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Biosynthesis of Rubber in Cultured Cells of *Sonchus oleraceus*†

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Rubber (*cis*-1,4-polyisoprene) was detected in the leaves of *Sonchus oleraceus* by ^{1}H-nuclear magnetic resonance spectroscopy. The rubber had a low molecular weight with a narrow molecular weight distribution centered around 1.0×10^5 Da. Callus cultures were initiated from the leaf parts of *S. oleraceus* and suspension cultures were established in Murashige and Skoog's medium with various concentrations of 1-naphthalene acetic acid (NAA) and benzylaminopurine (BA). The cell biomass was higher in the presence of 1-naphthalene acetic acid (0.1 mg/L) and benzylaminopurine (0.1 mg/L). The cultured cells contained trace amounts of rubber-like polymer identified by gel permeation chromatography.

Key Words: Natural rubber, Biosynthesis, Callus, *Sonchus oleraceus*.

INTRODUCTION

Natural rubber (*cis*-1,4-polyisoprene) from *Hevea brasiliensis* is the main commercial rubber source owing to its high productivity and excellent physical properties. A range of synthetic polyisoprenes are produced by the polymerization of isoprene monomer derived from petroleum. Some of these polyisoprenes exhibited *cis*-1,4-isoprene contents > 98 %. On the other hand, these synthetic rubbers are often green and vulcanized rubber is to some extent inferior to natural rubber. Therefore, natural rubber cannot be replaced completely with synthetic rubber in many applications.

The demand for natural rubber is increasing every year, particularly in developing countries. *H. brasiliensis* can be grown in relatively few regions, such as Southeast Asia and South America and the plant yields extractable commercial rubber after *ca.* 7 years. Given the limitations imposed by climate and access to arable land, it is difficult to envisage large increases in the production of natural rubber from *H. brasiliensis* from a viewpoint of environmental protection. More than 2,500 plant species have been shown to contain natural rubber[1], but only 2 species besides *H. brasiliensis*, Guayule (*Parthenium argentatum*) and Russian dandelion (*Taraxacum koksaghyz*), are used for natural rubber production. Guayule grows in semi-arid regions of Mexico and the United States and contains high-quality rubber without hypoallergenic latex in the stems[2,3]. Therefore, Guayule rubber is considered a promising plant to replace *H. brasiliensis* for commercial rubber production[4]. Moreover, an investigation of 100 plant species growing in the United States, which focused on biomass conversion and rubber formation, identified 12 species that might be suitable for development as crops for effective rubber production[5].

This study examined the rubber production potential of the herbaceous species, *Sonchus oleraceus. S. oleraceus* is a therophyte of the Asteraceae family and grows wild in many areas, including Europe and Japan. Furthermore, *S. oleraceus* can grow wild in severe habitats, such as roadsides. These features make *S. oleraceus* a potential alternative rubber resource.

Secondary metabolites can be biosynthesized by anaplastic cells, such as cells of the callus, outside of a complete plant. This paper reports the potential for the larger-molecular-weight rubber production from cultivated *S. oleraceus* cells.

EXPERIMENTAL

Sonchus oleraceus plants growing in Kobe, Japan, were collected from May to July 2010. The plants were immediately lyophilized and pulverized in a mortar. The freeze-dried powder of the leaves and stems was washed with methanol and acetone for 24 h each to remove the low-molecular-weight compounds. The washed dry powder was extracted successively using the Soxhlet method with toluene at 110 ºC for 4 h. The residue obtained from toluene extraction was rinsed

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with methanol. The molecular weight (MW) and molecular-weight distribution (MWD) of the toluene-extractable fraction was determined by gel permeation chromatography (GPC).

Culture media: Murashige and Skoog's (MS) medium[6] including inorganic salts and organic components with 3 % sucrose (w/v) was used as the base medium. The pH was adjusted to 5.7 with HCl and NaOH. The medium was used in combination with various concentrations of 1-naphthalene acetic acid (NAA) and benzylaminopurine (BA) as an auxin and cytokinin, respectively. The solid medium contained 0.2 % (w/v) gellan gum as the gelling agent.

