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PLANT GENETICS

Clonal Micropropagation of a Rare Species *Hedysarum theinum* Krasnob. (Fabaceae) and Assessment of the Genetic Stability of Regenerated Plants Using ISSR Markers

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Abstract—In the present study, a protocol was developed for the in vitro propagation of a rare medicinal plant, *Hedysarum theinum* (tea sweetvetch), from axillary buds, and identification of the regenerants was performed with the use of ISSR markers. It was demonstrated that Gamborg and Eveleigh medium supplemented with 5 μM 6-benzylaminopurine was the best for *H. theinum* for initial multiplication. On the other hand, half-strength Murashige and Skoog (MS) basal medium supplemented with 7 μM α-naphthaleneacetic acid proved to be the best for explant rooting. Molecular genetic analysis of the *H. theinum* mother plants and the obtained regenerants was performed with six ISSR markers. Depending on the primer, four to ten amplified fragments with sizes ranging from 250 to 3000 bp were identified. Our results confirmed the genetic stability of regenerants obtained in five passages and their identity to the mother plant.

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INTRODUCTION

Tea sweetvetch (*Hedysarum theinum* Krasnob., family Fabaceae L.) is a perennial herbaceous medicinal plant, a rare, endemic alpine species having a central Asian–southern Siberian distribution range [1, 2]. This species has a unique composition of secondary metabolites that determine a wide range of drug actions, including anti-inflammatory, bactericidal, antispasmodic, immunoprotective, antioxidant, and other effects [3–5]. As a result of the practice of mass harvesting of herb raw material, tea sweetvetch in the most accessible populations is critically endangered. Extremely slow growth makes this species especially vulnerable. The roots are considered mature and suitable for harvesting at the age of 30 years. The medicinal properties of tea sweetvetch, its biological features, and its limited growing region are the reasons for developing effective methods of its propagation, including biotechnological approaches with the purpose of reproduction, conservation in vitro and in vivo in living collections, and further reintroduction. It known that cultivation of plants in artificial media may cause somaclonal phenotypic and genotypic variation, which arises from in vitro-induced stress [6, 7]. Therefore, a key aspect in the use of in vitro technologies for propagation of rare and endangered plant species is verification of the genetic identity of derived regenerants.

Today, molecular genetic methods based on the use of polymerase chain reaction (PCR) for amplification of specific DNA markers are one of the most accurate and reliable tools for the detection and investigation of somaclonal variation. They distinguish somaclones from the original genotype. Modern methods of genetic analysis, such as RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats), AFLP (Amplified Fragment Length Polymorphism), and SSR (Simple Sequence Repeats), are widely used to identify genotypes and certify species and cultivars [8–18]. We used the method of electrophoresis of inter-microsatellite regions of genomic DNA (ISSR analysis), since it is known that this method is the most reproducible, convenient, sensitive, and polymorphic among the methods of anonymous fragment analysis [19–21].

The objective of this study was to develop a method for in vitro propagation of *H. theinum* from axillary buds and to conduct genetic identification of the mother plant and the obtained regenerants with the use of ISSR markers.

MATERIALS AND METHODS

In Vitro Propagation

Initial *H. theinum* mother plants were selected from the Rare and Endangered Plant Species of Siberia collection plot (Central Siberian Botanical Garden of the Siberian Branch of the Russian Academy of Sciences). These plants were obtained by seeding the seeds taken from a natural population of *H. theinum* (Krasnaya

Mountain, the Republic of Gorny Altai) and were characterized by a high content of xanthones and flavonols [22].

The axillary buds of five adult generative *H. theinum* plants were used as explants for cultivation of the in vitro culture. The buds were sterilized with 0.1% sublimate ($HgCl_2$) for 15 min, followed by washing three times with sterile distilled water. The in vitro cultures were cultivated using the Murashige and Skoog (MS) medium [23], supplemented with 5 μM 6-benzilaminopurinom (BAP) and casein hydrolysate (200 mg/L). Further cultivation of the shoots of *H. theinum* was performed on either MS or B5 [24] media, supplemented with 5 μM BAP. The rooting was achieved on half-strength MS basal medium containing 7 μM of α-naphthalene acetic acid (NAA). The explant cultures were maintained at 24 ± 1°C under 16/8 h light/dark photoperiod and light intensity of 2 to 3 klux.

