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Colchicine-induced polyploidy has the potential to improve rubber yield in *Taraxacum kok-saghyz*

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ABSTRACT

Taraxacum kok-saghyz (TK), also known as rubber dandelion, is under development as a source of natural rubber but has not been fully domesticated. Rubber yield is highly variable and biomass is low compared with most crops. Therefore, it is imperative to apply breeding methods to improve rubber yield and accelerate the domestication process. One strategy to accelerate breeding is polyploid induction, which could improve rubber concentration and plant size. A gradient of colchicine concentrations ranging from 0% to 0.5% was used to treat approximately 5000 seeds for different periods of time (12 h to 96 h), followed by use of flow cytometry to confirm induced tetraploids. The optimal treatment of 0.1% colchicine for 48 h resulted in an induction efficiency of 56.6%. An early-screening strategy successfully identified 2-month old tetraploid seedlings based on atypical leaf morphology. Comparisons of leaf, stomata, root morphological traits as well as biochemical traits including rubber, resin and inulin/sugars concentration were made between tetraploids and diploids. A 47.7% increase in rubber concentration but a decrease in inulin/sugars concentration was observed in tetraploid plants. However, there was no significant difference in the amount of rubber per root system among the greenhouse-grown plants in this research, likely due to root stunting observed in tetraploids and treated diploids. Evaluation of progeny is needed to circumvent the confounding physiological impact of colchicine treatment. However, the increase of rubber concentration observed in tetraploids suggests that polyploid breeding has the potential to improve rubber yield in TK.

1. Introduction

Natural rubber is a critical resource that is commercially produced by *Hevea* (*Hevea brasiliensis*), a tree that has narrow genetic diversity, long breeding cycles and is restricted to specific tropical countries (Lieberei, 2007). Changes in landscape usage towards the production of more labor-efficient crops such as palm oil trees in these countries have contributed to unstable rubber prices (Basiron, 2007). These geographical restrictions, and monoculture of the *Hevea* crop amplify the potential impact of diseases, such as South American Leaf Blight (SALB) (Rivano et al., 2013) and white root rot (Irogue, 2012). It is unlikely that *Hevea* cultivation can expand sufficiently to meet accelerating natural rubber global demands due to economic and ecological constraints. *Taraxacum kok-saghyz* (TK), is an alternative source of rubber of similar quality to that produced by *Hevea*. TK is an obligate outcrossing dandelion species, which can be cultivated as an annual. Additionally, TK is adapted to grow in a wider range of temperate areas. Moreover, wild populations usually exhibit large variation in rubber production, which may be leveraged to develop improved varieties with different environmental specializations. However, TK has not been domesticated and competes poorly with weeds when grown in the field. In addition, accurate phenotyping is time consuming and costly, impeding the breeding and domestication process needed to turn TK into a rubber-producing crop. Moreover, its outcrossing features make it impossible to either fix traits or reproduce progenies with uniform genetic background for accurate multi-location phenotyping. Therefore, only limited breeding efforts (Kupzow, 1980; Tysdal and Rands, 1953; Warmke, 1944) have been carried out, and future TK breeding faces numerous challenges.

Diploid TK has a high rubber production potential because individual plants with up to 30% rubber have been observed and the rubber yield of plants with medium rubber concentration (~5–6%) in the planting box (not published) can reach up to 1138 kg dry rubber/ha/year, which is comparable with annual rubber yield from *Hevea* rubber trees. However, due to poor vigor and competitiveness in the field, we have not yet achieved comparable yields in the field. Rubber yield can be improved by increasing rubber concentration and/or root size and polyploid breeding strategies are commonly used in

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agricultural and horticultural crops as they often possess superior agronomic traits over their diploid counterparts (Liu et al., 2007). For example, polyploids may have larger leaves and flowers, thicker stems and roots, darker green leaves, an increased width-to-length ratio of the leaves, a more compact growth habit and a higher tolerance to environmental stress (Lavania et al., 2012; Liu et al., 2007; Shao et al., 2003). Furthermore, previous studies have demonstrated that genomic multiplication commonly increases the concentration of secondary metabolites (Dhawan and Lavania, 1996) as in cochin grass (*Cymbopogon flexuosus*) (Ammal and Gupta, 1966), Egyptian henbane (*Hyoscyamus muticus*) (Lavania, 1986), and Iranian poppy (*Papaver bracteatum*) (Milo et al., 1987). In addition, autotetraploids and triploids are frequently of low fertility, or may even be sterile, and this can be a desirable characteristic in clonally propagated plants as it reduces contamination of the environment by pollen or seed escapes (Dhawan and Lavania, 1996).

