

Drought stress alters rubber molecular weight and relative expression levels of key genes -involved in natural rubber production in rubber dandelion, *Taraxacum kok-saghyz*

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Abstract

Natural rubber (NR) is a vital raw material for many industries, but its main source, *Hevea brasiliensis*, is vulnerable to diseases and climate change. *Taraxacum kok-saghyz* (rubber dandelion, TKS) is an alternative source of NR that can grow in temperate regions. This study investigated the effect of drought stress on NR biosynthesis and quality in TKS roots. Drought stress didn't affect the total rubber content, but increased the molecular weight (Mw) of the rubber significantly. The highest Mw was observed under severe drought stress, which also induced the highest expression of CPT and SRPP genes, which are involved in NR production. The rubber from TKS roots (TNR) had a high Mw of 994,000 g/mol under normal irrigation and a low glass transition temperature (Tg) of below -60°C , indicating its industrial potential. Drought stress also increased the levels of proline, H_2O_2 , MDA, and antioxidant enzymes (CAT, APX, GPX) in TKS roots, indicating a drought response mechanism. These results suggest that TKS can produce good quality NR under drought stress conditions and can be cultivated in regions with water scarcity.

1. Introduction

Natural polyisoprenes are divided into two major groups namely, *cis* and *trans*- polyisoprene. Natural rubber (NR) is *cis*-1,4-polyisoprene, and is one of the most valuable biopolymers in the world and is used as a critical raw material in numerous products (Cherian et al. 2019). It is energetically expensive to biosynthesize, a terminal carbon sink, and is a secondary metabolite in plant cells. Although it is made in at least 2500 plant species its function in plants is poorly understood and disparate among species (Kim et al. 2003; Cherian et al. 2019). The tropical para rubber tree (*Hevea brasiliensis*) cultivated predominately in southeast Asia is the only significant commercial source of NR production in the world (Ahrends et al. 2015). The NR supply chain is insecure due to growing demand, price volatility, increasing worker costs, commerce policy, rain forest destruction moratoria, and susceptibility to many diseases (Salehi et al. 2022). Biological and geographical diversity is needed. Among rubber-producing species known to synthesize high molecular weight rubber, *Parthenium argentatum* (also called guayule), and *Taraxacum kok-saghyz* (also known as rubber dandelion, TK, TKS) have received growing attention in the past few years, as potential commercial sources of natural rubber (Ramirez-Cadavid et al. 2019; Salehi et al. 2021). These species provide a sustainable and renewable source of natural rubber that offers an alternative to Hevea rubber in semi-arid temperate regions, respectively, hence reducing dependency tropical rubber. Guayule rubber is especially attractive as it is hypoallergenic, unlike normal Hevea rubber which can trigger allergic reactions in some individuals (Rousset et al., 2021). TKS is an annual herbaceous plant, originating in Kazakhstan and neighboring countries, widely adapted to temperate regions, that makes rubber in its root laticifers similar in quality to natural rubbers from Hevea (Cornish et al., 2016; Ramirez-Cadavid et al., 2019). Drought stress is a major global challenge that affects the productivity and quality of crops. Drought triggers physiological alterations and metabolic impairment such as lipid membrane peroxidation, protein folding issues, metabolite degradation (Ilyas et al., 2020), accumulation of reactive oxygen species (ROS) and respiratory metabolism inhibition, which may result

from a reduction in antioxidants. To scavenge reactive oxygen, antioxidant enzymes are vital. Some of the enzymes that protect plants from oxidative stress are CAT, which breaks down hydrogen peroxide, APX, which reduces ascorbic acid, and GPX, which uses guaiacol as a substrate (Hasanuzzaman et al. 2020). Environmental factors such as low temperature and water scarcity may trigger the production of rubber in some plants (Cornish and Backhaus 2003). A key ingredient for making rubber is isopentenyl pyrophosphate (IPP), which can be produced through two distinct routes: the pathway of mevalonic acid (MVA) and the pathway of methylerythritol phosphate (MEP) (Cornish, 2001; Chow et al., 2012; Tang et al., 2016). The membrane-bound enzyme complex responsible for the synthesis of the NR polymer (rubber transferase, RT-ase) is not yet fully understood (Cherian et al. 2019). This study examined the expression levels of four key genes likely related to rubber biosynthesis in response to drought. These genes are *SRPP* (which binds to small rubber particles), *CPT1* (which catalyzes the formation of *cis*-prenyl chains) *HMGR* (hydroxy methylglutaryl coenzyme A reductase) and *HMGS* (hydroxy methylglutaryl coenzyme A synthase). The *SRPP* gene was first recognized as encoding a protein associated with rubber particles in *Hevea brasiliensis*. Over-expression of *SRPP3* increased polyisoprene content in TKS roots (Schmidt et al., 2010), whilst *SRPP3* RNA interference (RNAi) caused a significant decrease in rubber content and rubber molecular weight (Collins-Silva et al. 2012). *SRPP* is also thought to play a role in rubber particle stability. CPT (*cis*-prenyltransferase) associated with rubber particles is a key catalytic enzyme involved in the polymerization reaction (Oh et al. 1999). The aim of this study was to examine how water stress affects the genes related to rubber production and the natural rubber content in the roots of TKS plants. It also examined the molecular characteristics of NR in TKS, such as molecular weight, glass transition temperature and antioxidant enzymes activities, under greenhouse and field conditions. This is the first and novel study to explore the impact and mechanisms of drought stress on NR biosynthesis and quality in TKS, an alternative source of NR with potential for cultivation and utilization.