Plant material from the in vitro culture was fixed after each of five passages. For these purposes, the regenerant leaves were wrapped in filter paper and placed in ziplock plastic bags filled with silica gel. Identification of maternal genotypes and analysis of the regenerant genetic variation in the first five generations was conducted using ISSR analysis.

DNA Extraction and ISSR Analysis

Genomic DNA was extracted using the NucleoSpin Plant II kit (Macherey and Nagel, United States). The quality and quantity of DNA preparations were checked by spectrophotometry (Eppendorf, Germany). The purity of DNA preparations was checked using the ratio of the UV absorbance at 260 and 280 nm.

The experiments were performed using six ISSR primers preselected for the analysis of the *H. theinum* variability and characterized by high polymorphism of the amplified fragments and considerable representativeness of the ISSR patterns [25]. PCR was performed in 25 μL of a reaction mixture containing 2.7 mM $MgCl_2$; 1.25 mM of primers, 0.4 mM of mononucleotides; 1× PCR buffer; 1.5 units of *Taq* DNA polymerase (Medigen, Russia), and 50 ng of DNA template. The amplification conditions consisted of DNA denaturation for 1.30 min at 94°C, followed by 35 cycles of 0.40 min at 94°C; 0.45 min of primer annealing at 41 to 47°C; and 1.30 min at 72°C; with a final extension of 5 min at 72°C. PCR reactions were run in the C 1000 Thermal Cycler (Bio-Rad Laboratories, United States). The annealing temperature (T_{theor}) was calculated for each primer. The optimal annealing temperature (T_{opt}) was chosen empirically. The amplification products were separated by means of electrophoresis on 1.5% agarose gel in 1× TBE buffer. The generated ISSR fragments were stained with SYBR-Green (Medigen, Russia), visualized using the Gel Doc XR+ gel documentation system, and analyzed using the Image Lab Software (Bio-Rad Laboratories, United States). The sizes of ISSR fragments were estimated from the molecular size marker, a 1-kb DNA ladder (Medigen, Russia). Each amplified fragment was treated as dominant marker, and either the presence (1) or absence (0) of this marker was scored in the examined samples.

RESULTS

In the Central Siberian Botanical Garden (CSBG, Siberian Branch of the Russian Academy of Sciences, Novosibirsk), reintroduction studies in populations of *H. theinum* growing on Krasnaya Mountain are performed. In these studies, the planting material is represented by seeds and seedlings grown from seeds [26]. To obtain high-quality planting material, it will be expedient to use the outputs of biotechnology.

The used regime of surface sterilization of *H. theinum* axillary buds yielded 40% viable uninfected plant material. After culturing the explants on MS medium supplemented with 5 μM BAP, the axillary buds gave rise to shoots with odd-pinnate leaves, which corresponded to the virginile phase of plant growth of the given species. Further subculturing was accompanied by plant rejuvenilization, and only trifoliate leaves (juvenile phase of development) were observed (Figs. 1a, 1b).

Explants were cultured for five passages in media of the same composition (B5 supplemented 5 μM BAP). In the first three passages, propagation was carried out by dividing the shoot into single-node segments (multiplication rate constituted 2 pcs/explant). At the fourth and fifth subculturing, the development of adventitious buds at the base of the shoot was observed (multiplication factor constituted 9.2 ± 1.1 pcs/explant), which may provide evidence of an accumulation of growth regulators in the explant tissues (Fig. 1c). The obtained *H. theinum* microshoots were then transferred to the rooting media. Rhizogenesis was observed after three weeks of cultivation.

Amplification of the regenerant and mother plant DNA templates with different ISSR markers produced from four to ten fragments with sizes ranging from 250 to 3000 bp (Fig. 2 and table). Furthermore, all electrophoretic profiles of the ISSR patterns in regenerants were monomorphic and identical to the mother plant. Thus, no differences between the regenerants, or between them and the *H. theinum* mother plant, were observed.