In 1945, Warmke successfully induced tetraploids in TK through colchicine treatment and compared them to diploids in terms of rubber yield, fresh root weight, and reproductive pattern. He found a 3.3-fold increase in rubber yield in the field, but no significant change in greenhouses. Also, root size significantly increased among tetraploids. However, he detected tetraploids only by counting chromosomes, and comparing pollen size, seed size and leaf morphology, which could be subjective and prone to human error. As biotechnology developed, flow cytometry has become the most efficient and precise method for detecting changes in ploidy level (Dolezel et al., 1989). Samples are easily and rapidly prepared, there is no need to divide cells and only a few milligrams of tissue are needed, and it is a quick and reproducible method for determining the ploidy levels of large numbers of samples (Sakhanokho et al., 2009). Moreover, during the 1940's (and more recently), TK was often conflated with TB (*Taraxacum brevicorniculatum*) — a triploid *Taraxacum* species — because it coexists with TK in nature, is phenotypically similar to TK, especially at early stages, and can also produce small amounts of high-quality rubber.

With such situations in mind, we have developed and optimized a method of inducing TK tetraploid plants, and confirmed the ploidy levels using flow cytometry to ensure accurate ploidy determination. Moreover, we demonstrated that phenotypic screening can enrich for doubling events, allowing for the development of large populations requisite of an outbreeding plant species. Chemical components in roots including rubber, inulin and resin were compared between tetraploid, treated diploid and control diploid plants.

2. Materials and methods

2.1. Plant material and seed preparation

High rubber plants were harvested from plants grown from an OSU bulked seed lot of USDA TK germplasm collections (Hellier, 2011). Seeds derived from open-pollinating those high rubber parents were collected in summer 2016 and stored under low humidity in a seed storage room (4 °C). A total of about 5000 seeds were treated with a colchicine concentration gradient for different durations.

2.2. Colchicine treatment and preliminary screening for putative tetraploids

Twenty-five 1.5 ml Eppendorf centrifuge tubes were used, each one containing 60 seeds (0.02 g). The seeds were immersed for 12, 24, 48, 72 or 96 h at room temperature in 0% (control), 0.05, 0.1, 0.2 or 0.5% aqueous solutions of colchicine (Sigma-Aldrich Co. LLC) dissolved in 1% DMSO. After treatment, seeds were washed with distilled water five times, with each wash step lasting 5 min. The seeds then were transferred directly to Promix soil and germinated in a growth chamber with 90% humidity, 20 °C and a daylight period of 12 h. All the treatments were replicated three times. One week after treatment, the germination rate ($y_g = a/b*100\%$) was calculated from the number of germinated seedlings (a) divided by the total number of treated seeds (b). Four weeks after treatment, the survivorship ($y_s = c/b*100\%$) was measured by dividing the surviving plants (c) by the total number of treated seeds (b). Since a large population of seedlings (> 1500 germinated seeds) was generated following the seed treatment, we carried out a preliminary morphological screening of the two-month-old seedlings to identify a smaller population of putative polyploids. Plants with atypical leaf morphologies, i.e. darker green leaves, thicker leaves, rougher leaves or leaves with abnormal edges and prominent veins or veinlets were selected as putative polyploids, which were then tested by flow cytometry. The same number of seedlings with normal leaf morphologies were also selected.

