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Direct plant regeneration for assisting micropropagation technology of *Picrorhiza kurroa*

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Abstract
Direct regeneration protocol has been optimized for *P. kurroa* using shoot tips as an explant. Shoot tips were established in culture vessels containing Murashige and Skoog (MS) basal media with solid support supplemented with different regimes of plant growth substances. Highest shooting was observed at BAP ($0.5mgl^{-1}$) and Kn($1.0mgl^{-1}$) and this observation was extrapolated as 92.33±2.08 percentage towards shooting response based on average shoot number 24±1.73 and average shoot length 4.35±0.21(cm). Upon recording the data of rhizogenesis, highest rooting was observed at MS media supplemented with 2,4-D ($0.8mgl^{-1}$) and IAA($0.4mgl^{-1}$). This observation data of rooting was extrapolated toward 97±3.60 percentage as a highest rooting response based on average root number 19.33±2.08 and average root length 6.1±0.36(cm). In another study, MS basal medium supplemented with different plant growth substances with different soluble Poly Vinyl Pyrrolidone (PVP) 0-$1.0mgl^{-1}$ concentration was to combat with phenolics. It was found that PVP at ($0.25mgl^{-1}$) concentration has the most suitable effect in order to control adverse effect of phenolics. This study of direct regeneration shall also pave the way for *ex situ* conservation of *P. kurroa* besides, direct regeneration also reduces the possibility of mixing of germplasm during propagation and beneficial for minimizing culture genetic make-up.

Keywords: Regeneration, rhizogenesis, micropropagation, *Ex situ* conservation.

Introduction
India is one of the highest diversifying nations on the globe. Due to their unique climate and variety of ecosystems, the Himalayas constitute an important source of plant and other bioresources and possess an enormous diversity. Micropropagation technology is a major advantage for laboratory to land and vice versa with the advantage to maintain uniform germplasm in laboratory as well as in field to conserve elite plant material and to obtain a benefit of making optimum harvest potential of the particular plant germplasm of threatened categories. Germplasm conservation strategies with special reference for defined herb of scientifically recognized values are ought to be maintained by special maintenance efforts. By using various plant tissue culture techniques, the micropropagation technology carried out under aseptic and favourable conditions on growth media [1].

Sustaining the plant like *Picrorhiza kurroa* shall be reinforced which is compulsory for our resource's conservation. Many of our bioresources are about to extinct including *Picrorhiza kurroa*. However, Himalayan mountains are endowed with varieties of herbs, shrubs and tree as an established medicinal value plants with numerous medicinal properties which are now in high demand as raw materials for pharmaceutical companies. The effort of conservation required due to the overexploitation of plant resources.

Picrorhiza kurroa is one such medicinal herb [2] which is also commonly known as Kutki, these are harvested at an altitude of 3000 to 4500m [3, 4]. *Picrorhiza* species consists of many active constituents such as Picroside I, II, III, IV, V, 6- Ferulocylcatalpol, Veronicoside, Minecoside, Kutkoside, Kutkin, Vanillic acid, Apocynin, Picein, Androsin, 25-Acetoxy-2-β-glucosyloxy-3,16,20-trihydroxy-9-methyl-18-norlanost5,23-dien-22-one [5]. Due to the presence of active constituents having many properties for the treatment of many disorders like liver, upper respiratory tract, chronic diarrhoea, dyspepsia, scorpion sting [6, 7] and properties like choleretic, anti-inflammatory, hypoglycemic, immunomodulatory, cardiovascular, antihepatotoxic, hypolipidemic, antispasmodic, antiviral, purgative, neuritogenic, molluscicidal, anti-phosphodiesterase and leishmanicidal [8-12]. Due to extensive depletion of this plant from its natural habitat it was listed as vulnerable species in Red Data Book [13] and categorized it as endangered species as per IUCN (International Union for Conservation of Nature and Natural Resources) criteria.