DISCUSSION

Among the micropropagation techniques developed for the reproduction and conservation of rare and resource species, the method of axillary meristem activation is the most popular [27]. The method is based on the use of a natural method of plant propagation via the development of lateral buds. Since this

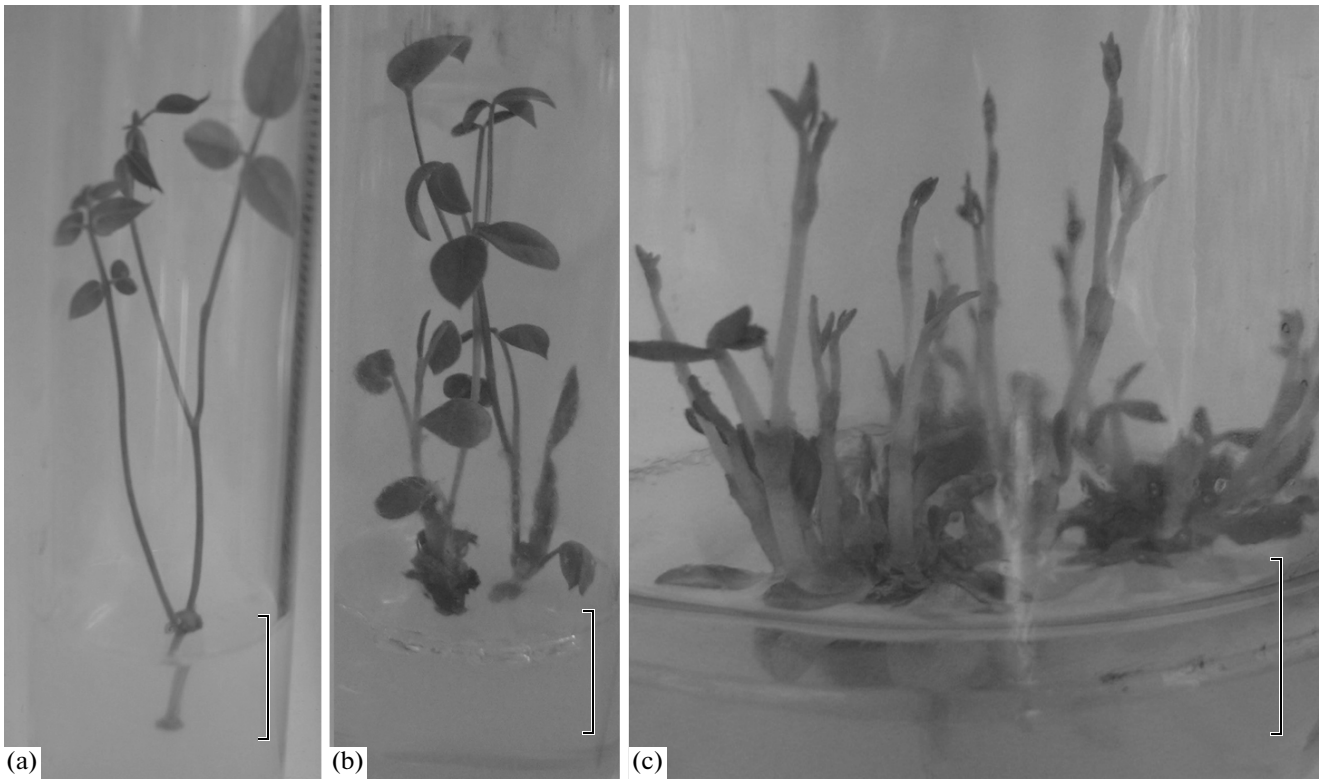

Fig. 1. Stages of clonal propagation of *Hedysarum theinum* from axillary buds. (a) regenerant at virginile phase (first passage); (b) regenerant at juvenile phase (third passage); (c) mass initiation of adventive buds (fifth passage). Scale: 1 cm.