2.3. Flow cytometric analysis

About 100 mg of fresh leaf tissue from each selected seedling was chopped with a razor blade in a 55 mm Petri dish containing 1.5 ml Galbraith's buffer (Loureiro et al., 2006) (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate (MOPS), 0.1% (*v/v*) Triton X-100, pH 7.0), a quarter of a tomato seed (lot#: D21OH101NC) with an estimated genome size of 2.05 Gb (2*n* DNA content) (Michaelson et al., 1991; Sato et al., 2012) was included as an internal control for each sample. The solution was then filtered through a 30 µm nylon mesh. The suspension of released nuclei was stained with 75 µl of propidium iodide (10 mg/ml) and 5 µl of RNase (1 mg/ml) for 15 min, followed by published procedures (Iaffaldano et al., 2017). The fluorescent intensity peak of samples, which is proportional to DNA content, was adjusted such that the control peak and expected diploid TK peak appeared between channel 50 and 100. The stained DNA was then excited by a green laser as a narrow stream of nuclei passed by a red-light detector in the Cyflow® Ploidy Analyzer (Partec, Munich, Germany). The peaks of test samples were compared with peaks derived from control tomato seeds to determine relative ploidy levels as either diploid, with a diploid genome size of 2.4 GB, tetraploid, or chimeric (having both diploid and tetraploid peaks). If the ratio of genome size of sample DNA to the tomato seed (standard) was double that of diploid TK control to the standard, then that sample was counted as tetraploid. Tetraploid seedlings were maintained and retested 4 months later, as described. Those seedlings that twice tested as tetraploid were grown for several months and evaluated for morphology and biochemical characteristics.

2.4. Morphological measurements

Leaf morphology traits, including number of leaves, leaf width (w), leaf length (l) and leaf shape index (l/w) were compared between treated diploids, control diploids and tetraploid plants at four months of age. Three leaves from 8 tetraploid individuals (a total of 8 tetraploids were obtained), 21 treated diploids and 25 control diploids were examined. In addition, the size and density of leaf stomata of five confirmed tetraploids, five randomly selected treated diploids, and five control diploids were measured on leaves, in triplicate. To make these observations, a thin piece of abaxial epidermis was mounted in tap water on a slide and immediately examined using a graduated eyepiece. The visual field area of the ocular was 4 mm^2 and 1 mm^2 under magnification of 20X and 40X, respectively (Xing et al., 2011). The stomatal density (Sd), stomatal length (Sl), and width (Sw) were measured under magnifications of 20× and 40×. The stomatal area (As) was calculated as follows (Xing et al., 2011): $As = 1/4 \times \Pi \times Sl \times Sw$, and total stoma area ($At$) was calculated as follows: $At = As \times Sd \times 100\%$ (Xing et al., 2011). Digital images were manually analyzed with ImageJ 1.50i (Wayne Rasband, National Institutes of Health, USA).

2.5. Analysis of biochemical components

The concentrations of inulin (with some smaller sugars), resin and

rubber were determined sequentially in diploid and polyploid plants at six to seven months of age using an Accelerated Solvent Extractor (ASE, ASE-350, Dionex Corp., Thermo Fischer Scientific Inc., Waltham, MA) by adapting a published method (Ramirez et al., 2017). Inulin/sugars, resin and rubber were usually extracted using water, acetone and hexane, respectively (Ramirez et al., 2017). The inulin/sugars was extracted using ASE parameters of N_2 pressure at 1500 psi; temperature at 95 °C; preheating time for 5 min; heat time for 5 min (automatic software default); static time for 20 min; flush volume of 150%; purge time for 60 s and 2 static cycles. The resin and rubber settings were as previously described (Ramirez et al., 2017).

2.6. Statistical analysis

Data combined from three independent biological replicates were used to analyze the effects of colchicine concentration and exposure time on induction rate, survival rate and tetraploid induction efficiency. The induction rate (y_i) was calculated as $yi = d/a*100\%$ where d represented the number of induced tetraploids, and a represented the number of germinated seedlings. Tetraploid induction efficiency was measured as described (Bouvier et al., 1994), where% efficiency ($y_e = y_s*y_i*100\%$) = % seedling survival (y_s) × % polyploid induction (y_i). Efficiency can range from 0 to 100, in which 100 indicates that all surviving seedlings were polyploid, whereas zero indicates that all treated seedlings either died or no polyploidy induction occurred. The Least Significant Difference (LSD) test was used for the mean separation with significant differences claimed at the different significance levels of 0.05, 0.01, and 0.001 using R software. Differences between mean values from diploid and tetraploid plants for the morphological and biochemical traits were evaluated using *t*-tests. All data were assessed for normality (Alva and Estrada, 2009) and homogeneity of variances prior to analysis (Schmetterer, 1964).