Due to these reasons the conservation of this valuable medicinal plant is necessary. *In vitro* propagation is carried out for this species, which has been facing a difficulty in regeneration by conventional methods like vegetative or seed due to various but obvious reasons. Another important use of micropropagation is to conserve the germplasm of threatened plants and for clonally uniform plant regeneration, in areas where destructive harvesting is prevalent which has led to a decrease in population. By making use of various differing *in vitro* culture techniques coupled with micropropagation strategies and thus conserving specific secondary metabolites production in *P. kurroa* has played a pivotal role, in reducing the population depletion of this important medicinal plant in natural habitats. [14-16]. Conventional propagation approaches through rhizomes, and stolons requires more time and large amount of plant parts whereas seeds limit its multiplication due to poor seed germination [17]. Maximum shoot frequency was reported in BAP(1.0mgl^{-1}) + Kn(0.75mgl^{-1}) combination where leaf and stem were used as explant for indirect regeneration [18]**.**The best response showed in the abaxial surface as compared to adaxial surface with respect to regeneration of aseptic culture [19].Callus culture supplemented with 1.0mgl^{-1} BAP, 0.5 mgl^{-1} Kn and 1.0mgl^{-1} GA3 showed highest frequency of shooting whereas highest rooting was observed with 2.5 mgl^{-1} IBA [20].Induction of shoot was achieved in MS media supplemented with 6 µM Kn and 10 µM IAA [21]. Present study was undertaken to develop a protocol for *in vitro* mass multiplication of *P. kurroa*. The present study for direct regeneration of *P. kurroa* from auxiliary buds require lesser time for regeneration of plantlet in *in vitro* condition from field collected plants maintained in germplasm evaluation facility at relatively low light intensity and ambient temperature.

Material and Methods

Collection of plant material

The *P. kurroa* plants were procured from Bageshwar district of Uttarakhand in the month of November /2019 from farmer's planted pots and established in Plant Germplasm Evaluation facility in controlled environment containment facility (25°C, 65.0% RH) at college of Basic Sciences and Humanities, G.B.P.U.A. & T., Pantnagar.

Plant material as an explant and their surface sterilization

Shoot tips were used as explants. Explants were washed to remove soil under running tap water and rinsed with 2-3 drops of Tween 20 under distilled water. Repeated washing of the explants was done with distilled water until the dirt from the explant were removed. After that the explants were treated with 0.1% Bavistin for 15 mins, in order to reduce the fungal load. Again, the explants were washed with distilled water for 2,3 times. The surface sterilization of explant with 0.1% $HgCl_2$ for 35 sec were done followed by washing of the treated explants with autoclaved distilled water for 3-4 time and again surface sterilized with 70% ethanol for 30 sec followed by rinsing of the explants thoroughly rinse with autoclaved distilled water under laminar flow air cabinet. After that the explants were air dried air dry of explant on petri plate in order to avoid any contamination. Now explant is ready for culture.

Inoculation of explant and shoot induction

Explant (1.5 cm) was inoculated on MS medium having 3% sucrose and 0.4% agar with pH ~ 5.8. To avoid browning of the initiated cultures due to phenolics, medium was supplemented with 0.05% PVP (Poly Vinyl Pyrolidine) soluble polymer. The medium was supplemented with different concentration and combination of growth regulators BAP(0-1.0mgl^{-1}) and Kn (0-1.0 mgl^{-1}). After every 15 days sub-culturing of the culture was done for proper growth and development.

Shoot generation

After initial establishment of the aseptic cultures, shoots were allowed to proliferate from the shoot tip at different concentration of hormones containing media BAP, Kn. Sixteen different MS medium combination with growth regulators were tried for shoot multiplication**.** After every 15 days subculturing was done in fresh media with different combinations of growth regulators having BAP (0-1.0 mgl^{-1}), Kn (0-1.0 mgl^{-1}) and IBA (0.5 mgl^{-1}).

Root generation

For root induction, Well developed *in vitro* shoot cultures were implanted into MS media containing different concentration of hormones 2,4-D (0-1.0 mgl^{-1}) and IAA (0-1.0 mgl^{-1}) for induction and development of roots**.**

Culture conditions

Light and temperature is the major environmental factors which affects the tissue differentiation. Cultures grow well within wide range of photoperiod, light intensities, and optimal temperature. All cultures were incubated under 16/8hours light/dark photoperiod provided by fluorescent tubes with light intensity of 27-33.75µ mol sec^{-1} and 25±2 °C temperature and 60-70% humidity.

Bio-hardening and Acclimatization of plantlets

In vitro propagated plantlets were washed carefully before transferring to potting media in order to remove the sticking agar from the roots of the plantlets for avoiding fungal infection. Transplanting of *in vitro* regenerated plantlets were done in plastic pots containing mixture of autoclaved soil: sand: vermiculite (1:1:1) and placed in the Plant Germplasm Evaluation Facility (25°C, 65.0% RH) at College of Basic Science and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar. The pots were covered with polythene bags and irrigated twice a week with ½ MS medium to maintain high humidity.