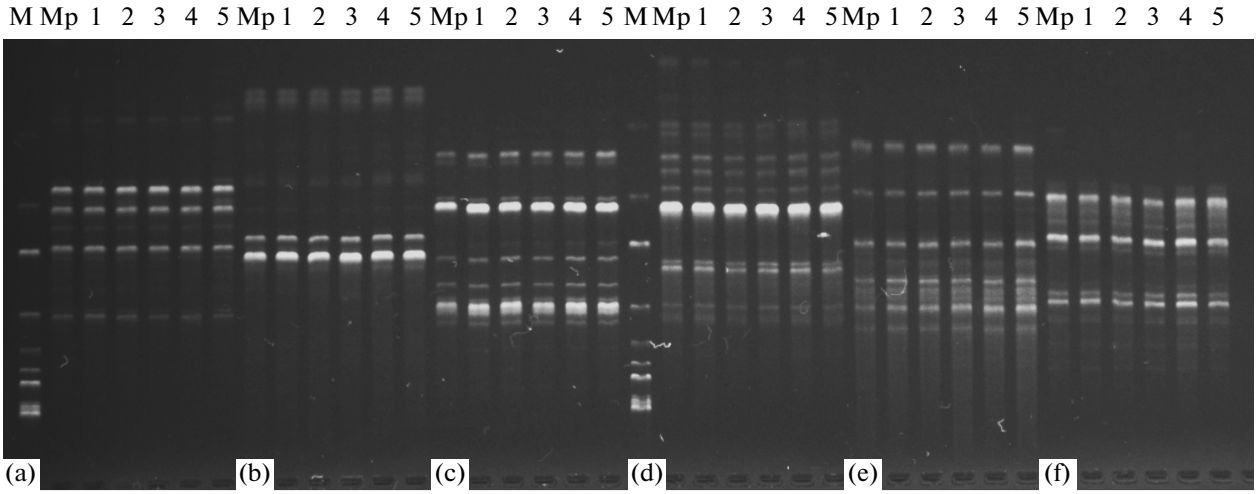

Fig. 2. Electrophoregram of ISSR fragments obtained by amplification of the *Hedysarum theinum* maternal plant and regenerant DNA with primers: (a) 17898A; (b) 844A; (c) HB14; (d) 844B; (e) 17899A; (f) 17898B. M, molecular size marker; Mp, mother plant; 1 to 5, regenerants of the first to fifth passages.

technology is used as a pathway of plant development from preexisting meristems, it is the most reliable for producing progeny characterized by the absence of genetic and epigenetic variations [28–30], i.e., identical to initial plants [31].

The selected nutrient media and cultivation conditions made it possible to successfully propagate *H. theinum* from axillary buds. Furthemore, a subsequent tendency to rejuvenate explant tissue (a transition from the virginile phase of development to the juvenile) was observed during cultivation, along with an increase in the multiplication factor, as a result of adventitious bud initiation and development.

ISSR analysis of genomic DNA confirmed genetic the identity of the *H. theinum* mother plant and regenerants developed from the in vitro cultivated axillary

Characteristics of primers used in the study

Primer	Nucleotide sequence, 5'–3'	Annealing temperature, °C		Number of amplified fragments
		T_{theor}	T_{opt}	
17898A	CACACACACACAAC	42	45	4
844A	CTCTCTCTCTCTCTCTAC	54	44	6
HB14	CTCCTCCTCGC	38	42	10
844B	CTCTCTCTCTCTCTCTGC	56	41	10
17899A	CACACACACACAAG	42	47	10
17898B	CACACACACACAGT	42	47	10

buds. Genetic analysis (including ISSR) was used earlier to confirm the stability of regenerated plants [8, 9, 12, 16].

There are a number of factors that can affect regenerated plant stability. These factors include the type of explants used, the duration of in vitro culturing, and plant genotypes [32, 33]. Our studies showed that the duration of the culturing period (five months) and changes in the explant tissues resulting in *de novo* bud initiation (adventitious shoot formation) did not affect the genotype of *H. theinum*. Similar analyses of the genetic variation of regenerants obtained in the in vitro cultures were carried out on some plant species, including *Prunus dulcis* (Mill.) D.A. Webb., *Curcuma amada* Roxb., *Curcuma longa* L., and *Bixa orellana* L. [17, 34], and others.

Thus, this study is the first analysis of the genetic variation of mother plants and regenerants obtained in five passages performed for the rare medicinal species tea sweetvetch. The analysis was conducted with six ISSR primers. Depending on the primer used, four to ten amplified fragments with sizes ranging from 250 to 3000 bp were identified. The electrophoresis patterns of ISSR fragments showed the identity of the genotypes of initial plants and the regenerants obtained.

Based on the results of the study, a recommended protocol was developed for efficient in vitro propagation of *H. theinum*. The isolated axillary buds were cultured on B5 medium containing 5 μM BAP, which made it possible to obtain 9.2 shoots per explant. At the stage of rooting, half-strength MS medium containing 7 μM NAA was used. The developed protocol for *H. theinum* clonal micropropagation can be used to create a bank of in vitro and living collections for the reintroduction of valuable genotypes and populations, as well as for plantation cultivation of this species characterized by medicinal properties.

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