3. Results

3.1. Survival rate and early screening of potential polyploids

The germination rates of the colchicine-treated seeds, especially at higher concentration levels, were much lower than controls, and emergence was delayed. Moreover, the survivorship of seedlings, ranging from 0 to 42%, was greatly inhibited by colchicine treatment, especially at concentrations above 0.2%, decreasing as the concentration and duration time increased. The highest lethality was observed in 0.5% 96 h (Table 1). Two months after colchicine treatment, some seedlings had atypical leaf morphology (i.e. wrinkled leaves, rough leaf edges, thick leaves (Fig. 1). Flow cytometry confirmed that 8 of 10 preselected seedlings, with atypical leaf morphology, were tetraploid. This morphological screen allowed the screening of over 3000 seedlings germinated from colchicine treated seed to be sorted with a much smaller number being analyzed for ploidy level.

3.2. Flow cytometric analysis

The flow cytometric analysis indicated three different kinds of ploidy level, diploid, tetraploid and chimeric (Fig. S1). The number of induced tetraploids differed among the colchicine treatments (Table 1). The highest induction rate was in 0.2% colchicine for 48 h followed by 0.1% colchicine for 48 h. The 0.1% colchicine for 48 h treatment also gave the highest induction efficiency. No tetraploid was obtained from the 0.5% dose at any treatment duration. Eight tetraploids were identified among seedlings of all seed treatments.

3.3. Leaf and stomata morphological characteristics

The effects of polyploidy were evaluated morphologically, using traits known to be affected by ploidy level (Liu et al., 2007; Ye et al., 2010). Young tetraploids (2-month old) grew more slowly than the untreated diploid plants of similar age. Six months after the colchicine treatment, the tetraploid plants had fewer leaves than the diploids, but their leaves were wider and thereby had a reduced leaf index (length/width) (Fig. 2A–D; Table 2). Tetraploid leaves were also thicker and darker green in color than diploid leaves (Fig. 2A–D). Stomatal length (*l*), width (*w*), stomata area (*As*), and total stomatal area (*At*) were significantly greater in tetraploid plants than in diploids (Table 2; Fig. 2E–F). However, fewer stomata per unit leaf area were observed in tetraploid than diploid plants (Table 2; Fig. 2E–F). The average stomatal frequency in control diploids was 56.9/mm^2, in treated diploids was 43.0/mm^2 and in tetraploids was 35.7/mm^2 (Table 2). The tetraploid seeds were also larger than diploids (Fig. 2G).

3.4. Comparisons of root morphology and biochemical components between diploid and tetraploid plants

Significant differences were observed among tetraploids, treated diploids and control diploids for fresh plant weight, fresh root weight, dry root weight, rubber concentration inulin/sugar concentration, rubber, resin and inulin/sugar per root (Table 3). However, there were no significant differences in rubber per plant root system among these greenhouse-grown plants.

4. Discussion

Colchicine is highly toxic and high concentrations cause plant cell death because this antimitotic agent blocks spindle fiber development and modifies the differentiation process (Trojak-Goluch and Skomra, 2013). Even though the treatment of seeds with various colchicine concentrations permitted a moderate germination (emergence) frequency (up to 43%) (Table 1), many of these seedlings grew very poorly, especially from the higher concentration treatments or the longer treatment times. The elongation of the radicle was inhibited and root hairs were rarely formed, thereby severely compromising the ability of many seedlings to develop further. This was also observed by other researchers (Liu et al., 2007), who developed tetraploids from seeds of the plane tree (*Platanus acerifolia*); none of their tetraploid seedlings successfully grew into tetraploid plants due to poor root development.