2. Materials and methods

2.1. Experimental design and drought stress treatments

TKS seeds of accession number W6 35164 were acquired from the USDA-ARS (Regional Plant Introduction Station, Washington State, U.S.A.). Germination rates were improved to above 80%, by first soaking the seeds in water for 20 h, followed by 2% KNO₃ for 4 h. Each seed was rinsed twice with distilled water and sown in a growth tray that had drain holes and a mixture of perlite, cocopeat, and peat moss in equal parts. Thereafter, when plants reached the six-leaf stage, they were transferred to pots containing clay loam mixed with gravel (2mm to 5mm) at a ratio of 3:1, about 24% sand and the soil content include sand (0.05 mm to 2 mm), 45% silt (0.002 mm to 0.05 mm) and clay 31% clay (< 0.002mm). Potted plants were grown in the research greenhouse of the College of Agricultural and Natural Resources, University of Tehran, Karaj, at 35° N and 50° E, with 25°C day and 16°C night temperatures and a 16 h day and 8 h dark photoperiod. The plants were watered and fertilized (NPK 20-

20-20) about 4 months until 50% of plants reached sexual reproduction. Additional seedlings were planted in the field with clay loam soil.

2.1.1. Drought stress treatment in the greenhouse

The experiment followed a Randomized Complete Block Design (RCBD) method with three blocks. Each block had six plants in it. When 50% of the plants began flowering, plants were exposed to the following treatments at three levels of water availability: 30% of field capacity (FC) (severe stress), 60% of FC (mild stress) and 90% of FC (sufficient-irrigation treatment) (Fig. 1.a). After 21 days, plants were taken out of the pots and the samples were collected simultaneously from both tissue of root tips (two cm in length) and leaves of TKS, with a total of 15 leaves and the samples were rapidly frozen in liquid nitrogen and then kept at -80°C for analysis.

2.1.2. Drought stress treatment in the field

Soil moisture measurements were used to guide irrigation treatments according to the percentage of maximum allowable depletion (MAD) of the total available water of the soil (Merriam 1966). Drought stress treatments were based on the 10, 40 and 70% MAD of total available soil water, representing the control, mild and severe drought stress, respectively. The amount of soil water content was estimated using a TDR probe (TDR Model 100) system.

2.2. Physiological measurements

2.2.1. Leaf extract preparation

Leaf samples (0.2 g) were chopped and powdered in liquid nitrogen using a mortar and pestle then moved to tubes with a capacity of 2 ml. Thereafter, 1 ml of extraction buffer consisting of (Tris-HCl(C₄H₁₁NO₃HCl) (Merck, Germany), 1M, pH 6.8 and 2% polyvinyl pyrrolidone (PVP) (Merck, Germany) was added and mixed well. The extract underwent centrifugation at 4°C with a force of 22,500 xg for half an hour. The liquid fractions were separated and kept at -20°C for later enzyme activity assays.

2.2.2. Evaluation of malondialdehyde (MDA) content

The Peever and Higgins method based on the thiobarbituric acid (TBA) reaction was used to measure the level of malondialdehyde (MDA), a marker of lipid peroxidation. First, 0.1 g of fresh TKS leaves were homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 10,000 xg for 5 min. Then, 1 ml of the supernatant was mixed with 4 ml of 20% TCA containing 0.5% (w/v) TBA and the mixture was heated at 95°C for 30 min. After being cooled on ice, the mixture was centrifuged again at 10,000 xg for 15 min and the absorbance of the supernatant was measured at 532 and 600 nm. (Peever and Higgins 1989).

2.2.3. Measurement of proline level

To measure the free proline in the fresh leaves of TKS under different treatments, the acid ninhydrin method was applied. A 0.5 g sample of fresh leaves was homogenized with 10 ml of 3% sulfosalicylic acid on ice. The homogenates were centrifuged at 22,500 xg for 15 min at 4°C and filtered through filter paper. The filtrates were collected in 15 ml Falcon tubes and mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid. The mixture was incubated in a water bath at 65°C for 1 h and then cooled on ice. After adding 4 ml of toluene to each tube and vortexing for 20 sec, the absorbance of the toluene layer was measured at 520 nm using a plate reader device (EON Biotek, Highland Park, Winooski, Vermont, USA). The proline concentration of the samples was estimated from the absorbance curve against proline concentrations of standard solutions. Finally, the amount of proline was determined from the following equation in terms of micrograms per gram of dry weight (Bates et al., 1973; Kramer, 1969).

$$M = \frac{C \times V}{W}$$

2.2.4. Evaluation of H₂O₂ content

To measure the H₂O₂ content in fresh leaves, the method of (Velikova et al. 2000) was followed. A 0.5 g sample of leaf tissue was ground in 2.5 ml of 1% TCA solution and spun at 15,000 xg for 15 min. Then, 0.25 ml of the clear liquid was combined with 0.25 ml of 10 mM potassium phosphate buffer (pH 7) and 0.5 ml of 1M potassium iodide solution. The mixture's absorbance at 390 nm was read by a plate reader and matched to a standard curve of H₂O₂ solutions ranging from 2 to 10 mM.

2.2.5. Determination of catalase (CAT) content

10 µl of leaf extract (section 2.2.1) was mixed with 1,990 µl reaction buffer comprised of 50 mM potassium phosphate buffer and 50mM H₂O₂ CAT enzyme activity was assayed using a spectrophotometer at 240 nm.

2.2.6. Determination of ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) activity was measured following the method of Nakano and Asada, (1987). The assay mixture contained 50 mM potassium phosphate buffer, 0.5 mM ascorbic acid and 1mM H₂O₂. A 10 µl aliquot of leaf extract was added to 1990 µl of the assay mixture and the decrease in absorbance at 290 nm due to the oxidation of ascorbic acid by APX was recorded spectrophotometrically. The activity was expressed as $\Delta A_{290} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$.

2.2.7. Determination of guaiacol peroxidase (GPX) content

The method of Chance and Maehly, (1955) was followed to assay guaiacol peroxidase (GPX) activity. The reaction buffer was composed of 25 ml potassium phosphate (10 mM), 25 ml H₂O₂ and 25 ml guaiacol. Leaf extract (10 µL) was added to 1,990 µL of this reaction buffer, mixed well and the GPX activity was spectrophotometrically determined at 470 nm.

