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TDZ Plays Key Role in Shoot Regeneration from Different Explants of *Picrorhiza kurroa*: An Endangered Medicinal Herb of Western Himalayas

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Authors' contributions

This work was carried out in collaboration between both authors. Author VP raised aseptic shoot cultures, recorded the observations and was involved in data analysed and manuscript writing. Author AB analysed the data and helped in editing of manuscript. Both the authors read and approved the final manuscript.

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ABSTRACT

Picrorhiza kurroa plants were collected from its natural habitat. *In vitro* plants were raised from the leaves of high yielding collection screened in an earlier study. Leaves, roots and internodal segments were cultured for 15 days. The effect of thidiazuron (1-phenyl-3-(1, 2, 3- thiadiazol-5-yl) urea; TDZ) pretreatment for 15 days on regeneration potential of different explants *viz.* leaves, roots and internodes of *Picrorhiza kurroa* was studied. Regeneration potential varied significantly with the type of explant. Regeneration response of 100% with 46.25 shoots per explant was obtained in leaf segments of 2.0 cm length pretreated with 0.5 µM TDZ for 15 days and then transferred to 2.32 µM kinetin (Kn) containing MS basal medium. In case of root explants maximum shoot number (17.12) was obtained on 0.5 µM TDZ pretreated for 15 days and then to 3.64 µM Kn. Maximum shoots per explants (12.33) were obtained in case of internodes pretreated with 0.5 µM TDZ for 15 days and transferred to 1.16 µM Kn. Regenerated shoots from different explants developed *in vitro* rooting on

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MS basal medium within 7-8 days. Conclusively, an efficient and repeatable protocol for rapid regeneration from different explants and *in vitro* rooting has been developed in *P. kurroa* which can be effectively used for its conservation.

Keywords: Picrorhiza kurroa; kinetin; leaves; shoot regeneration; stem internodes; thidiazuron.

1. INTRODUCTION

Picrorhiza kurroa Royle ex Benth, an endangered, perennial, medicinal herb of western Himalayas, is the main source of many naturally occurring biologically active compounds such as Picrosides I, Picrosides II and kutkosides. These compounds are responsible for the hepato-protective [1,2,3], anti-inflammatory [4], anti-asthmatic [2,5], anti-cancerous [6,7], anti-mutagenic [8], anti-periodic [9] and immunomodulatory [10] properties.

In view of the increasing demands for the above mentioned compounds in traditional as well as modern systems of medicine, there has been extensive over-harvesting of the *P. kurroa* from their natural habitats [11]. Thus, it is now enlisted as an endangered species in the Red Data Book of the International Union for Conservation of Nature and Natural Resources (IUCN) [12]. Besides over-harvesting, narrow distribution range, small population size [13] and poor natural regeneration [14] have been reported to be the major factors for the rapid dwindling of *P. kurroa* plants. Thus, *P. kurroa* is one of the 37 species of western Himalayas that require urgent propagation for stability and conservation. Development of new conservation methods such as *in vitro* regeneration and micropropagation of the plant has thus gained immense importance. Although work has been taken up in past by some workers [11,15,16,17,18,19], there is ample scope for improvement in the existing shoot regeneration systems. It is a well known fact that the success of any *in vitro* shoots regeneration system is largely dependent upon the type of explants used [20]. Therefore, the present study was undertaken to evaluate the regeneration capacity of different explants i.e. leaf, internode and root from *in vitro* raised plants of *P. kurroa*. Genetic stability of regenerated shoots was also assessed.

2. MATERIALS AND METHODS

2.1 Plant Material

P. kurroa plants were collected from its natural habitat at Rohtang pass (4000 m amsl, 32°23′ N, 77°15′ E, India) and maintained in the experimental farm of CSIR-Institute of Himalyan Bioresource Technology, Palampur, H.P, India (1300 m amsl; 32°06′ N, 76°33′ E). The method of Patial et al. (2012) [11] was used to raise *in vitro* plants from the leaves of high yielding collection screened in an earlier study. The plants maintained under *in vitro* conditions (25 ± 2°C and 70 μMm^{-2}s^{-1} photon flux density) served as the source of explants in the present study.