Colchicine treatments caused low germination and survival rates, which were around or below 50%. This is likely a result of colchicine toxicity preventing some treated seeds from emerging. In preliminary experiments, treated TK seed, germinated in petri dishes with water, had germination rates above 80% while untreated seed were above 90%. However, due to the large numbers of seeds treated, it was more practical to directly sow treated seeds. A low induction rate was also seen in our research, in which the highest induction rate of 4.92% was obtained by treatment with 0.2% colchicine for 24 h, followed by a 3.77% induction rate from treatment with 0.1% colchicine for 48 h. The optimal treatment also was 0.2% 24 h for seeds of Madagascar periwinkle (*Catharanthus roseus L.*) (Xing et al., 2011). Low overall survivorship and induction rate also have previously been reported (Lam et al., 2014; Liu et al., 2007) and may be due to residual colchicine in the seed coat inhibiting later seedling growth. Higher polyploid induction efficiencies, recovering near-normal development, have been first reported in *in vitro* cultured tobacco (*Nicotiana tabacum*) (Murashig and Nakano, 1966), and more recently, in guayule (*Parthenium argentatum*) (Hashemi et al., 1989), *Alocasia* (Thao et al., 2003), and crape myrtle (*Lagerstroemia indica* L.) (Ye et al., 2010). Therefore, other *in vitro* or *in vivo* induction strategies may improve the efficiency of TK tetraploid induction even though *in vitro* induction strategies may be more appropriate for limited starting materials.

Given the low efficiency of the seed induction technique, it was imperative to develop a high throughput screening method in order to rapidly eliminate as many of the unsuccessfully induced diploid

Table 1
Germination, survival and induction rates calculated after treating diploid TK seeds with colchicine.

Colchicine Conc. (%)	Treatment time (*h*)	No. of germinated seedlings (*a*)	% germination rate (y_g)	No. of surviving seedlings (y_s)	% surviving rate (y_s)	No. of induced seedlings (*d*)	% induction rate (y_i)	% Induction Efficiency (y_e)
0.05	12	88	37.78	77	42.78	0	0.00	0.00
	24	86	47.78	85	47.22	0	0.00	0.00
	48	63	35.00	43	23.89	1	1.59	37.92
	72	43	23.89	40	22.22	0	0.00	0.00
	96	58	32.22	44	24.44	0	0.00	0.00
0.10	12	67	37.22	55	30.56	0	0.00	0.00
	24	67	37.22	37	20.56	1	1.49	30.68
	48	53	29.44	27	15.00	2	3.77	56.60
	72	56	31.11	17	9.44	0	0.00	0.00
	96	62	34.44	16	8.89	1	1.61	14.34
0.20	12	68	37.78	31	17.22	0	0.00	0.00
	24	61	33.89	17	9.44	3	4.92	46.45
	48	43	23.89	4	2.22	0	0.00	0.00
	72	29	16.11	6	3.33	0	0.00	0.00
	96	63	35.00	2	1.11	0	0.00	0.00
0.50	12	48	26.67	32	17.78	0	0.00	0.00
	24	36	20.00	3	1.67	0	0.00	0.00
	48	10	5.56	5	2.78	0	0.00	0.00
	72	74	41.11	2	1.11	0	0.00	0.00
	96	30	16.67	1	0.56	0	0.00	0.00
0	12	80	44.44	96	53.33	0	0.00	0.00
	24	125	69.44	133	73.89	0	0.00	0.00
	48	100	55.56	104	57.78	0	0.00	0.00
	72	91	50.56	145	80.56	0	0.00	0.00
	96	112	62.22	122	67.78	0	0.00	0.00

% germination rate ($y_{g=}a/b$*100%) = germinated seedlings (*a*)/treated seeds (*b*) (a week after treatment).
% surviving rate ($y_s = c/b$*100%) = surviving seedlings (*c*)/treated seeds (*b*) (a month after treatment).
% induction rate ($yi = d/a$*100%) = induced polyploid seedling (*d*)/germinated seedlings (*a*) (2 months after treatment).
% induction efficiency ($y_e = y_s*y_i$*100%) = % seedling survival (y_s) * % polyploid induction (y_i) (2 months after treatment).

