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Rapid in vitro propagation of *Clematis heynei* M. A. Rau: An important medicinal plant

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Abstract

The propagation of *Clematis* species has been studied for last 2-3 decades, but problems related to rooting have limited the conventional methods to fulfil commercial needs. The present investigation describes an efficient micropropagation protocol for *Clematis heynei*, an important medicinal plant. Multiple shoot production was successfully achieved from axillary buds using nodes as explants. The highest organogenic response was obtained from the nodal segments cultured on MS medium enriched with Kinetin (3 mgL^{-1}) and indole-3-acetic acid (0.4 mgL^{-1}). Microshoots were transferred for rooting in variety of auxins and its concentrations; in which maximum root induction was occurred in Indole-3-acetic acid (1 mgL^{-1}). Through sequential hardening process, well-rooted plantlets were established in the field conditions and exhibited 88 % survival with normal morphology and growth characteristics. This protocol is a successful and efficient biotechnological approach to the micropropagation of *C. heynei*.

Key words: Acclimatization, Growth regulators, Medicinal plant, Micropropagation, Nodal explant, Rooting

Abbreviations: BA - N6–benzyladenine; KN – Kinetin; TDZ – Thidiazuron; IAA - Indole-3-acetic acid; IBA - Indole-3-butyric acid; NAA - α-naphthaleneacetic acid; MS - Murashige and Skoog medium.

Introduction

Plants are valuable source of a vast array of chemical compounds. They synthesize and accumulate extractable organic substances in quantities sufficient to be economically useful as raw materials for various commercial applications. Industrialization coupled with urbanization is constantly putting pressure on natural resources. Due to depletion of habitat and ruthless collection, medicinal plants are on the verge of extinction. Hence, the conservation of these valuable genotypes is imperative. Plant tissue culture technology holds great promise for micropropagation, conservation, and enhancement of the natural levels of valuable secondary metabolites and to meet pharmaceutical demands and reduce the *in situ* harvesting of natural forest resources (Bapat et al., 2008).

Clematis L. (Ranunculaceae) is a medicinally important genus with more than 250 species of climbing and erect or ascending perennial herbs (sometimes woody) widely distributed through the temperate regions, chiefly in the Northern Hemisphere (Rehder, 1940). *Clematis heynei* Roxb is a somewhat woody climber very sparsely distributed in deciduous forests of Western Ghats, India. In the Indian system of medicine 'Ayurveda' this plant is used to eliminate malarial fever and headache. Roots are given orally for secretion of bile. Leaf paste is applied externally for itches, in wounds and skin allergies. The traditional medicine practitioners give the root decoction orally or small pieces placed in mouth in bilious vomiting. Leaf juice for treating boils leprosy, blood diseases and cardiac disorders. Decoction of root is given every night with boiled rice water to the children as anthelmintic (Mahekar and Yadav, 2006). *Clematis* species has many different pharmacological effects such as antibacterial, anti-inflammatory, antitumor, analgesic and diuretic functions (Song et al., 1996). Reports on the chemical components of genus *Clematis* have been scarce up to now and mainly refer to triterpenoid saponins (Song et al., 1996; Liu et al., 2009; Huang, 2002; Zhi et al., 2010).

Many investigators successfully isolated some of the secondary metabolites from the species of

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Clematis. Clemontanoside-C, a new hedragenin based saponin was isolated from the stem of *Clematis montana* (Thapliyal and Bahuguna, 1993). From the aerial part of *Clematis tibetana*, two new hedragenin 3, 28-O-bisdesmosides called clematibetosides A and C, and a new gypsogenin 3, 28-O-bisdesmoside called clematibetoside B have been isolated (Kawata et al., 2001). Protoanemonin has been isolated from the Australian 'Headache Vine' *Clematis glycinoides* (Southwell and Tucker, 1993).

Many horticultural varieties are grown as ornamental purposes following extensive breeding programmes. Vegetative propagation of some species and their cultivars, notably *C. heynei, C. armandii, C. florida* and *C. texensis* is difficult because cuttings are difficult to root (Dirr, 1990; Kreen et al., 2002). Hence, certain varieties of *clematis* can however be difficult to propagate by cutting which is one reason for the development and commercial use of *in vitro methods*. In recent years, there has been increased interest in tissue culture techniques, which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered, and threatened medicinal plants like *Clematis gouriana* (Naika and Krishna, 2008) and *Stevia rebaudiana* (Anbazhagan et al., 2010). Reports on *in vitro* propagation of *Clematis* species were limited (Leifert et al., 1992; Luttman et al., 1994; Kreen et al., 2002; Naika and Krishna, 2008). A literature survey indicated that the *in vitro* regeneration protocol for this medicinal climber was not yet standardized. In view of its medicinal importance and the lack of tissue culture reports, the present study reports the prime protocol for regeneration of *C. heynei* from nodal explants.