2.2 Optimization of Regeneration System for Different Explants

In order to improve the regeneration potential of *P. kurroa*, different explants such as leaves, roots and internodal segments were studied. These were cultured on MS basal [21] medium containing 3% sucrose and 0.5 μM TDZ (MST) for 15 days as optimized earlier by Patial et al. [11].

2.3 Optimization of Leaf Size in Term of Length

In vitro leaves of four different lengths i.e., 1.0, 1.5, 2.0 and 2.5 cm were selected for optimization of regeneration response. The selected leaves were then transversally excised into lower, middle and upper segments. A total of six segments from two leaves were obtained. Each of these were placed in 90 mm Petri-plates such that the adaxial surface of three segments and the abaxial side of remaining three segments touched the medium containing 0.5 μM TDZ (MST). However, only two segments i.e., upper and lower were obtained from leaves that were 1.0 cm long. Hence, only two segments of 1.0 cm leaves were cultured. After 15 days of treatment, the leaf segments were transferred to 2.32 μM kinetin (Kn) containing medium for further response. Data recording was initiated after 45 days of culturing. Data on percentage of responsive explants and total number of shoot developed per explant were recorded.

2.4 Regeneration from Root Segments

Roots of *in vitro* raised plantlets were cut transversally into 1.0 cm long segments. As in

case of leaves, the segments were cultured on MST for 15 days. Thereafter, these were transferred to MS medium containing 1.16, 2.32, 3.48 and 4.64 µM Kn. MS0 served as control. All the cultures were incubated at 25 ± 2 °C under a 16 hour light and 8 hour dark period at photosynthetic photon flux density (PPFD) of 70 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Phillips Trulite). Data on percentage of responsive explants, number of shoots formed per explant and shoot height was recorded at regular 15 day interval for 45 days.

2.5 Internodal Segments

For use as explants, 1.0 cm long segments between two adjacent nodes were excised from the *in vitro* raised shoots. These were cultured for 15 days on MST. Thereafter, the internodal segments were transferred to MS medium containing 1.16, 2.32, 3.48 or 4.64 µM Kn and incubated under culture lab conditions as described above. MS0 served as control. Data on percentage of responsive explants and number of shoots developed per explant were recorded at 15 day regular interval for 45 days.

2.6 Rooting of *In vitro* Raised Microshoots

In vitro raised shoots (2.5 cm long) were individually transferred to MS basal medium for rooting as reported earlier by Patial et al. (2012) [11]. These were then incubated under culture lab conditions as mentioned above.

2.7 Hardening of *In vitro* Raised Plants

Plants with well developed root systems (6-7 roots) in MS basal medium were acclimatized as per the report of Patial et al. [11]. For this, the plants were first kept at 15ºC for 10 days. After 15ºC cold treatment, leafy shoots (2.5 cm long) rooted on MS0 were first submerged in sterile distilled water for 30 minutes to loosen the medium attached to their roots. Thereafter, the plantlets were washed thoroughly under running tap water, and treated with streptomycin sulfate (0.02%; w/v) and Bavistin (0.02%; w/v) for 5-6 min. These were then planted in 6 inches pots containing autoclaved sand and covered with Magenta jars for 15 days. These were maintained under poly-house conditions. The plantlets were watered at 3 day interval and also nourished with Hoagland solution.

2.8 Statistical Analysis

The experiments with four replicates per treatment were repeated thrice. The data obtained was analyzed by General Linear Model for Main effect Analysis of Variance (ANOVA) followed by Duncan's multiple range test using STATISTICA version 7 (Statsoft Wipro, Bangalore, India).