Fig. 1. Pre-screening of two-month old seedlings for evaluation of putative tetraploids. The 2-month-old seedlings with atypical leaf morphology on the upper two photographs were selected as putative tetraploid plants and were later confirmed by flow cytometry analysis; The bottom two figures showed seedlings at 4-month-old stage. 2×: diploids; 4×: tetraploids.

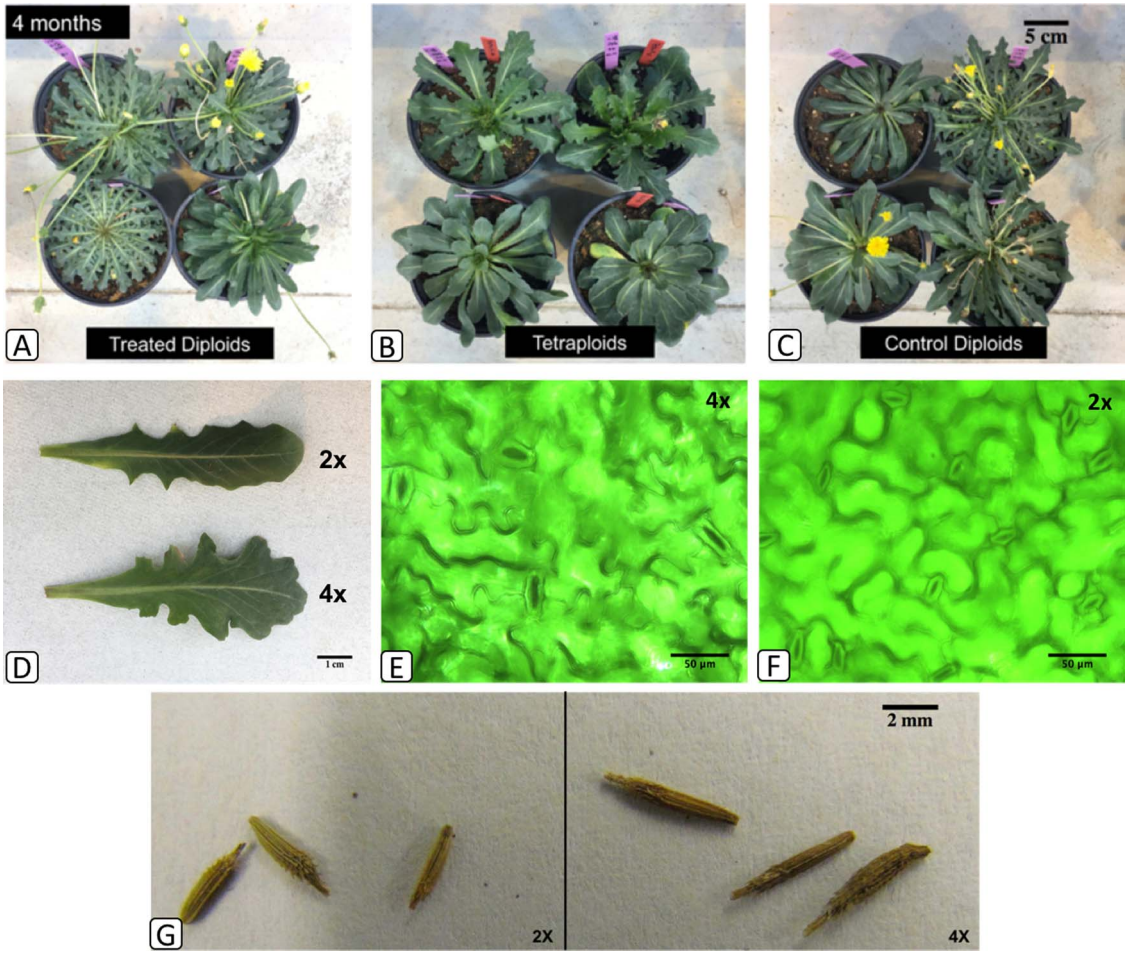

Fig. 2. Morphological comparisons of tetraploid and diploid plants. (A–C) 4- month-old plants in three groups; (D) leaf morphology comparison; (E–F) stomata size comparison; (G) seed comparison.

Table 2
Comparisons of nine morphological traits among three groups.