Material and Methods

Plant material and explant disinfection

The plants collected from the wild source were identified and maintained in the Botanical garden of Department of Botany, Shivaji University, Kolhapur (MS) India. Tender nodal segments of *C. heynei* from mature plants were used as explants. The explants were washed in running tap water for at least 20 min followed by repeated washings with a detergent, labolene (5%, v/v). Surface sterilization of the explants was done with mercuric chloride (0.1%, w/v) for 7 min before being washed thoroughly (3-4 times) with sterile double distilled water.

Culture media and culture conditions

Nodal explants measuring approximately 1 cm with single node were cultured on sterile nutrient medium (Murashige and Skoog, 1962), major and minor salts, vitamins, FeEDTA and inositol (100 mgL^{-1}) supplemented with different growth regulators. Sucrose (3%, w/v) was added as a carbon source and pH of the medium was adjusted to 5.8 ± 0.1, prior to the addition of Clarigel (0.22%). All the media were autoclaved at 1.08 kg cm^{-2} for 20 min. Cultures were maintained at 25 ± 1^0C with 16-h photoperiod.

In vitro shoot multiplication and root induction

Nodal segments were aseptically transferred onto the MS medium supplemented with different plant growth regulators. At the establishment stage, the MS basal medium containing 3% sucrose, 0.2% w/v Gelrite was supplemented with 0.5 mg/L^{-1}were used. For shoot multiplication MS medium supplemented with varying concentrations of BAP, KN, TDZ and IAA were used either alone or in combinations. MS medium without growth regulators served as control. Subculturing was done once at 4 weeks interval. Data on explant response, number of shoots/node explant and length of shoots was recorded after 4 weeks of growth. Rooting of the *in vitro* derived plant accomplished on half strength MS basal medium supplemented with various concentrations and combinations of IBA and NAA. Data were recorded on rooting percentage, mean number of roots/shoot and mean root length after 4 weeks of culture.

Acclimatization and transfer of plantlets to green house

Plantlets with well developed roots and shoots were removed from the culture tubes were cleared of traces of medium to avoid pathogenic contamination. The plantlets were then transferred to small plastic pots containing sterile garden soil, river sand and vermicompost either alone or in combination. The pots were covered with polythene bags and were watered with ¼ MS basal salt solution every alternate day for first week. The plantlets were maintained in a culture room at 25±2 °C for 2 weeks before being transferred into pots containing soil. The 6-8 weeks old plantlets were transferred stepwise to the natural conditions. Survival percentage was recorded after transfer of plants to their natural habitat within 8 weeks.

Statistical analysis

All experiments were set up in completely randomized design. Data presented in the table are treatments of 20 replicates and all experiments were repeated twice. Data were analyzed using analysis of variance (ANOVA) and means were compared using Dunnets multiple range test.

Results and Discussion

The morphogenic response of nodal explants of *Clematis heynei* was shown in almost all the

treatments of BA, TDZ KN and IAA (Table 1). Nodal explants cultured on MS medium without growth regulators showed no sign of bud break even after four weeks. Nodal bud culture has been used as a tool for propagation of various endangered and medicinal plant species such as *Clematis filamentosa* (Shao and Yu, 2005) and *Frerea indica* (Desai et al., 2003). Of the three cytokinins tested, BA and KN were more effective compared to TDZ. The new shoots were developed adjacent to the axillary shoots. The frequency of axillary shoot proliferation and the number of shoots per explant increased with increasing concentration of BA up to some extent, (Table 1). BA (4 mgL^{-1}) showed positive response during shoot regeneration with long and healthy shoots (Figure 1 a). Efficacy of BA during shoot induction and proliferation has been reported for *Crataeva magna* (Benniamin et al., 2004), *Aloe vera* (Kalimuthu et al., 2010) and *Centaurea zeybekii* (Kurt and Erdag, 2009).