Result and Discussion

Regeneration of plantlets

Direct organogenesis from shoot tip

Shoot responses, average shoot number and average shoot length were increased with increasing concentration of hormone (0-0.5 mgl^{-1}) but further increasing concentration of hormone (0.75-1.0 mgl^{-1}) decreases shooting responses. The individual shoot tips were transfer to MS medium supplemented with various concentrations of BAP (0.0-1.0 mgl^{-1}) and Kn (0.0-1.0 mgl^{-1}). Among various treatments, when MS medium supplemented with only BAP (1.0 mgl^{-1}) resulted in shooting percentage (30±2.0%) while in presence of only Kn (1.0 mgl^{-1}) resulted in shooting percentage (49.66±2.51%). When BAP and Kn were used in combination the highest shooting percentage (92.33±2.08%) was recorded at BAP (0.5 mgl^{-1}) and Kn (1.0 mgl^{-1}) [Fig.1(a-d), Table 1].

Table 1: Effect of BAP and Kn on the shoot regeneration of *Picrorhiza kurroa* (Mean±SE)

S. No.	Concentration of growth regulators (mgl^{-1})		Response (%)	Average shoot number/ apical meristem	Average shoot length(cm)
	BAP	Kn			
1.	0.0	-	-	-	-
2.	0.25	-	-	-	-
3.	0.5	-	-	-	-
4.	0.75	-	-	-	-
5.	1.0	-	30±2.0	11±1.0	1.3±0.2
6.	-	0.25	-	-	-
7.	-	0.5	-	-	-
8.	-	0.75	42.66±1.52	10.66±1.15	1.46±0.15
9.	-	1.0	49.66±2.51	12.33±1.52	1.96 ±0.20
10.	0.25	0.25	-	-	-
11.	0.5	0.25	23.66±2.51	7±1.0	1.16±0.20
12.	0.75	0.25	53±2.0	15±1.0	1.86±0.25
13.	1.0	0.25	61.66±1.52	18±1.0	2.66±0.15
14.	0.25	0.5	30±2.0	5.66±0.57	1.36±0.23
15.	0.5	0.5	52±2.64	13.33±1.52	2.36±0.15
16.	0.75	0.5	64.33±2.08	19.33±1.15	3.36±0.25
17.	1.0	0.5	80.33±1.52	22±2.0	4.16±0.20
18.	0.25	0.75	52±2.64	16.33±2.08	1.66±0.15
19.	0.5	0.75	74.66±2.08	19 ±2.0	3.3±0.17
20.	0.75	0.75	64.33±1.52	17.33±2.51	3.26±0.23
21.	1.0	0.75	31.33±1.52	7.66±1.52	1.2±0.2
22.	0.25	1.0	49.66±1.52	15.66 ±1.15	2.66±0.15
23.	0.5	1.0	92.33±2.08	24±1.73	4.35±0.21
24.	0.75	1.0	74.66±2.08	21±2.64	3.83±0.20
25.	1.0	1.0	41.66±1.52	9 ±1.0	1.56±0.15