Morphological traits	4×	Treated 2×	Control 2×
Stomata density (*Sd*, $n*mm^{-2}$)	6.80 ± 4.23A	12.93 ± 5.20B	12.13 ± 3.92B
Stomatal length (*Sl*, $mm*10^{-2}$)	3.15 ± 0.38A	2.19 ± 0.21B	2.25 ± 0.31B
Stomatal width (*Sw*, $mm*10^{-2}$)	1.66 ± 0.40A	1.06 ± 0.25B	1.06 ± 0.15B
Stomatal area (*As*, mm^2*10^{-2})	0.4 ± 0.01A	0.02B	0.02B
Total stomata area (*At*, mm^2)	0.29 ± 0.18A	0.23 ± 0.11A	0.23 ± 0.08A
Leaf index (*l*/*w*)	2.55 ± 0.31A	4.07 ± 0.23B	4.32 ± 0.25B
Number of leaves	9.57 ± 1.11A	14.57 ± 0.84B	15.48 ± 0.96B
Leaf width (*w*, cm)	2.18 ± 0.14A	1.53 ± 0.10B	1.37 ± 0.11B
Leaf length (*l*, cm)	5.72 ± 0.29A	5.27 ± 0.38A	5.96 ± 0.27A

Different small-case/capital letters indicate the significance of the difference between two mean values at the p = 0.05/0.01 level, respectively, as tested by two-sample *t*-test.

seedlings as possible before embarking on time-consuming and costly cytological analyses. Abnormal leaf morphological traits such as dark green and thicker leaves (Amma et al., 1984; Liu et al., 2007), prominent veins or veinlets (Amma et al., 1984) and wrinkled leaf edges (Liu et al., 2007) usually found in polyploid plants were used to screen young seedlings. TK seedlings with abnormal leaf morphology were confirmed as tetraploids at an 80% rate using flow cytometry. This method is much faster than using stomatal length and density as indicators of ploidy for early population screening, without impairing selection efficiency (Liu et al., 2007; Ye et al., 2010).

Tetraploid TK were confirmed when the ratio of the nuclei mean for tomato seed (G1 peak)/TK sample (G2 peak) was approximately 2.44, which is twice the ratio of tomato/TK diploid controls. This ratio was not always exactly 2.44 probably due to a combination of the following reasons. The DNA-protein (chromatin) packing may be tighter and more condensed in tetraploids than diploids (Rabinovitch, 2010), or the staining time may be too short, as polyploids have more DNA which needs to be stained. As tetraploids have greater DNA mass, it is expected that they will exhibit wider fluorescence distributions relative to diploids; however, our method was able readily to detect doubling events, as these are large-scale changes. In some scans, peaks were poorly defined requiring manual selection of peak range, leading to variable S phase distribution, thereby affecting the means ratio, as has been previously noted (Yan et al., 2016).

Polyploid crops are deployed in agriculture and horticulture as they often possess superior agronomic traits over their diploid counterparts, such as larger leaves and flowers, thicker stems and roots, and a higher tolerance to environmental stress (Liu et al., 2007; Shao et al., 2003; Xing et al., 2011; Ye et al., 2010). Wider leaves and an increased leaf area index were apparent in our TK tetraploids. In addition, stomatal length and density have been shown to be reliable indicators of ploidy in a number of species (Dhawan and Lavania, 1996), and the measurement is simple, largely non-destructive, and does not require specialized equipment. Our results were in agreement with previous studies of stomatal characteristics at different ploidy levels in *Hevea* (Amma et al., 1984), stevia (*Stevia rebaudiana*) (de Oliveira et al., 2004) and crape myrtle (Ye et al., 2010), where authors noted that diploid

Table 3
Comparisons of plant growth traits and biochemical components among three groups.