3. RESULTS

3.1 Effect of Explant Type on Regeneration

In the present study, length and surface of leaves were also optimized along with different explants (roots and internodal segments). Although shoot regeneration response was observed in all the explants, the variations in time taken for shoot bud initiation, origin of shoot bud(s) and rate of multiplication was significant.

3.2 Optimization of Leaf Size

Among the three sizes of leaves and their segments (i.e., lower, middle and upper), 2.0 cm long leaves supported the maximum number of shoots that were more than 0.5 cm in height (Table 1). As above, maximum regeneration was recorded in the abaxial surface of middle segments; irrespective of leaf size (Fig. 1A). Of the two segments of 1.0 cm leaves however, numerous tiny shoots were obtained only from lower segments, irrespective of abaxial or adaxial surfaces (Table 1).

Among the two media tested, the number of shoots formed on MSK was invariably higher than that on MS0, irrespective of leaf size. However, the only exceptions were the abaxial surface of lower segments of 1.5 cm leaves (Table 1).

3.3 Root Segments

The root segments were more responsive as compared to internode segments. Swelling was observed in the root segments after 9-10 days of incubation on MST. This was followed by callusing at the cut ends after transfer to medium containing various concentrations of Kn (1.16-4.64 µM) (Romman et al. 2013). While only one end of the root segments showed callusing after 4 days of culture (Table 2, Fig. 1B), there was profuse callusing at both the ends after 8 days.

Development of shoot buds occurred at both the ends after 15 days in all concentrations of Kn, except at 4.64 μM (Fig. 1C). Rather, vitrification was observed at 4.64 μM Kn (Fig. 1D). Maximum numbers of shoot buds were obtained at 3.48 μM Kn after 45 days (Table 3, Fig. 1E). Although, MS basal medium supported callusing, the number of responsive explants with shoot bud formation was low as compared to medium supplemented with Kn (Table 3).

Increase in the number and height was recorded only when the above obtained shoots were sub-cultured on to MS medium containing respective concentrations of Kn. The height of the shoots growing on MS medium containing different concentrations of Kn or MS0 were at par after 15 and 30 days. However, after 45 days on medium containing Kn, there was significant increase in shoot height at all concentrations except 4.64 μM (Table 3). While rooting was initiated after 30 days of growth on MS0, all concentrations of Kn promoted rooting after 45 days. There was a decrease in root number with increasing concentrations of Kn. The highest number of roots (4.63 ± 0.24a) was invariably recorded on MS0 (Table 3).

3.4 Internodal Segments

Callusing was observed at both the cut ends of internodal segments after 12 days of culture on MST (Fig. 1F). When after 15 days, the calli were transferred to MS basal medium containing 1.16 to 4.64 μM Kn, shoot bud formation was recorded within 8-9 days in 100% explants (Table 4, Fig. 1G). The percentage of responsive explants showing shoot regeneration was slightly lower at 4.64 μM Kn and on MS0 (Table 4). On considering the number of shoot buds formed per explant, a maximum of 12.33 shoots were recorded at 1.16 μM Kn after 45 days (Fig 1H). Moreover, there was simultaneous but continuous initiation of shoot buds with increase in shoot height (Fig. 1I). With increasing concentrations of Kn, there was a steady decline in shoot number (Table 5). Shoot bud development was lowest (3.33 in number) on MS0 after 45 days (Table 5).

3.5 Rooting of *In vitro* Raised Microshoots

Shoots (2-3 cm) were transferred to PGR free MS medium (MS0), rooting was observed after 4 weeks in all the shoots (Fig. 2A). Size of the leaf also found to increase in case of MS basal medium.

