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Conservation of *Colutea gifana*, a rare and potential ornamental species, using *in vitro* method

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Abstract

In vitro methods provide a variety of tools to supplement traditional methods for collection, propagation and preservation of endangered plant species. In this study, an efficient protocol was developed for *in vitro* propagation of *Colutea gifana*, a rare and endangered plant species with limited reproductive capacity that grows in a narrow area of Iran. Single node explants were used for a series of experiments to select the appropriate disinfection method and growth regulators for establishment, proliferation and rooting stages. Explants showed the highest establishment percent after treatment with 2% Sodium hypochlorite (NaOCl) for 15 min, cultured in MS medium containing 2.2 µM 6-benzylaminopurine (BA) and 1 µM indole-3-butyric acid (IBA). In proliferation stage, 8.8 µM of BA was more effective cytokinin than Kinetin (Kin) and Thidiazuron (TDZ) for growth induction of axillary shoots. *In vitro* rooting of proliferated shoots was induced in half-strength MS medium in all concentrations of both tested auxins i.e. IBA and α-naphthalene acetic acid (NAA). Eighty percent of the plantlets were successfully acclimatized to *ex vitro* conditions, showed normal development. These plants were used to replenish declining populations in the collection sites and conserve *C. gifana* from extinction.

***Keywords*:** conservation, extinction, Fabaceae, germplasm, micropropagation

Introduction

In vitro propagation of endangered plants offer considerable benefits for the rapid multiplication of species that are at risk, have limited reproductive capacity and exist in threatened habitats (Fay, 1992). These methods are essential components of plant genetic resources management and become increasingly important for conservation of rare and endangered plant species (Sudha et al., 1998; Benson et al., 2000; Iankova et al.,2001; Bhatia et al., 2002;). This is especially important since *in vitro* propagation allows the establishment of cultures from the minimum amount of starting material coupled with the possibility of further multiplication (Benson, 2000). In combination with another *in vitro* culture technique, referred to as slow growth, it is possible to establish long term collections of germplasm with minimal resources (Watt et al., 2000). Similarly, these techniques facilitate the application of genetic manipulation procedures (Knapp et al., 2001; Ueno et al., 1996).

Colutea gifana Parsa is a local endemic plant in N Khorasan province, NE Iran. This species is a dwarf shrub with yellowish-green branches, glabrous paired leaves and inflorescences that average 1.5-2.5 cm long with 4-5 yellow flowers (Rechinger, 1984) that has a high potential to be used as a new ornamental plant. Due to the limited extent and occurrence of natural populations, this species is considered vulnerable (Jalili and Jamzad, 1999) and facing extinction in the medium-term future, therefore needs urgent *in situ* and *ex situ* conservation. Furthermore, as low seed set makes problems in seed propagation of this species, *in vitro* multiplication might be used as an alternative method for reintroduction of this species into the natural environment (Wochock, 1981) and reducing the risk of extinction (Nadeem et al., 2000;Chandra et al., 2006). However, the members of Fabaceae family are generally recalcitrant under *in vitro* conditions (Trigiano et al, 1992; Jha et al, 2004). This study was conducted to develop a protocol for *in vitro* propagation of this endemic and rare plant, to help with its future *in situ* and *ex situ* conservation.

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Materials and Methods

Plant material and preparation of explants

Young shoots (20 cm long) were collected from six *Colutea gifana* shrubs in N Khorasan province in May 2009. Collected shoots were washed with running tap water for 1 h and surface sterilized with 70% ethanol (30s) and different concentrations and time intervals of sodium hypochlorite (NaOCl) and mercuric chloride ($HgCl_2$) (Table 1). After rinsing three times with distilled water, the terminal buds and leaves were removed. Single node explants with 2 cm long were cultured in Murashige and Skoog basal medium (Sigma-Aldrich, USA; pH adjusted to 5.8) with 2.2 µM BA and 1 µMIBA. Cefotaxime at 100 mg/l concentration was also used in some disinfection treatments to evaluate the effect of this treatment for further contamination control (Table 1). Cultures were maintained at 24±1ºC and illuminated by white fluorescent tubes (40 µmol m^{-2} s^{-1}) 16 h per day. Fifteen replicates were used for each treatment. Number of surviving explants and explants with developing axillary shoots were recorded after four weeks of culture. For the proliferation stage, developed axillary shoots were cultured on MS medium supplemented with BA (2.2, 4.4, 8.8 or 17.7 µM), Kinetin (2.2, 4.4, 8.8 or 17.7 µM) or TDZ (0.5, 1.1, 2.2 or 4.5 µM) and IBA (1 µM). After transferring to the proliferation medium, number of shoots per explant and shoot length were recorded after four weeks. At rooting stage, well-developed shoots, approximately 4 to 6 cm in length, were cultured on half-strength MS medium supplemented with IBA or NAA (1.3, 2.7, 5.4or 10.8 µM).