2.3. RNA isolation and reverse transcription

Following the MIQE guidelines (Bustin et al. 2009), real-time PCR was conducted. Root tip tissue (100 mg) from TKS was collected and the sample was pulverized into a fine powder using liquid nitrogen and a cold mortar and pestle. The improved CTAB method Zarei et al. (2012) was used to isolate and purify total RNA. A 1% agarose gel and Nanodrop ND-1000 were used to check the quality and quantity of total RNA, respectively. To remove any possible genomic DNA contamination (confirmed by PCR), the total RNA was digested with DNase I, RNase-Free DNase Set (Fermentas, Waltham, MA). Then, using a cDNA synthesis kit (RP1300 Reverse transcription Kit made by SMOBIO Taiwan), the first strand cDNA was generated from the total RNA following the protocol provided by the manufacturer.

Primer design and quantitative real-time PCR (qRT-PCR)

The corresponding primers of the selected genes (Fig. 2), *CPT1* (KM401657.1), *HMGR3* (KT899404.1), *HMGS1* (KT899404.1) and *SRPP2* (KT899433.1), were designed using Oligo7 primer analysis software. Primers were checked with the Oligo-analyzer and NCBI/Primer-BLAST web tools software. Table 1 shows the forward and reverse primer sequences used for qRT-PCR. Real time PCR was performed for 40 cycles with SYBR green Master Mix 2X in a Rotor-gene 6000 series software (QIAGEN's real-time PCR system) following the manufacturer's instructions. Each cycle consisted of three steps: 95°C for 15 sec, 59–65°C (based on the annealing temperature from PCR gradient results) for 20 sec, and 72°C for 20 sec. The relative expression of the different genes was then calculated. All the reactions were repeated with three technical replicates for statistical analysis and relative gene expression was calculated using REST software (REST 2009 V2.0.13).

Table 1
Specific primers used in qRT-PCR

Target Genes	Gene bank accession number	Primer sequences (Sequence in 5'-3' direction)
<i>CPT1</i>	KM401657.1	F: GTGTCATAGCTTCTCGCCCAA R: ATGGTGACGTACTTAACTCCGAT
<i>HMGS1</i>	KT899404.1	F: CCATAGGACTCGCACAAGATTGC R: CGATTACCGTTTCACTCCCGACTTC
<i>SRPP2</i>	KT899433.1	F: TTTGCTGATAATAAGGTTGCCCA R: CTTTTACGGTTTCCACTACACCA
<i>HMGR3</i>	KT899404.1	F: CCGTTTTCAACAAATCAAGCCGAT
<i>Actin</i>	KT965025.1	R: ACCATGTTCATCCCCATTGCATC F: GTATCCATGAGACCACCTACAAC R: GTCAGCAATACCAGGGAACATA

2.4. TKS root tissue preparation for rubber extraction

TKS roots from three different irrigation regimes were harvested from greenhouse-grown TKS plants. The plants were removed from their pots, thoroughly washed with clean water, dried at room temperature for about 1 h and finally aerial organs of plant was separated from the crown of plant by a cutter. The roots were sliced and oven-dried at 50°C for 4–7 days (Fig. 1.b). Dried roots were cut into 2 mm pieces. Finally, the chopped dried roots were kept in a zipped bag at 4°C in the dark until TNR extraction.

2.5. Extraction and characterization of TNR

TNR was extracted using sequential solvent extraction with acetone and hexane (Zhang et al. 2019) Roots harvested from both the greenhouse and the field with a weight of approximately 0.1 g per sample were stirred for 24 h in acetone (20 ml acetone/g ground root), then centrifuged at 2,800 xg for 10 min. After decanting the supernatant, the pellet was washed with acetone two times. Each pellet was then mixed in hexane for 6 h (20 ml hexane/g ground root). The slurry was centrifuged at 2,800 xg for 10 min. Each supernatant was transferred to a pre-weighed glass petri dish and dried in a 50°C oven for 2 h (Fig. 3) until the solvent had completely evaporated. The extracted TNR was weighed. TNR samples were left in the petri dishes, covered and sealed in plastic, and the samples were kept in a light-free environment at 4°C until further analysis.

2.5.1. FTIR analysis of NR

15 to 20 mg of extracted of rubber were stretched on KBr cells (Guo et al. 2015; Nandiyanto et al. 2019) and analyzed by Fourier transform infrared (FTIR) spectroscopy using an ALPHA II, Brucker device.

2.5.2. GPC analysis of NR

The molecular weight and polydispersity of extracted TNR was assessed through gel permeation chromatography (GPC). TNR (3 mg) was dissolved overnight in tetrahydrofuran (THF) at 25°C with moderate shaking. The solutions were filtered through a PTEF filter (0.45 µm) and injected (100 µl) into a GPC (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA) with a RI detector and two PLgel mixed-C columns (10 µm, 300 mm × 7.5 mm) in series. The flow rate and the temperature were 1 ml/min and 35°C, respectively. Polystyrene standards (ranging from 580 to 19,560,000 molar mass) were used to estimate molecular weights.

2.5.3. DSC analysis of NR

The thermal responses of TNR samples (10 mg) to heat were analyzed by differential scanning calorimetry (DSC) (Perkin-Elmer DCS model 8000) under a nitrogen atmosphere. To erase their thermal memory, the rubber samples were kept at 30°C for 10 min. Then they were warmed up from – 80°C to 180°C at a speed of 10°C/min (Junkong et al., 2017).

2.6. Statistical analysis

The results of physiological parameter measurements and total rubber content were analyzed using SAS (9.4) software. The least significant difference (LSD) test was used to compare the means of data at (p

≤ 0.05 and $p \leq 0.01$) significance levels. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was applied to statistically analyse the relative expression levels of each selected gene with three technical replicates.