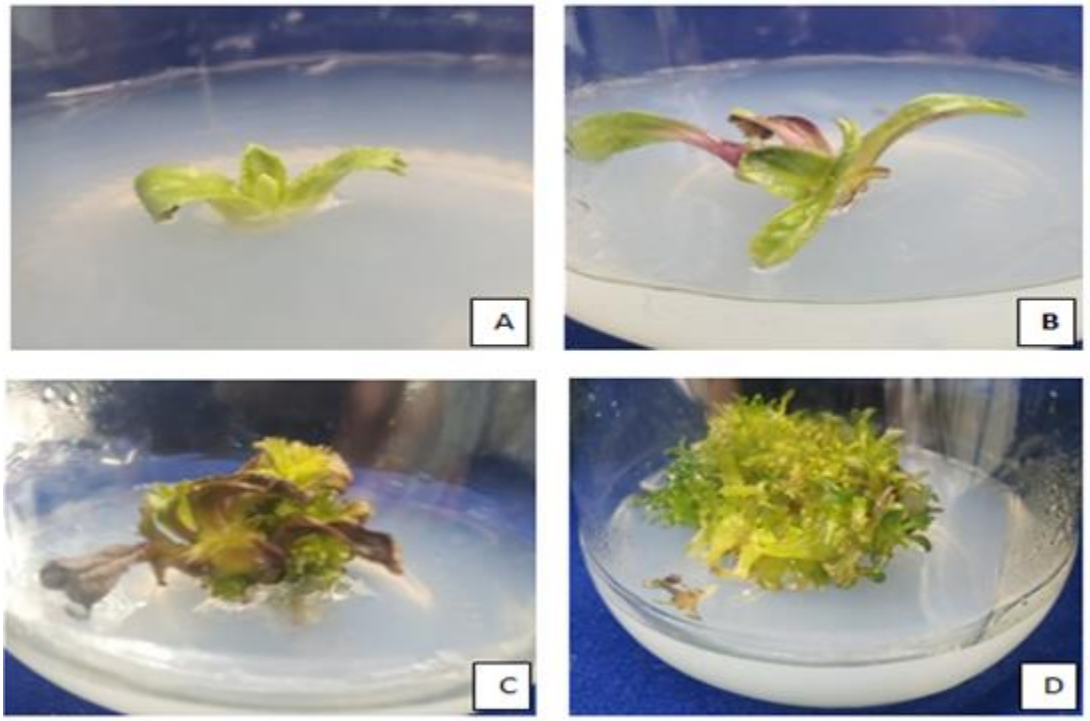

Fig 1: Shoot bud differentiation and regeneration on MS medium containing BAP (0.5 mg l^{-1}) and kinetin (1.0 mg l^{-1}) (A) shooting initiation after 1 week (B) shoot growth after 2 week (C) after 3 week (D)after 6 week of culture

Effect of Polyvinyl pyrrolidone to control the effect of Phenolic oxidation

Explant(1.5cm) were cultured aseptically and transferred to the growth medium at photoperiod of 16/8 hours with 27-33.75 µ mol sec^{-1} light intensity at 25°C temperature. Different concentrations of PVP (0-1.0 mgl^{-1}) were subjected to the MS basal media. Data of survival and death of explants due to phenolic oxidation (browning) were recorded after 20-25 day. It was found that the 0.25 mgl^{-1} concentration of PVP was most suitable concentration to control adverse effect of phenolics (Figure 2 C). The absence of PVP (0.0 mgl^{-1}) on the medium causes browning of callus and same effect seen at concentration of 0.8 mgl^{-1}, 1.0 mgl^{-1}. (Figure 2 (A), (E), (F))