After 5 weeks, rooted shoots were gently washed with distilled water and transferred to plastic cups with drainage holes containing a 1:1 mixture of perlite and fine sand for the first month of acclimatization stage. For the initial seven days, plantlets were covered with a transparent cup and irrigated with a half-strength MS solution (sucrose free) every other day. After seven days, plantlets were exposed to the atmosphere and irrigated with both distilled water and a half-strength MS solution. Surviving plantlets were transferred to larger containers with a 1:1 mixture of perlite and peat before moving plants to outdoor and collection sites.

Data analysis

Collected data were subjected to analysis of variance (ANOVA) using SAS (SAS Institute, Inc., Cary, NC) software. Mean values were separated according to Duncan's multiple range test at probability of 0.05 level.

Results

Explants showed the highest establishment rate (less contamination and higher growth) after a 2% NaOCl disinfection treatment for 15 min. Cefotaxim didn't show any positive effect on contamination control (Table 1). Although antibiotic treatments are the most widely used method to eliminate bacterial contaminations, they are not always effective against different bacterial strains and several cases of their toxicity for plants have been reported (Cassels 1997; Falkiner 1997).

Bud breakage and growth of shoots were noticed in the pre-existing axillary meristems of nodal explants in the presence of all tested cytokinins (BA, Kinetin, TDZ) in MS medium.BA was more effective cytokinin considering shoot numbers and at concentration of 8.8 µM resulted in the highest multiplication rate with average values of 5.3 shoots per explants (table 2). The superiority of BA over the other cytokinins has been also reported in micropropagation of some species from Fabaceae family (Rout, 2005; Prakesh et al, 2006). As BA concentration increased, shoot growth became distorted, showed deformed leaves, pale color, and hyperhydricity, which may be related to the excess of cytokinin (Arora et al., 2009). An inhibitory effect of high concenteration of BA has also been reported in *Pterocarpus santalinus* L. from Fabaceae (Prakash et al, 2006). TDZ was less effective for shoot proliferation in this experiment, although it has been reported that induces high rates of axillary shoot proliferation in some Fabaceae species, such as *Swainsona Formosa* (Jusaitis 1997), *Glycine max* (Kaneda et al, 1997) and *Swainsona salsula* (Yang et al, 2001).

Table 1. Effect of different disinfection treatments on infection percent and growth of *C. gifana* explants.

Treatment	Infection (%)	Growth (%)
3 min 0.1% $HgCl_2$	0	37.5
6 min 0.1% $HgCl_2$	7.2	14.2
3 min 0.1% $HgCl_2$ + Cefotaxim	11.7	30
6 min 0.1% $HgCl_2$ + Cefotaxim	33.3	16.6
20 min 1% NaOCl	0	40
30 min 1% NaOCl	5	5
30 min 1% NaOCl + Cefotaxim	27.2	0
10 min 2% NaOCl	0	26.3
15 min 2% NaOCl	0	70
15 min 2% NaOCl + Cefotaxim	0	36.8

Table 2. Effect of different cytokinins on axillary shoot proliferation.

Cytokinin (µM)	Sprouting (%)	Shoot number	Shoot length (cm)	Mean number of nodal segment
BA				
2.2	92	2.3 b	0.5 ab	1.6 d
4.4	89	4.6 a	0.8 a	2.0 cd
8.8	100	5.3 a	0.8 a	2.9 bc
17.7	100	3.8 a	0.5 ab	1.7 cd
Kinetin				
2.2	100	1.0 b	0.6 ab	2.1 cd
4.4	100	1.3 b	0.3 b	2.4 bcd
8.8	93	1.0 b	0.8 a	2.8 bcd
17.7	100	2.4 b	0.8 a	2.8 bcd
TDZ				
0.5	93	1.8 b	0.7 ab	2.3 bcd
1.1	100	1.2 b	0.9 a	2.2 bcd
2.2	93	2.1 b	0.5 ab	2.6 bcd
4.5	86	1.2 b	0.8 a	4.0 a

*Values within each column followed by the same letters are not significantly different by the Duncan Multiple Rang test at 0.05% probability level.