Table 1. Effect of leaf size on shoot regeneration response

Media	Leaf size (cm)	Leaf surface					
		Abaxial			Adaxial		
		Lower segment	Middle segment	Upper segment	Lower segment	Middle segment	Upper segment
MS0	1.0	30.50 ± 0.64^{d}	0.00 ± 0.00^{l}	20.00±0.47gh	20.75 ± 0.47^{f}	0.00 ± 0.00^{l}	16.50 ± 0.28^{hij}
	1.5	23.50 ±0.95^{gh}	24.50 ± 0.28^{g}	17.50 ± 0.28^{jk}	16.00 ± 0.40^{ij}	20.50 ± 0.28^{f}	13.00 ± 0.40^{k}
	2.0	26.50 ± 0.64^{f}	33.00 ± 0.40^{c}	28.00 ± 0.40^{ef}	16.75±0.47^{ghij}	23.00 ± 0.40^{d}	16.75 ± 0.28^{hij}
	2.5	20.75 ± 0.47^{i}	22.50 ± 0.28^{h}	16.50 ± 0.28^{k}	17.00 ±0.40^{ghi}	21.25 ± 0.62^{f}	13.50 ± 0.64^{k}
MSK	1.0	35.00 ± 0.40^{b}	0.00 ± 0.00^{l}	24.75 ± 0.25^{g}	23.50 ±0.64^{cd}	0.00 ± 0.00^{l}	20.75 ± 0.47^{f}
	1.5	22.75 ± 0.62^{h}	29.50 ± 1.19^{de}	18.25 ± 1.03^{j}	17.75 ±0.47^{gh}	21.50 ± 0.50^{ef}	13.25 ± 0.62^{k}
	2.0	33.00 ± 0.70^{c}	46.25 ± 0.25^{a}	24.75 ± 0.40^{i}	25.75 ± 0.47^{b}	31.25 ± 0.62^{a}	22.75 ± 0.25^{de}
	2.5	27.50 ± 0.64	31.25 ± 0.47^{c}	19.00 ± 0.40^{j}	18.00 ± 0.40^{g}	24.50 ±0.28^{bc}	16.00 ± 0.40^{ij}

The values are mean ± SE; Mean values having different superscript lowercase letters are significantly different according to Duncan's multiple range test at $P \leq 0.05$

Table 2. Regeneration response of root segments on MS medium containing different concentrations of Kn after 15 days of pre-treatment with 0.5 μM TDZ

MS + Kn (μM)	Response		
	4 d	8 d	12 d
0.00	Callusing at one end	Profuse callusing at one end	Callusing at one end
1.16	Callusing at one end	Callusing at both ends	Callusing at both ends
2.23	Callusing at one end	Callusing at both ends	Callusing on entire root segments
3.48	Callusing at one end	Callusing at both ends	Callusing on entire root segments
4.64	Callusing at one end	Vitrification along with callus	Vitrification along with callus

Table 3. Growth of adventitious shoot buds on MS basal medium supplemented with various concentrations of kinetin

Kn (µM)	Days	Response		
		Shoot number	Shoot height (cm)	Root number
0.00	15	3.62 ± 0.13^{i}	0.19 ± 0.00^{e}	0.00 ± 0.00^{e}
	30	4.12 ± 0.31^{h}	0.38 ± 0.03^{d}	2.50 ± 0.29^{b}
	45	4.50 ± 0.00^{h}	1.88 ± 0.13^{c}	4.63 ± 0.24^{a}
1.16	15	5.75 ± 0.14^{g}	0.20 ± 0.00^{e}	0.00 ± 0.00^{e}
	30	9.50 ± 0.29^{e}	0.41 ± 0.03^{d}	0.00 ± 0.00^{e}
	45	12.00 ± 0.41^{d}	2.33 ± 0.06^{b}	2.13 ± 0.13^{b}
2.32	15	8.50 ± 0.20^{f}	0.21 ± 0.00^{e}	0.00 ± 0.00^{e}
	30	12.12 ± 0.43^{d}	0.43 ± 0.04^{d}	0.00 ± 0.00^{e}
	45	13.75 ± 0.14^{c}	2.38 ± 0.13^{b}	1.88 ± 0.31^{c}
3.48	15	13.50 ± 0.20^{c}	0.22 ± 0.00^{e}	0.00 ± 0.00^{e}
	30	14.50 ± 0.20^{b}	0.38 ± 0.03^{d}	0.00 ± 0.00^{e}
	45	17.12 ± 0.31^{a}	3.00 ± 0.13^{a}	1.38 ± 0.24^{c}
4.64	15	5.12 ± 0.31^{gh}	0.21 ± 0.00^{e}	0.00 ± 0.00^{e}
	30	5.12 ± 0.24^{gh}	0.35 ± 0.05^{d}	0.00 ± 0.00^{e}
	45	5.75 ± 0.24^{g}	1.18 ± 0.10^{d}	0.88 ± 0.13^{d}