Callus induction and maintenance: Surface-sterilized leaf stems from *S. oleraceus* were excised into small pieces and placed in Murashige and Skoog's medium supplemented with 1-naphthalene acetic acid and benzylaminopurine at 23 ºC in the dark to induce callus formation. Each callus was sub-cultured under the same conditions. Subsequently, the callus was cultured in a 200 mL Erlenmeyer flask containing 100 mL of Murashige and Skoog's medium supplemented with various concentrations of 1-naphthalene acetic acid (0.1 to 2 mg/L) and benzylaminopurine (0.1 to 2 mg/L). The flasks were incubated on a rotary shaker at 130 rpm. After 2 weeks, the water layer was separated from the suspension culture with a callus by centrifugation at 5,000 g. After adding the organic solvents (toluene:hexane, 1:1) to the supernatant, the polymer fraction was extracted by liquid-liquid extraction (toluene: hexane, 1:1). The polymer fraction derived from the callus was extracted by the Soxhlet method with methanol (2 h) followed by toluene for 2.5 h. The molecular weight and molecular weight distribution of the toluene-extractable fractions were determined by GPC.

Measurements: The ^{1}H nuclear magnetic resonance (NMR) spectra were obtained at 400 MHz using TMS as an internal standard. The molecular weight and molecular weight distribution of the polymer were determined by GPC using a column packed with polystyrene gel. The measurements were taken using tetrahydrofuran as an eluent with a flow rate of 1.0 mL/min at 40 ºC. Commercially-obtained polystyrene standards were used for column calibration.

RESULTS AND DISCUSSION

Callus induction: After *ca.* 3 weeks of culture on Murashige and Skoog's medium supplemented with 1-naphthalene acetic acid and benzylaminopurine, callus initiation was observed from the leaves of *S. oleraceus*. The callus from the explants was a bubble type and completely distinguishable from the explants. Callus formation was observed in all media tested, regardless of the 1-naphthalene acetic acid (from 0.1 to 2.0 mg/L) and benzylaminopurine (from 0.1 to 2.0 mg/L) concentrations.

Effect of 1-naphthalene acetic acid and benzylaminopurine on callus formation: Fig. 1 shows the relative growth of callus transferred to a fresh medium. The highest degree of callus formation showed *ca.* 10-fold relative growth after 3 weeks in Murashige and Skoog's medium containing 1-naphthalene acetic acid (0.1 mg/L) and benzylaminopurine (0.1 mg/L). After 3 weeks, the growth rate decreased, possibly due to depletion of the culture medium.

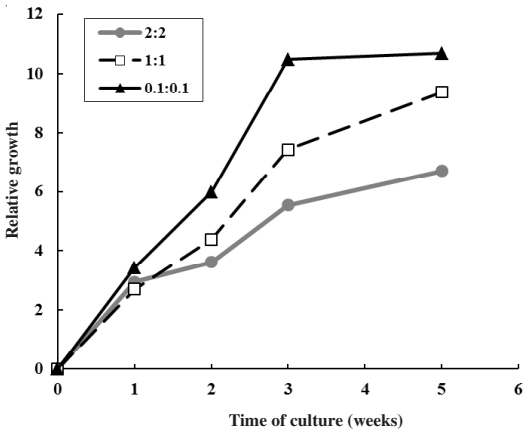

Fig. 1. Changes in relative callus growth in cultures of *S. oleraceus*. 2:2, 1-naphthalene acetic acid (2.0 mg/L) and benzylaminopurine (2.0 mg/L); 1:1, 1-naphthalene acetic acid (1.0 mg/L) and benzylaminopurine (1.0 mg/L); 0.1:0.1, 1-naphthalene acetic acid (0.1 mg/L) and benzylaminopurine (0.1 mg/L)

Structural characterization of the toluene-extractable fraction from the leaves of *S. oleraceus*: The molecular structure of the major polyisoprene in the toluene-extractable fraction from the leaves of *S. oleraceus* was determined by ^{1}H NMR spectroscopy (Fig. 2). The 3 main ^{1}H NMR peaks for the isoprene units in the cis configuration were observed at 1.66 (CH_3-), 2.02 (-CH_2-) and 5.10 (=CH-) ppm, confirming that the plant extract was composed of rubber (*cis*-1,4-polyisoprene).