3. Results

3.1. Physiological measurements

Drought stress significantly increased the levels of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and free proline in *T. kok-saghyz* leaves in both greenhouse and field conditions (Fig. 4). MDA is a product of lipid peroxidation caused by reactive oxygen species (ROS) such as hydroxyl radical (OH), superoxide ion ($O_2^{\bullet-}$) and H_2O_2 (Gill and Tuteja 2010). The highest accumulation of MDA was observed under severe water deficit (30%) in both locations, indicating higher oxidative damage to the membrane lipids. H_2O_2 concentration also increased under drought stress, but the highest level was found under mild water stress rather than severe stress. Free proline, a compatible osmolyte that protects plants from dehydration and osmotic stress, accumulated under even mild water stress in both locations (in the greenhouse ($p \leq 0.01$) and field ($p \leq 0.05$)). The antioxidant enzyme activities of guaiacol peroxidase (GPX), catalase (CAT) and ascorbate peroxidase (APX) also increased significantly under drought stress in both locations (Fig. 4). GPX activity reached the highest level under severe stress in the field and under mild stress in the greenhouse, while CAT and APX activities reached the highest levels under severe stress in the field ($p \leq 0.01$, < 0.01 and < 0.05 , respectively).

3.2. Gene expression analysis

Among the selected genes, the most notable effects of drought stress were suppression of *HMGS1* under both stress levels (Fig. 5) and increased expression of *SRPP4* under the most severe drought treatment. *CPT1* appeared to slightly increase and *HMGR* to slightly decrease under severe drought. The expression of the other genes was not affected by drought.

3.3. Rubber concentration

The rubber concentration in the *TKS* roots seemed to increase under drought stress at the 30% and 60% levels in both field and greenhouse conditions, but the difference was not statistically significant (Fig. 6). The expression of *HMGS*, a key enzyme for the production of the rubber monomer IPP, was downregulated in the root tips under stress (Fig. 5). The rubber content in *TKS* dry roots from the wild collection was consistent with our results, ranging from 4-4.5% (Zhao et al. 2022) (Fig. 6).

3.4. Molecular weight distribution of TNR

Drought stress may have increased the molecular weight of the extracted TNR and decreased the polydispersity (Table 2). The molecular weight of TNR under normal irrigation was almost the same as the natural rubber from *H. brasiliensis* (Ramirez-Cadavid et al. 2017).

Table 2
Molecular characteristics of TNR extracted from single samples from each greenhouse treatment

Sample	Mw (g/mol)	Mn (g/mol)	Mp (g/mol)	PDI
Control (90%)	0.99*10 ⁶	4.12*10 ⁵	1.04*10 ⁶	2.41
Mild stress (60%)	1.31*10 ⁶	9.08*10 ⁵	1.17*10 ⁶	1.45
Severe stress (30%)	1.12*10 ⁶	6.30*10 ⁵	1.04*10 ⁶	1.77

3.5. Differential scanning calorimetry (DSC)

TNR's glass transition temperature (T_g) was measured by DSC and shown in Fig. 7. The T_g value was -63.02°C and the heat capacity at the T_g was $0.402 \text{ J/g}^{\circ}\text{C}$.

3.6. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR confirmed that the TNR sample was *cis*-1,4, polyisoprene (Fig. 8). The main peaks observed below 1,000 are the unique peaks for *cis*-1,4 bonds (Nandiyanto et al., 2019; Chen et al. 2013), wave number 835 cm^{-1} .

4. Discussion

The results indicated that drought stress triggered oxidative stress and osmotic adjustment in *TKS* leaves, as manifested by the elevated levels of MDA, H_2O_2 , and free proline. These responses are consistent with those observed in other rubber-producing plants such as *H. brasiliensis* under water deficit conditions (Wang 2014). MDA is a product of lipid peroxidation and membrane damage caused by ROS, which are produced by various metabolic pathways under stress conditions (Gill and Tuteja 2010). The higher accumulation of MDA under severe water deficit implies that the antioxidant defense system was insufficient to cope with the excessive ROS generation. H_2O_2 is one of the main ROS that can act as a signaling molecule or a toxic agent depending on its concentration and location (Elstner, 1991). The highest level of H_2O_2 under mild water stress may suggest a higher activation of the respiratory burst oxidase system in the plasma membrane, which generates superoxide ions that are converted to H_2O_2 by SOD (Verma et al. 2019). Alternatively, it may indicate a lower removal capacity of H_2O_2 by antioxidant enzymes such as GPX, CAT and APX under mild stress compared to severe stress. Free proline is a well-known compatible solute that accumulates in plants under various abiotic stresses such as drought, salinity, and low temperature (Tarabih and El-Eryan 2020; Jiang 2020). Proline accumulation can result from several factors, such as reduced protein synthesis, enhanced proline biosynthesis from glutamate or ornithine, decreased proline degradation or catabolism, and increased protein hydrolysis (Götz et al. 2018; Tarabih and El-Eryan 2020; Jiang 2020). Proline can play multiple roles in plant stress tolerance, such as stabilizing membranes and proteins, scavenging ROS, maintaining cellular redox balance, regulating osmotic potential, and acting as a signaling molecule (Shafi et al. 2019; Ghosh et al. 2022). Drought