Rooting and Acclimation

Root initiation was observed within 10–15 d in all treatments expect in auxin free medium (data not shown). Micro-cuttings cultured on half strength basal MS medium with IBA or NAA produced normal roots without forming callus. The highest frequency of root initiation (average of 55%) was observed in half strength MS medium supplemented with IBA in comparison to NAA after five weeks (table 3, figure1D). No significant differences were found among treatments when comparing the number of roots and root length (table 3). Rooted plantlets were successfully acclimatized to growth chamber conditions and survival percentage was 80%. Acclimatized plantlets were healthy and well developed, phenotypically similar to the parental stock and showed active growth (figure 1F). These plants were successfully established in the collection sites (N Khorasan province) and continued to grow.

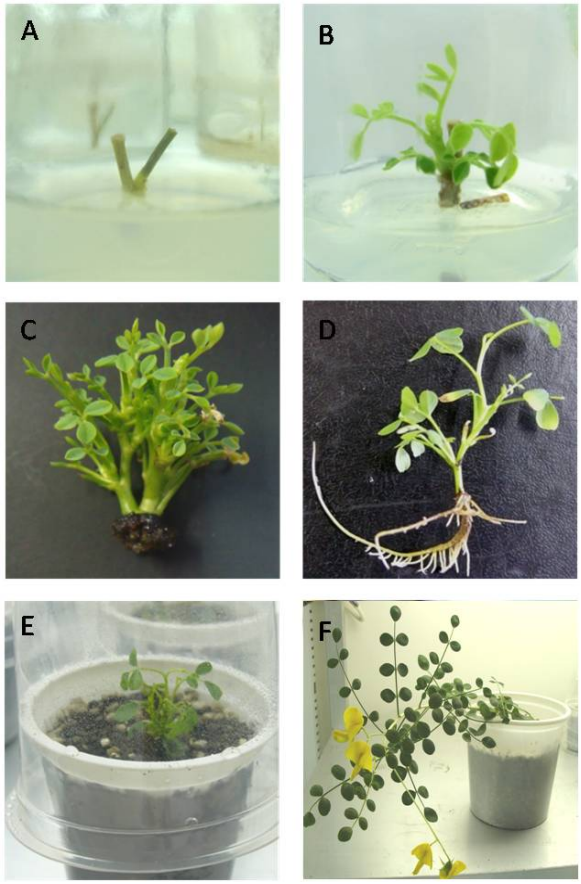

Figure 1. Complete micropropagation cycle of *Colutea gifana*: (A, B) Shoot initiation from nodal segments; (C) Multiple shoot regeneration; (D) Root development; (E, F) Acclimatization stages.

Table 3. Effect of different auxin treatments on rooting percent and root growth on 1/2 MS medium.

Auxin (µM)	Rooting (%)	Number of root	Mean root length/shoot (cm)
IBA			
1.3	60	2.2 a	2.1 a
2.7	50	2 a	2.7 a
5.4	50	2.9 a	2.5 a
10.8	60	2.5 a	1.7 a
NAA			
1.3	40	2.5 a	1.6 a
2.7	40	1.8 a	1.6 a
5.4	40	2.4 a	1.1 a
10.8	50	2.7 a	1.1 a

*Values within each column followed by the same letters are not significantly different by the Duncan Multiple Rang test at 0.05% probability level.

Discussion

This paper presents a protocol for micropropagation of *C. gifana*, which is the first report for this species. It is important to note that the morphology of *in vitro* plantlets showed a true-to-type growth habit, both *in vitro* and when transferred to *ex vitro* growth conditions. There was no evidence of callus formation at proliferation stage and shoot multiplication occurred from pre-existing axillary shoot primordia that eliminated the risk of genetic instability. Our future, long-term research will concentrate on the *ex vitro* transfer stage for the possible introduction of this species as a new ornamental plant and assessing the value of the protocol for micropropagation of the other *Colutea* species. To date, *ex vitro* transfer has been based on the use of commercially available substrates, however more consideration must be given to the environment-specific needs of this species. In the wild, *C. gifana* proliferates in

serpentine soil, which is derived from ultramafic bedrock. It may therefore, be appropriate to simulate the environmental requirements of *C.gifana* during acclimatization stage. The developed protocol would be useful for large-scale multiplication of *C. gifana*, *ex situ* and *in situ* conservation and sustainable utilization of this endemic and endangered plant species.

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