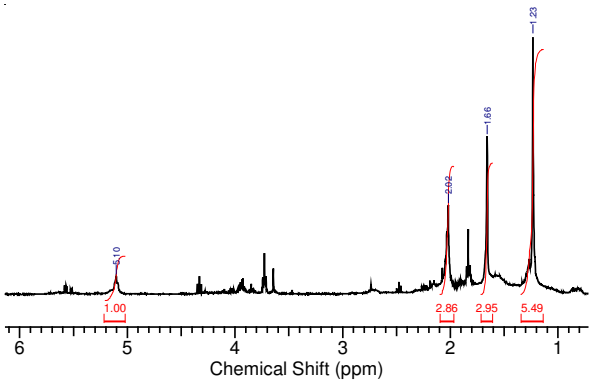

Fig. 2. ^{1}H NMR spectra of toluene-extractable fraction from leaves of *S. oleraceus*

Molecular weight and molecular-weight distribution of the extracts from the plant and cultivated callus: As shown in Fig. 3, the polyisoprene fraction extracted with toluene from the leaves of *S. oleraceus* showed a unimodal molecular weight distribution, whereas Hevea rubber showed a bimodal distribution with peaks at 1.0-2.0 × 10^5 and 1.0-2.5 × 10^6 Da[7]. The ratio of the low- to high-molecular-weight peaks varied according to the clone of *Hevea*. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) were estimated to be 0.5 × 10^5 and 1.5 × 10^5, respectively. Compared to the rubber from sunflower and goldenrod (both therophytes)[8], *S. oleraceus* produced approximately twice the rubber of the molecular weight.

Hevea rubber from the same clone at different ages (1 month to 3 years) exhibited a bimodal molecular weight distribution rich in low-molecular-weight forms[9]. The ratio of

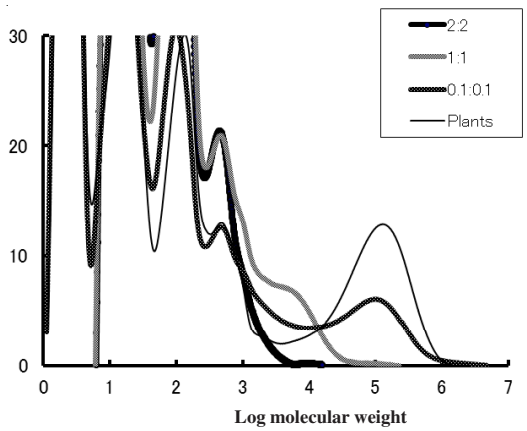

Fig. 3. Molecular-weight distribution (MWD) of toluene-extractable fractions from cultures of *S. oleraceus*. 2.2, 1-naphthalene acetic acid (2.0 mg/L) and benzylaminopurine (2.0 mg/L); 1:1, 1-naphthalene acetic acid (1.0 mg/L) and benzylaminopurine (1.0 mg/L); 0.1:0.1, 1-naphthalene acetic acid (0.1 mg/L) and benzylamino-purine (0.1 mg/L); Plants, *S. oleraceus* leaf

low- to high-molecular-weight peaks decreased with increasing age of the tree. The rubber from therophytes was vastly different from *Hevea* rubber in terms of the size and polydispersity, suggesting that the molecular weight of rubber in the therophytes is controlled by a simpler mechanism than in *Hevea* trees.

Interestingly, extracts from the callus cultivated in the suspension medium with 1-naphthaleneacetic acid (0.1 mg/L) and benzylaminopurine (0.1 mg/L) showed a similar molecular weight distribution to that of the rubber from *S. oleraceus* leaves. This strongly suggests that rubber can be produced from callus cultures. In cultures with 1-naphthalene acetic acid (1.0 mg/L) and benzylaminopurine (1.0 mg/L), a polymer with a maximum molecular weight of 2×10^4 was observed. On the other hand, no polymer was produced in cultures with 1-naphthaleneacetic acid (2.0 mg/L) and benzylaminopurine (2.0 mg/L).