Morphological and biochemical traits	4×	Treated 2×	Control 2×
Fresh plant weight (g)	10.68 ± 2.19A	17.87 ± 1.49B	27.97 ± 1.08C
Fresh root weight (g)	6.47 ± 0.88A	10.38 ± 0.64B	14.54 ± 0.53C
Dry root weight (g)	1.37 ± 0.26A	2.99 ± 0.19B	4.28 ± 0.16C
Rubber concentration (mg/g)	57.58 ± 7.01A	34.58 ± 4.59B	30.13 ± 3.66B
Rubber per root (mg)	78.81 ± 26.03A	100.06 ± 17.04A	129.67 ± 13.59A
Resin content (mg/g)	27.45 ± 7.32A	33.48 ± 4.63A	32.37 ± 4.11A
Resin per root (mg)	38.02 ± 23.54A	90.23 ± 14.89AB	125.75 ± 13.23B
Inulin content (mg/g)	33.41 ± 4.42A	47.32 ± 2.79B	37.72 ± 2.36B
Inulin per root (g)	54.20 ± 33.10A	133.13 ± 20.93AB	173.55 ± 17.69B

Different small-case/capital letters indicate the significance of the difference between two mean values at the p = 0.05/0.01 level, respectively, as tested by two-sample *t*-test.

species had the highest stomatal density and the smallest stomatal size. All of them concluded that stomatal parameters represent a rapid and efficient method for screening putative polyploid plants and confirming ploidy levels. However, large founding populations of polyploids are probably required for a highly heterozygous species like TK. The rapid early screening method we developed to select putative tetraploids based on leaf morphology, seems to encompass all true polyploids and so allows much larger numbers to be screened before confirmation by flow cytometry. Only 20% of plants passing through the morphological screen proved to be misidentified morphologically aberrant diploids.

In addition, rubber concentration was significantly higher in tetraploid TK than in diploids, suggesting that polyploidy has the potential to improve rubber yield. However, it should be noted that the data in this paper were gathered from generation zero (G0) tetraploids, and it is clear that residual effects of colchicine persisted when the data from treated and untreated diploids were compared (Table 3). G0 treated diploids showed a significant decrease in root biomass as well as a reduction in stomatal density compared with untreated diploids. Thus, the stunting of TK tetraploid roots also was likely caused by residual colchicine, or indirect persistent adverse effects of treatment. The higher rubber concentration but decreased root biomass in tetraploid G0 plants than in diploid plants (Table 3) meant that rubber yield per root system did not significantly differ from untreated diploids. There was no significant difference in resin concentration among the three groups, and so resin per root system was determined by root size. In contrast to our tetraploids, Warmke found that the colchicine-induced, greenhouse-grown, G0 tetraploids had significantly larger roots but lower rubber concentration (Warmke, 1945). However, the less advanced ploidy confirmation techniques and rubber quantification methodologies available in the 1940′s may weaken the reliability of their results, and the diploid germplasm used differed. It is clear that polyploid plants must be interbred and propagated through multiple generations to escape toxic effects. Only then can meaningful field trials be attempted, reliable estimates of rubber yield and quality be obtained, and the true potential of polyploid TK be determined.

In general, due to the self-incompatibility of TK, the genetic background of TK seeds is heterozygous and complex. This complexity may confound effects caused by chromosome doubling. Therefore, less diverse seed lots may be useful for future research. Also, comparisons can be made between progenies derived from treated diploids and tetraploids to eliminate lingering toxic effects of colchicine or by interaction between toxicity and ploidy. This may be challenging because tetraploid TK may have high levels of sterility and low seed set rate (Warmke, 1945). However, this may be overcome by acquiring larger sample sizes using the methods we have described. Furthermore, environmental conditions may require optimization to improve pollen production and seed.

5. Conclusion

Tetraploid breeding in TK is a promising methodology to enhance rubber yield in the field. Our phenotypic seedling screen now permits the development of large polyploid populations, which are required because of the high heterozygocity of TK. Atypical leaf morphology was proven effective to preliminarily identify rare tetraploid seedlings among thousands of diploids, leading to an 80% confirmation rate by flow cytometry analysis. Root rubber concentration increased in tetraploids while inulin/sugar concentration decreased. Tetraploid progeny produced through multiple generations will need to be evaluated to determine the impact of tetraploidy on root growth and rubber yield in the absence of residual toxic colchicine effects. Tetraploid induction may help quickly realize metabolic and agronomic improvements in this potential rubber crop.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2017.11.010.

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