A maximum of 6 shoots were produced when MS medium was supplemented with KN (3.0 mgL^{-1}). Thus, BA and KN resulted better in terms of period required for bud break, percent bud break, number of shoots regenerated per explant. This indicates the cytokinin specificity of nodal explants of *C. heynei* for multiple shoots formation. Most of the *Clematis* species showed good response with BA during shoot multiplication and regeneration (Leifert et al. 1992; Kreen, 2000), but *C. heynei* was well responded to combination of KN and IAA. MS medium supplemented with KN (3.0 mgL^{-1}) in combination with IAA (0.4 mgL^{-1}) was found to be optimal for multiple shoot regeneration. A maximum of 11.1 shoots produced from the nodal bud after 4 weeks of inoculation on the same media composition (Figure 1 b). A synergistic effect of cytokinins in combination with auxins was reported for *Clematis gouriana* (Naika and Krishna, 2008) and *Stevia rebaudiana* (Anbazhagan et al., 2010). Combination of KN and IAA showed advantageous effects on rate of shoot bud proliferation but, they reduced the shoot length. A harvest of 15-17 sturdy microshoots was obtained after third subculture on MS medium supplemented with KN (3 mgL^{-1}) with IAA (0.4 mgL^{-1}). TDZ was found to be pessimistic during the shoot multiplication. Thidiazuron (3mgL^{-1}) produced maximum of 3 shoots per node culture and the shoots produced were stunted and vitrified. There was intermittent callus formation at the junction of root and shoot during increasing concentrations of TDZ (Table 1). The endogenous levels of growth regulators in the members of Ranunculaceae might be responsible for the observation of variation in the response and growth regulator requirement for *in vitro* shoot regeneration.

Table 1. Effect of different growth regulators on shoot multiplication of *Clematis heynei* on modified MS medium for 28 days.

Growth regulators (mgL-1)				Multiplication (%)	Number of shoots per explant ± S.E.	Shoot height (cm) ± S.E.	Callus production
BA	KN	TDZ	IAA				
GR free				00	00	00	
1				65	1.3±0.3**	8.2±1.3**	
2				80	2.1±0.3**	8.1±1.3**	
3				90	3.4±0.6**	10.1±0.9**	
4				95	4.4±0.6**	9.2±0.8**	
5				75	2.6±0.6**	7.4±1.0**	
	1			70	1.4±0.3^{ns}	5.6±0.9**	
	2			85	2.6±0.4**	6.1±0.7**	
	3			90	5.5±0.7**	6.1±0.6**	
	4			80	2.7±0.5**	5.6±1.0**	+
	5			75	1.5±0.2^{ns}	5.4±0.9**	+++
		1		75	0.9±0.1^{ns}	7.3±1.2**	+
		2		80	1.8±0.3**	6.8±0.9**	++
		3		85	2.8±0.5**	6.5±0.9**	
		4		85	2.2±0.4**	6.4±1.0**	
		5		80	1.8±0.3**	6.1±0.8**	
	3		0.1	80	3.7±0.9**	5.9±0.7**	
	3		0.2	85	4.2±0.8**	5.9±0.7**	
	3		0.3	90	5.2±0.6**	5.8±0.8**	
	3		0.4	95	11.1±1.1**	6.0±0.6**	
	3		0.5	75	3.5±0.6**	5.5±0.8**	

ns Non significant; ** Significant; + Minute; ++Moderate; +++ Maximum

Table 2. Effect of different growth regulators on *in vitro* rooting of *Clematis heynei* on modified MS medium for 28 days.

Growth regulators (mg l^{-1})		Rooting %	Number of roots per shoot ± S.E.	Root length (cm) ± S.E.
IBA	NAA			
GR free		00	0.0	0.0
0.1		85	4.2±0.4**	3.0±0.4**
0.5		85	6.0±0.9**	3.1±0.4**
1.0		95	8.2±0.9**	3.2±0.3**
1.5		80	3.7±0.6**	2.5±0.3**
2.0		70	1.8±0.3^{ns}	1.6±0.4**
	0.5	80	2.5±03**	2.3±0.4**
	1.0	95	4.8±0.4**	2.4±0.2**
	1.5	80	3.7±0.4**	2.2±0.3**
	2.0	70	2.6±0.4**	1.8±0.4**
	2.5	70	2.2±0.3**	1.6±0.3**
1.0	0.2	85	2.3±0.2**	2.1±0.3**
1.0	0.4	95	6.1±0.5**	2.6±0.3**
1.0	0.6	85	4.0±0.4**	2.1±0.3**
1.0	0.8	80	2.6±0.3**	2.1±0.3**
1.0	1.0	65	1.8±0.3**	1.8±0.3**