The values are mean ± SE; Mean values having different superscript lowercase letters are significantly different according to Duncan's multiple range test at P ≤ 0.05

Table 4. Effect of kinetin concentrations on the regeneration response of internodal segments pre-treated with 0.5 µM TDZ for 15 days

Kn (µM)	Responsive explants (%)	Remarks
0.00	84.14	Initiation of indirect shoot buds from both the ends of internodal segments
1.16	100.00	
2.23	100.00	
3.48	100.00	
4.64	85.19	

Table 5. Effect of different concentrations of Kn on shoot regeneration

Kn (µM)	Average number of shoot buds/explant		
	15 d	30 d	45 d
0.00	0.67 ± 0.33^{c}	1.00 ± 0.58^{d}	3.33 ± 0.33^{d}
1.16	5.00 ± 0.58^{a}	8.33 ± 0.33^{a}	12.33 ± 0.88^{a}
2.32	4.33 ± 0.33^{a}	6.33 ± 0.88^{b}	8.66 ± 0.88^{b}
3.48	2.33 ± 0.33^{b}	3.33 ± 0.3^{c}	5.66 ± 0.33^{c}
4.64	1.33 ± 0.33^{bc}	2.33 ± 0.33^{c}	4.32 ± 0.33^{cd}

The values are mean ± SE; Mean values having different superscript lowercase letters are significantly different according to Duncan's multiple range test at P ≤ 0.05

3.6 Hardening of *In vitro* Raised Plants

The survival of plants was 100% under polyhouse conditions. These were healthy under polyhouse conditions (Fig. 2B).

4. DISCUSSION

In vitro cultures of *P. kurroa* have been used in different biotechnological applications, such as micropropagation [11,15,17,18,22], genetic transformation [23,24,25] and for the understanding of picrosides biosynthetic pathway [1,26,27,28]. Present study was aimed at improving the regeneration potential of different explants (leaf, internode and root) of *P. kurroa* using TDZ pretreatment. There are very few reports on regeneration of *P. kurroa* [11,15,16, 17,18,19]. TDZ with its cytokinin like activity [29,30] have also been reported for its usage in *in vitro* regeneration of many other medicinal plants [31,32,33,34,35,36]. In the present work, the regeneration efficiency of leaves, roots and internodal segments has been compared. It can be inferred from the present study that the distinctive regeneration response of different explants (with respect to shoots number) under similar PGR treatment and incubation condition may be the consequence of varied endogenous hormone level in the respective explants. The best response in the form of shoot number was observed in leaf explants (46.25 ± 3.52^{a})