stress induced an increase in the antioxidant enzyme activities of GPX, CAT and APX in *TKS* leaves, suggesting a strengthened defense mechanism against ROS-induced oxidative damage. GPX reduces H_2O_2 to water using guaiacol as an electron donor, while CAT decomposes H_2O_2 to water and oxygen without requiring any cofactor. APX uses ascorbate as an electron donor to reduce H_2O_2 to water and regenerates ascorbate from monodehydroascorbate by MDHAR or DHAR (Song et al. 2005). These enzymes may have different roles and regulation in the antioxidant system under varying levels of drought stress (Sofo et al. 2015; Laxa et al. 2019). GPX may be more important for scavenging low levels of H_2O_2 under mild stress, whereas CAT and APX may be more effective for removing high levels of H_2O_2 under severe stress. The higher activity of GPX in the field than in the greenhouse under severe stress may also indicate a higher adaptation of *TKS* to natural conditions than to artificial conditions. *SRPP4*, which belongs to a stress-related protein category (Berthelot et al. 2014), was overexpressed under severe drought. *SRPP* has been shown to stabilize rubber particles and increase rubber production in guayule under drought stress (Dong et al. 2021). The co-localization of *CPT* with *SRPP* implies that they may also be upregulated by drought stress, as reported in *T. brevicorniculatum* (Schmidt et al. 2010; Post et al. 2012; Hemmerlin et al. 2012; Cherian et al. 2019). *CPTs* are involved in sterol and dolichol synthesis (a 15 to 23-mer of *cis*-polyisoprene) and cell membrane formation. Dolichol plays a role in drought stress tolerance in *Arabidopsis* (Zhang et al. 2008) and in the antioxidant mechanisms of cell membranes, so drought stress may increase *CPT* gene expression through the enhancement of antioxidant enzyme activities (Bergamini 2003). This work reveals new aspects of the molecular mechanisms underlying rubber biosynthesis and drought stress response in *TKS*. The expression levels of key genes involved in rubber biosynthesis, such as *HMGS1*, *SRPP4* and *CPT1*, were altered by drought stress, indicating that *TKS* may have a sophisticated regulation of rubber production under unfavorable environmental conditions. However, the increase in rubber concentration under drought stress could not be explained by enhanced rubber biosynthesis or reduced root biomass under stress, as the whole root weight data was inconclusive (Ferraris 1993). Moreover, the downregulation of *HMGS* suggests that the rubber biosynthesis pathway may be inhibited by drought stress in *TKS*. The rubber content in *TKS* dry roots from the wild collection may reflect the natural variation of rubber production among different genotypes or environmental conditions. The DSC results are in agreement with previous reports that showed similar M_w values for TNR, e.g., 1.4×10^6 g/mol (Cornish et al. 2013; Musto et al. 2016), suggesting that drought stress does not affect the polymerization of rubber monomers in *TKS*. The main finding of this paper is that drought stress does not decrease the total rubber content in *TKS* roots, but rather improves the quality of the extracted rubber for industrial applications. This may be attributed to the increased stability of rubber particles under stress, as evidenced by the higher expression of *SRPP4* (Fig. 5). The T_g of TNR was comparable to that of Hevea NR, which was reported to be -62.8°C by Jitsangiam et al. (2021). This indicates that the molecular mobility and chain flexibility of TNR were similar to those of Hevea NR, despite the different sources and extraction methods of the natural rubber (Rattanapan 2016). The FTIR peak at 835 cm^{-1} corresponds to the *cis*-1,4 bond stretching vibration of polyisoprene (Chen et al. 2013; Bayu et al. 2019), confirming that the TNR sample was mainly composed of *cis*-1,4 polyisoprene, which is the characteristic structure of natural rubber. The other peaks at 1377, 1449, 1668 and 3070 cm^{-1} are related to the CH_2 and CH_3 groups of the isoprene units (Chen et al., 2013).

5. Conclusions

TKS was able to survive mild and severe stress in both greenhouse and field conditions without a significant loss in rubber properties. This suggests that TKS may be a suitable crop for semi-arid temperate regions or those regions that experience transient drought periods. Key metabolic indicators responded as expected as stress-related enzymes and genes changed in activity and expression, respectively. Further research is needed to determine best agricultural practices, such as planting date, planting density and harvest date, and the commercial viability of TKS farming in different parts of Iran.

Declarations

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Ethical Approval

Not applicable

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Seyed Shahab Hedayat Mofidi: Data curation, Writing- Original draft preparation, **Mohammad**

Reza Naghavi: Supervision, Conceptualization, Methodology, **Manijeh Sabokdast:**

Visualization, Investigation, **Parisa Jariani:** Software, Validation, **Katrina Cornish:** Writing-