ns Non significant; ** Significant

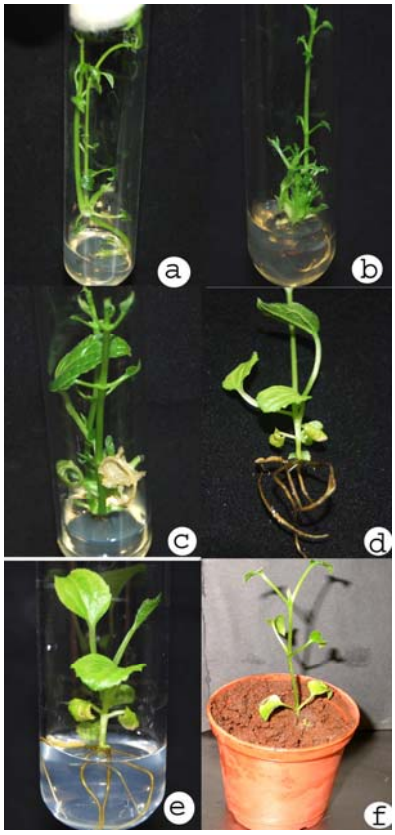

Figure 1. Direct organogenesis in *C. heynei*; a: Shoot multiplication on MS medium containing BA (4.0 mgL-1); b: Multiple shoot induction and proliferation on MS medium + KN (3.0 mgL-1) + IAA (0.4 mgL-1); c: Leaf abscission in higher concentrations of BA; d: Shoots with well developed roots on MS medium with IBA (1.0 mgL-1); e: *In vitro* rooting on MS medium + IBA (1.0 mgL-1) + NAA (0.4 mgL-1); f: Acclimatized *C. heynei* plantlet after three weeks.

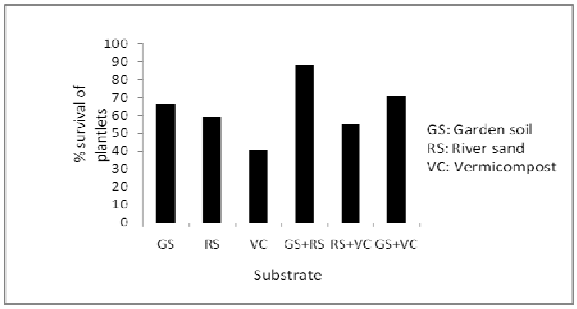

Figure 2. Effect of different planting substrates during acclimatization of *Clematis heynei*. GS: Garden Soil; RS: River Sand; VC: Vermicompost.

During the present study, the micropropagated shoots exhibited leaf abscission (Figure 1 c). Similar phenomenon has also been reported during the *in vitro* multiplication of *Gymnema sylvestre* (Komalavalli and Rao, 2000). Leaf abscission occurred at the basal node region of the microshoots. Nevertheless, leaf abscission did not affect the multiplication of shoots.

During vegetative propagation stem cuttings of *C. heynei* was able to induce little numbers of shoot but, they failed to induce roots. To overcome rooting problem, *in vitro* regenerated shoots were harvested from the clump when they attained a length of more than 4-5 cm with 3-4 leaf primordia. They were rooted only upon transfer to half strength MS medium containing various auxins, whereas no rooting was noted in hormone free half strength MS medium. Half strength MS medium supplemented with different auxins at different concentrations showed varied effect of rooting

(Table 2). Among the auxins tested, IBA was the most effective for inducing roots and was followed by NAA. Half strength MS medium fortified with IBA (1 mgL^{-1}) with sucrose (3% w/v) was found to be optimal for *in vitro* root induction. After 4 weeks of incubation, it was observed that about 8-9 roots were emerged out from basal cut end of the microshoots (Figure 1 d). Effectiveness of IBA in rooting has been reported in medicinal plants viz. *Gymnema sylvestre* (Komalavalli and Rao, 2000) and *Hemidesmus indicus* (Sreekumar et al., 2000). MS medium containing NAA (1 mgL^{-1}) was capable of producing up to 5 roots per shoot. Roots produced on the NAA supplemented media were whitish in colour, fragile and reduced in height compared to IBA. The effectiveness of NAA in rooting has been reported for a few plant species such as *Decalepis hamiltonii* (Reddy et al., 2001). However, the combinations of two different auxins IBA (1 mgL^{-1}) + NAA (0.4 mgL^{-1}) had the cumulative effect during root induction which fashioned in to 6-7 roots per shoot (Figure 1 e).

Shoots with well-developed roots transferred directly to mini pots containing different substrates, revived growth within 15 d (Figure 1 f). Among the various substrates used for the acclimatization of the *C. heynei* and the plantlets exhibited 88% survival in mixture of garden soil and river sand (Figure 2). Plantlets transferred subsequently to field conditions grew well and exhibited morphological characters similar to wild plants. They developed sturdy roots and flowered normally (Figure 3).

Figure 3. Flower of *C. heynei.*

Conclusions

In the present investigation, a high frequency of multiple shoot induction and proliferation leading to complete propagation was achieved in *C. heynei* through nodal bud segments. This protocol imparts highly repeatable, successful and rapid technique that can be utilized for the commercial propagation and *ex situ* conservation of this medicinal plant. It is important to note that the morphology of the *in vitro* plantlets of *C. heynei* showed a true-to-type growth habit, both *in vitro* and when transferred to *ex vitro* growth conditions.

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