3): most of the rubber was of low molecular weight with a mean Mw $\pm$ s.e. (n=4) of 68,543 $\pm$ 4,527 for *cv.* 573 and 68,180 $\pm$ 2,265 for *cv.* 4049. However, both cultivars also had some higher Mw rubber, 573 considerably more than 4049 (see the shoulder on Fig. 3 – this was a distinct peak on the original chromatogram for 573 and a shoulder for 4049, which largely disappears on the differential plot shown here). The mean molecular weight of this minor peak was 611,225 $\pm$ 100,947 for *cv.* 573 and 509,950 $\pm$ 123,071 for *cv.* 4094.

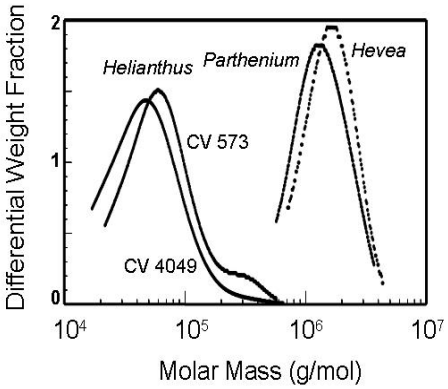

Figure 3. Molecular weight distribution of latex rubber purified from two cultivars of *H. annuus* and compared with latex rubber from *P. argentatum* and *H. brasiliensis.*

Optimization of tissue culture and regeneration protocols of non-transgenic materials led to rapid improvements in regeneration efficiencies. The number of total shoots regenerated from 200 mature seeds was increased from 70 to 120.

Optimization of the wounding method used for *Agrobacterium*-mediated transformation increased transient GUS expression from only 25% to a maximum of 40% in both split apexes and proximal cotyledon explants from mature seeds. In these trials, at least six stable transformation events were obtained from 88,000 seeds. The presence of the transgene was confirmed with PCR testing.

Transgenic plants containing genes that are part of the rubber biosynthesis pathway were successfully transferred to pots in a biocontainment greenhouse and fertile seed was produced. A study is currently underway to evaluate one transgenic sunflower line to determine if the line produces more rubber than non-transformed controls.

Discussion

Currently, *H. annuus* production as an oilseed crop is not profitable in many areas of the country. In Colorado, crop enterprise budgets for 2000 showed *H. annuus* production to range in net losses to the farmer from $8.35US/ha ($3.38/acre) to as high as $231.95US/ha ($93.87/acre), depending on irrigation and location (Dalsted et al., 2001). Under various production scenarios for more recent estimated production costs and returns, losses for irrigated and dryland conditions were as high as $370.72 US/ha ($150.03/acre) and, under ideal market and production conditions, a profit of $160.64 US/ha ($65.01/acre) was possible (Tranel et al., 2005). If farm gate prices for transgenic, rubber-producing *H. annuus* were comparable to alfalfa hay prices ($77.14US/Mt, 9.0 Mt @ $77.14 = $694.26US/ha) ($70/ton,

4 tons @ $70 = 280.00/acre) the value of rubber-producing *H. annuus* would be greater than the highest value in Colorado for irrigated *H. annuus* of $668.65US/ha ($270.60/acre). Looking from another standpoint, the average price for raw rubber is approximately $2.98 US/kg ($1.35/lb) and with an initial sunflower rubber production of 896 kg/ha (800 lbs/acre) the value for the rubber would be $2670 US/ha ($1080/acre). Currently, sunflower grown for oil under irrigation can yield upwards of 2,800 kg of seed/ha (2,500 lbs /acre). At an average oil content of 40% and a price for oil at $0.44 US/kg ($0.20/lb) the value of sunflower oil after processing the seed (personal communication, Larry Kleingartner, 2001, National Sunflower Association) is $494/ha ($200.00/acre). Thus, the production of rubber from sunflower has more profit potential and cost flexibility, both for the farmer for growing the crop and for the processor, than for irrigated sunflower currently produced for oil. Moreover, sunflower produced for oil and rubber under dryland conditions would be less profitable than yields of irrigated sunflower.

Rubber-producing crop plants ideally are fast-growing, produce high biomass, and are annual crops. Annual crops can be more readily included in on-farm crop rotations and farming systems and planted and plowed-out in response to market needs and farmer production considerations. Perennial cropping systems, such as those for trees, that must remain in place for many years are not well suited to changing market needs and price fluctuations.

We have successfully developed a sunflower transformation system based on explants from mature seeds and have carried metabolically engineered transformants through the T2 generation. Transformation efficiency rates remain extremely low which will continue to impede the development of sunflower as a rubber-producing crop.

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