The effect of the source of nitrogen in cultivation media on the production of secondary metabolites was examined by analyzing the cultures with various concentration ratios of NH_4^+ and NO_3^- ($[NH_4^+]/[NO_3^-]$)as follows: 0 mM/60 mM, 5 mM/55 mM, 10 mM/50 mM and 20 mM/40 mM (Fig. 4). Higher-molecular-weight polymers were observed in the presence of 0 and 5 mM NH_4^+. On the other hand, the polymers formed in the presence of 10 and 20 mM NH_4^+ showed a broad molecular weight distribution with a peak at 1.0×10^4. These two different molecular weight distributions indicate 2 control mechanisms for the molecular weight, one of which might be inhibited by NH_4^+ at concentrations > 10 mM.

Fig. 5 shows the molecular weight distributions of a polymer extracted from the aqueous fraction of callus cultivated in suspension medium. The polymer was clearly detected in the aqueous fraction after 4 weeks of culture, exhibiting a broad molecular weight distribution with peaks at 0.5×10^4 and 1.0×10^5. The higher peak was detected in the polymer extracted from the cultivated callus. The presence of polymer in the aqueous fraction suggests 2 possibilities *i.e.*, the polymer might be secreted by cells or the polymer may be released by cell rupture.

Conclusion

Several features of the therophyte *Sonchus oleraceus* suggest that it might be an alternative source of natural rubber.

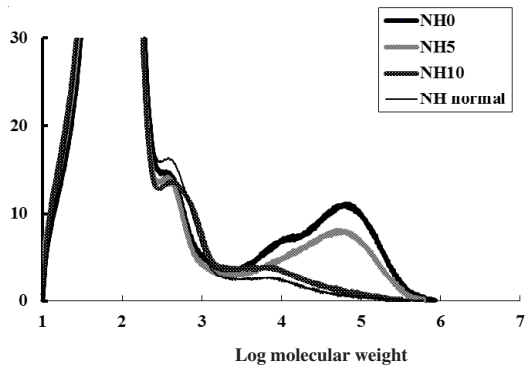

Fig. 4. Molecular-weight distribution (MWD) of toluene-extractable fraction in cultures of *S. oleraceus*. NH0, NO^- (60 mM) and NH^+ (0 mM); NH5, NO^- (55 mM) and NH^+ (5 mM); NH10, NO^- (50 mM) and NH^+ (10 mM); NH normal, NO^- (40 mM) and NH^+ (20 mM)

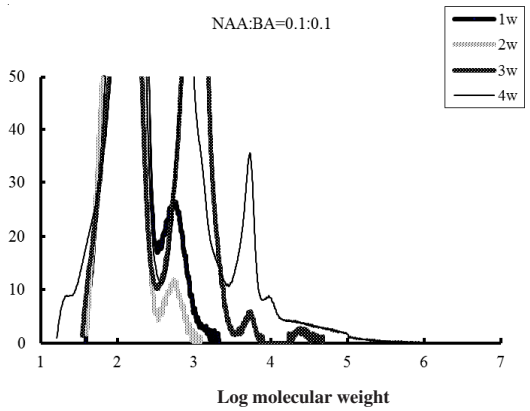

Fig. 5. Molecular-weight distribution (MWD) of toluene-extractable fraction from aqueous fraction in cultures of *S. oleraceus*. 1W, 1 week of culture; 2W, 2 weeks; 3W, 3 weeks; 4W, 4 weeks

Polymers were extracted from *Sonchus oleraceus* and from callus cultures grown under a range of conditions. Rubber was obtained from *S. oleraceus* and from callus cultures of the plant and the polymer characteristics could be modified by adjusting the levels of auxin and cytokinin (In this study, 1-naphthalene acetic acid and benzylaminopurine were used as the auxin and cytokinin, respectively). In addition, changes in the nitrogen source in the culture medium lead to changes in polymer formation. *S. oleraceus* may be a viable source of natural rubber and has the potential for biosynthetic production in culture.

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