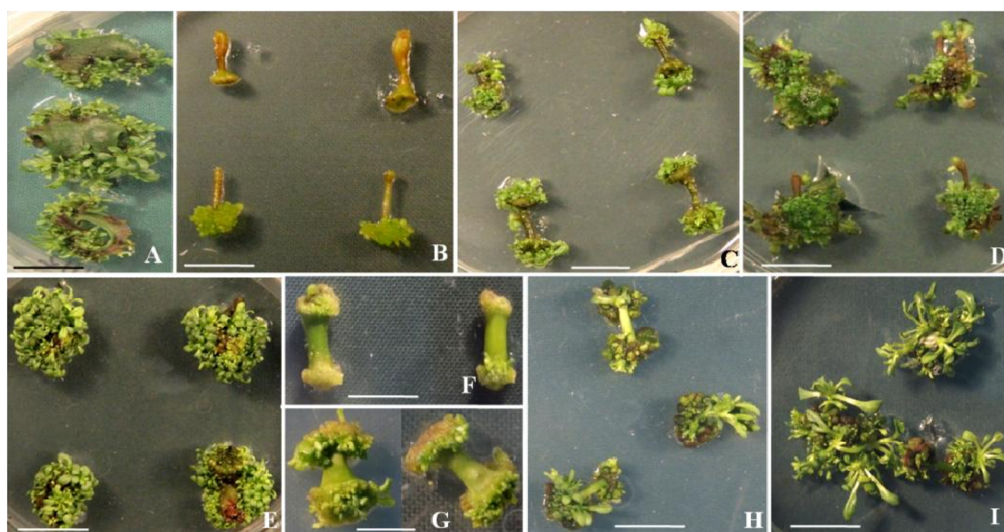

Fig. 1. *In vitro* regeneration from leaf, root and internodal segments: (A) shoot bud regeneration from abaxial surface of the leaf segments on 2.32 µM Kn, (B) callus formation at one end of the root segments after 4 days of transfer to 3.48 µM Kn , (C) shoot bud formation at both the ends of the root segments after 8 days of culture on 3.48 µM Kn, (D) vitrification of shoot buds regenerated from root segments at 4.64 µM Kn after 12 days, (E) Profuse shoot buds development from root segments after 12 days of culture on 3.48 µM Kn, (F) Callus formation at both the cut ends of internodal segments at MST after 12 days, (G) shoot bud initiation on callus within 9 days after transfer to 1.16 µM Kn, (H) growth of developed shoot buds on1.16 µM Kn after 15 days, (I) initiation of profuse shoot buds on 1.16 µM Kn

Fig. 2. *In vitro* rooting of plants and growth in sand under polyhouse after one month of transfer: (A) rooting on MS basal medium, (B) hardened plants growth in sand

followed by root (17.12 ± 0.31[a]) and internodal segments (12.33 ± 0.88[a]). Lal et al. [37] and, Sood and Chauhan [17] had reported highest regeneration frequency of 87% and 70% respectively, from the leaf explants. While 100% regeneration has been achieved from leaf explants in the current study. The increase in the regeneration efficiency and shoots number may be the effect of TDZ pretreatment. Enhancement in the regeneration potential using TDZ was reports in *Vigna mungo* [38], *Cannabis sativa* [39], *Jatropha curcas* [40], *Rhododendron sichotense* Pojark. and *Rhododendron catawbiense* [41] etc. Leaf of 2.0 cm size with abaxial surface in touch with medium showed highest number of shoots (46.25 ± 3.52). Earlier reports say MS medium supplemented with auxin was reported to support *in vitro* rooting

[14,15,17]. In our study, however, 100% rooting was observed after one month in MS0. Thus, it may be concluded that the micropropagation protocol thus developed for *P. kurroa* is a reliable system for mass propagation and hence germplasm conservation.

5. CONCLUSION

The present study reveals the importance of TDZ pretreatment in improving the regeneration ability of different explants of *P. kurroa*. Leaf explants showed better response as compared to root and internodal segments. Leaf size and surface also influence the regeneration response. Leaf explants of 2.0 cm size reported to have the highest regeneration frequency from abaxial surface i.e., 100% with 46.25 ± 3.52[a] shoots/explant. The plantlet regeneration and development method described in the present study can be successfully employed for the conservation and sustainable utilization of *P. kurroa* - an endangered medicinal herb of western Himalayas.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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