Reviewing and Editing

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

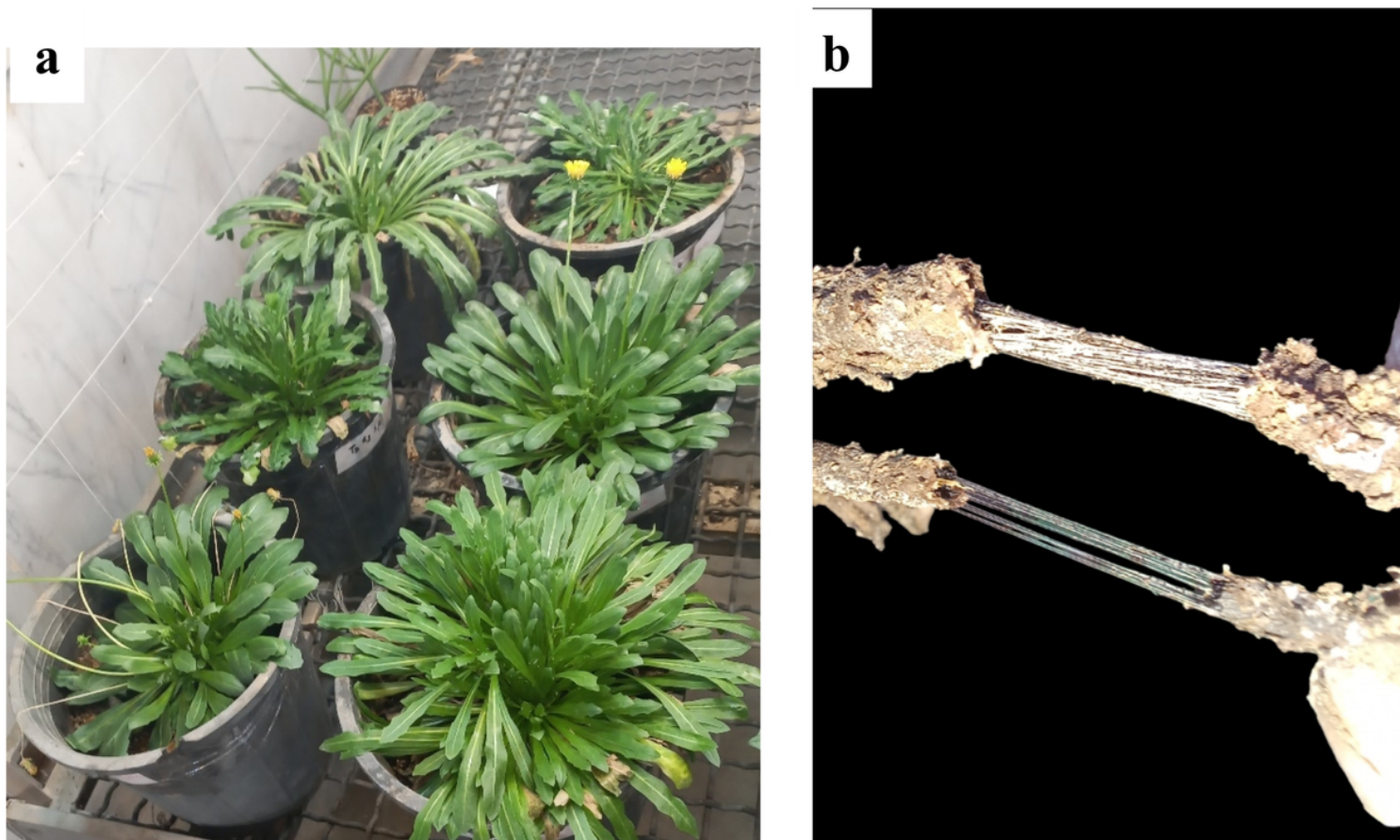


Figure 1

(a); *T. kok-saghyz* plants and (b); Natural rubber (TNR) strands stretched between broken root sections



Figure 3

Plant (left) and dried TNR in a petri dish (right)

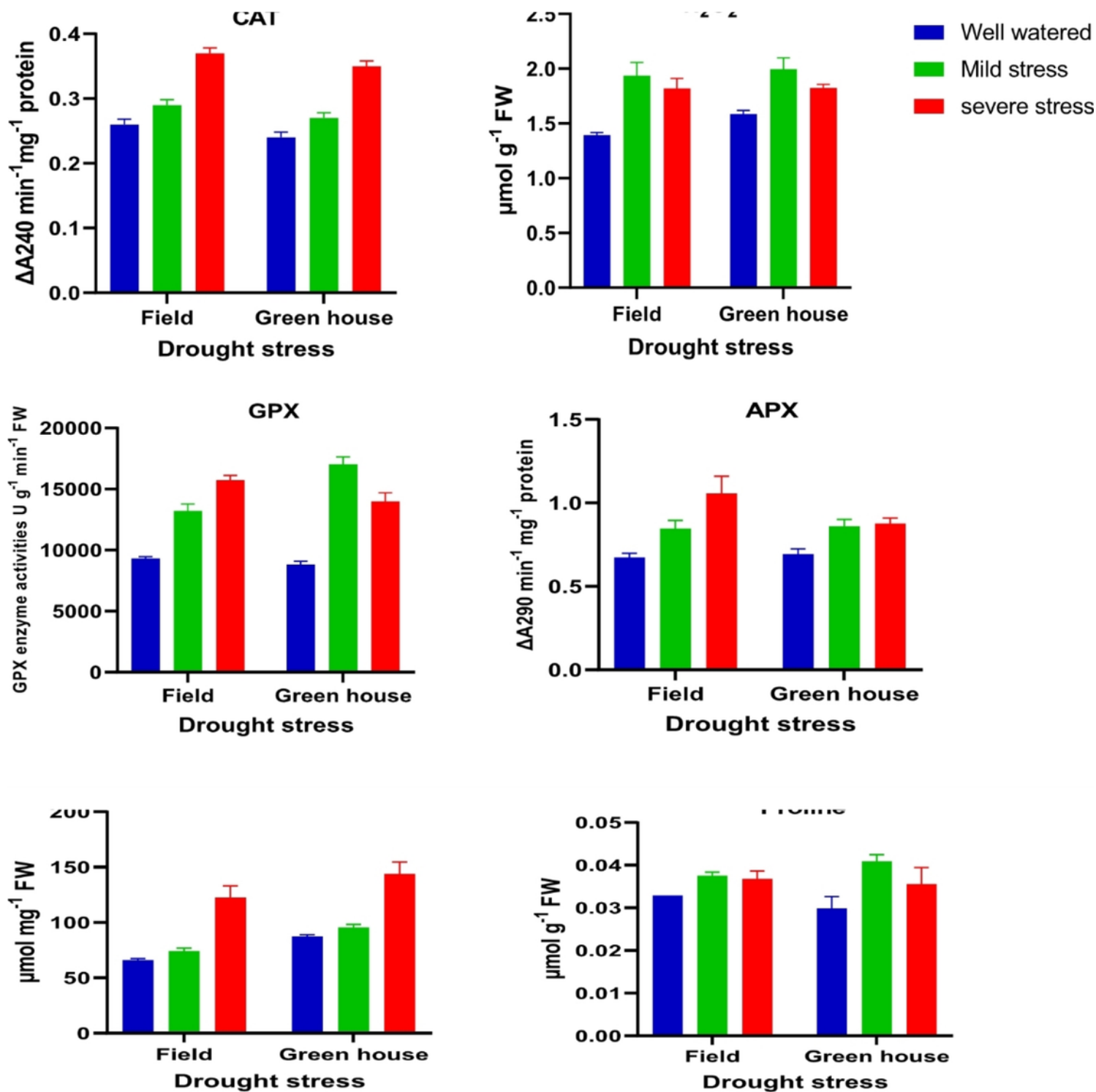


Figure 4

Catalase (CAT) activity, hydrogen peroxide (H₂O₂), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), malondialdehyde (MDA) and proline content in leaves harvested from TKS plants grown under different levels of drought stress. Each value is the mean of 3 replicates $\pm$ SE.

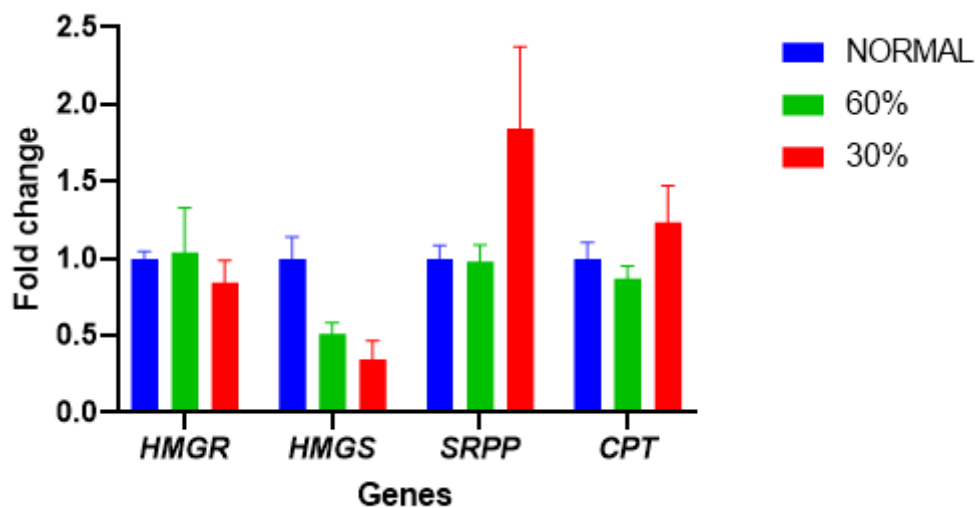


Figure 5

Relative expression of selected genes putatively involved in natural rubber biosynthesis in TKS root tips under different drought stress treatments. Each value is the mean of 3 replicates $\pm$ SE

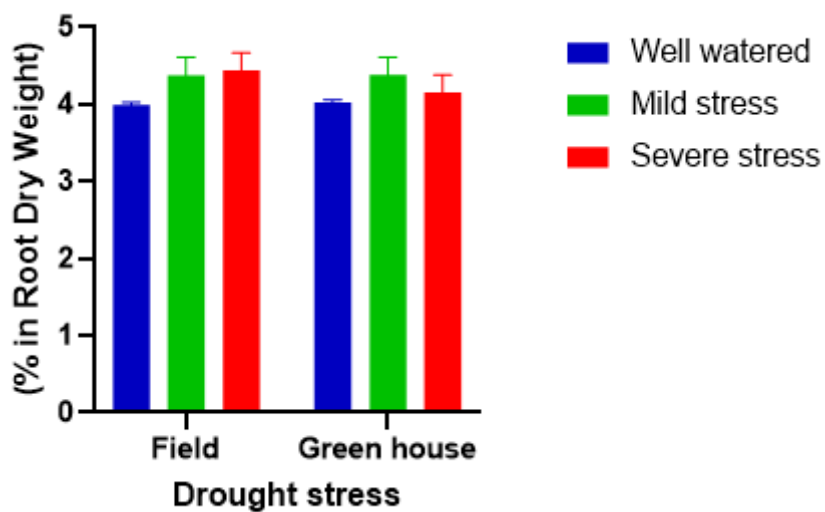


Figure 6

Rubber concentration in TKS roots. Each value is the mean of 3 replicates $\pm$ SE

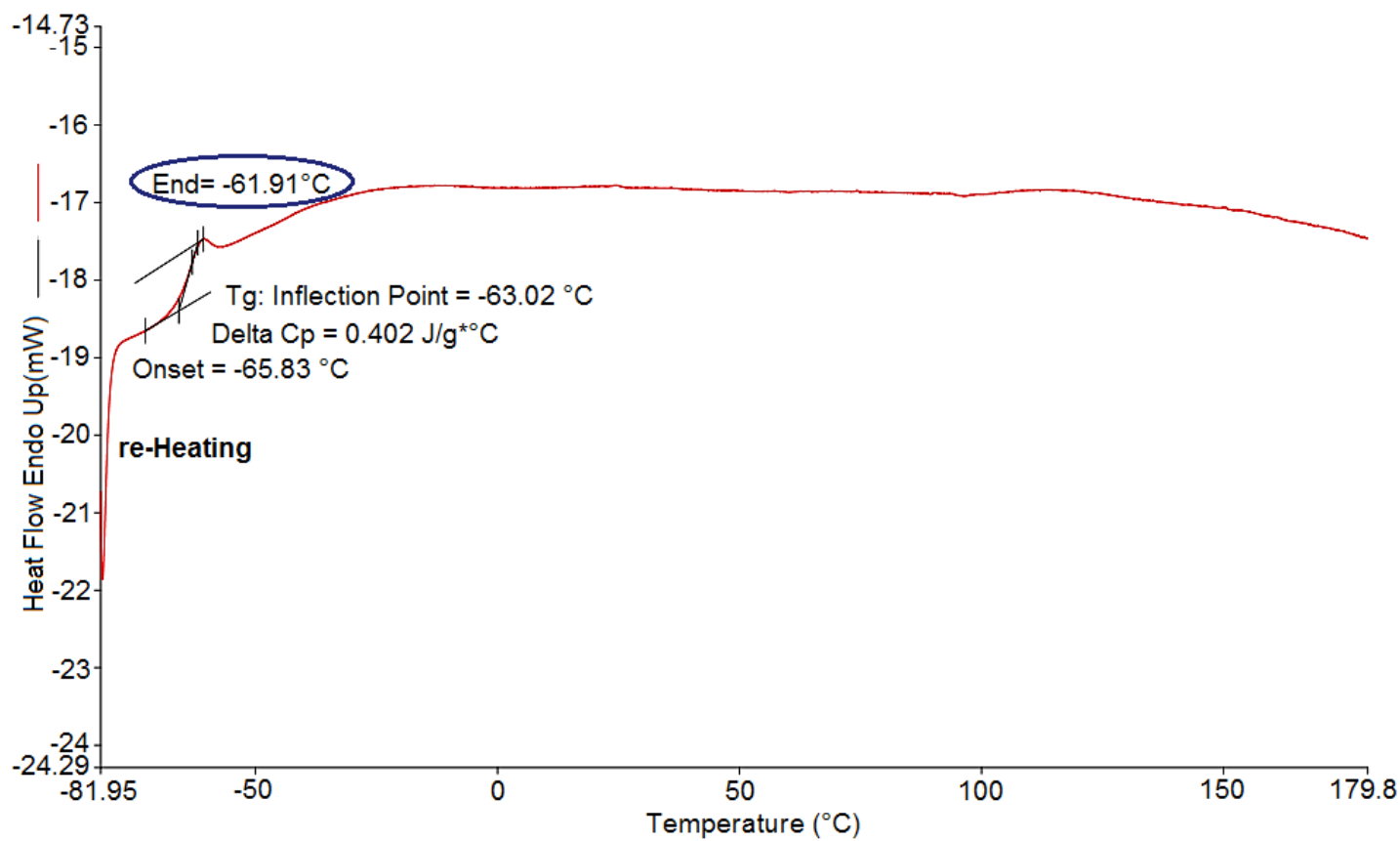


Figure 7

Differential scanning calorimetry (DSC) analysis of NR

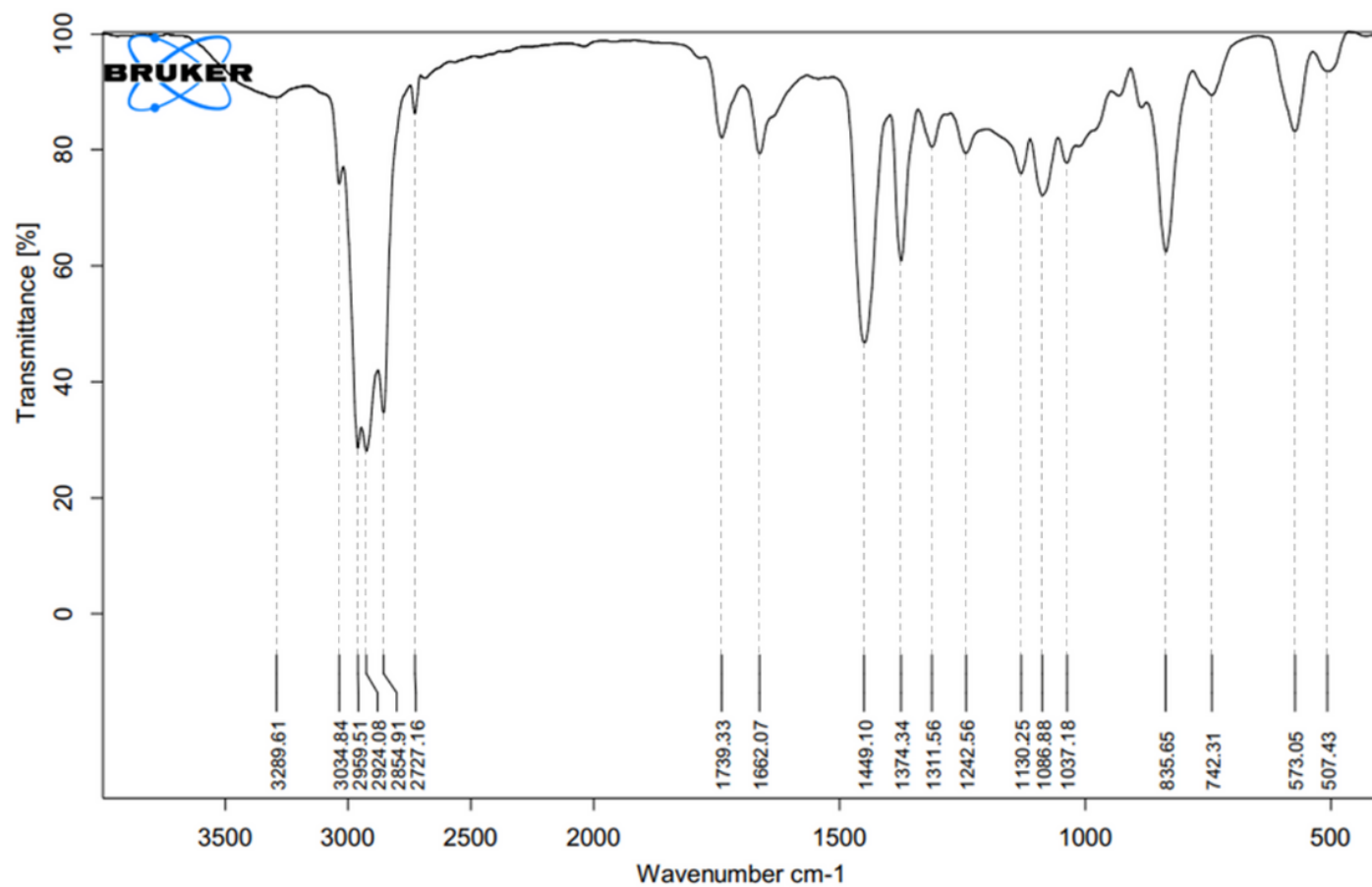


Figure 8

FTIR analysis of TNR