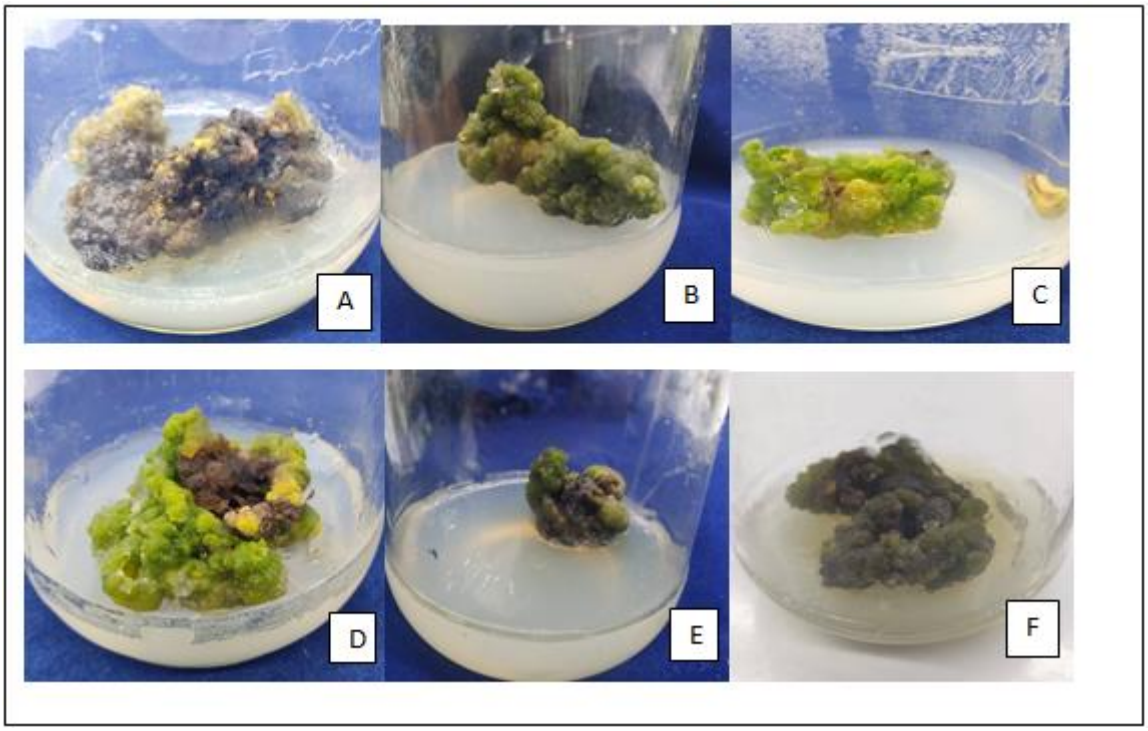

Fig 2: Effect of different concentration of PVP on callus culture (A) 0.0mgl^{-1} (B) 0.1mgl^{-1} (C) 0.25mgl^{-1} (D) 0.5mgl^{-1} (E) 0.8mgl^{-1} (F) 1.0mgl^{-1} PVP treatment

Induction of root from *in vitro* grown shoot

Root regeneration from *in vitro* grown culture when transferred onto MS media supplemented with different combinations of 2,4-D and IAA was observed.

By using different combination and concentration of hormones (2,4-D and IAA) onto media it was found that with the increasing of concentration of hormones (0.0-1.0 mgl^{-1}) the average root number and average root length increased but further increase of concentration decreases its responses. In absence of IAA and increased concentration of 2,4-D (0-1.0 mgl^{-1}), responses seen in 0.6 mgl^{-1} and 1.0 mgl^{-1}. whereas in absence of 2,4-D and increased concentration of IAA (0-1.0 mgl^{-1}) responses seen only in concentration of 0.8 mgl^{-1}. Highest response % (97±3.60), average shoot number (19.33±2.08), average shoot length (6.1±0.36) at 2,4-D (0.8 mgl^{-1}) and IAA (0.4 mgl^{-1}) (Fig.3) further increase of concentration of hormone decreases the responses (Table 2).

Table 2: Effect of 2, 4-D and IAA on rooting of after 45 days of culture (mean ± SE)

S. No.	Concentration of growth regulators(mgl^{-1})		Response (%)	Average root number	Average root length
	2,4-D	IAA			
1	0.0	0.0	-	-	-
2	0.2	-	-	-	-
3	0.4	-	-	-	-
4	0.6	-	42.33±2.08	11±1.0	1.96±0.25
5	0.8	-	-	-	-
6	1.0	-	30.33±1.52	13.33±2.08	1.86±0.15
7	-	0.2	-	-	-
8	-	0.4	-	-	-
9	-	0.6	-	-	-
10	-	0.8	35.66±2.30	11±1.0	1.8±0.2
11	-	1.0	0	0	0
12	0.2	0.2	21.33±2.0	6±1.0	1.53±0.15
13	0.4	0.2	43.66±1.52	11.66±1.15	2.96±0.20
14	0.6	0.2	55.33±2.08	13.33±1.52	3±0.20
15	0.8	0.2	35.66±2.51	8.66±0.577	2.13±0.15
16	1.0	0.2	-	-	-
17	0.2	0.4	42±1.73	6±1.73	3.23±0.32
18	0.4	0.4	51.66±1.52	12.66±2.51	4.23±0.25
19	0.6	0.4	71.66±2.08	23±1.41	4.73±0.20
20	0.8	0.4	97±3.60	19.33±2.08	6.1±0.36
21	1.0	0.4	0	0	0
22	0.2	0.6	64.66±4.16	15.33±1.52	4.93±0.20
23	0.4	0.6	-	-	-
24	0.6	0.6	94.33±4.93	22.66±2.51	6.53±0.25
25	0.8	0.6	61.66±3.21	18.33±1.52	4.66±0.15

26	1.0	0.6	41.33±3.78	11±2.64	3.43±0.20
27	0.2	0.8	69.66±1.52	18.33±3.05	5±0.3
28	0.4	0.8	55±3.0	11.66±1.52	3.03±0.25
29	0.6	0.8	46.33±3.78	7±1.0	2.53±0.15
30	0.8	0.8	32.33±3.21	6.66±1.52	2±0.2
31	1.0	0.8	18.66±1.52	4.66±0.57	1.03±0.25
32	0.2	1.0	0	0	0
33	0.4	1.0	48±4.58	12.66±2.51	4.4±0.26
34	0.6	1.0	0	0	0
35	0.8	1.0	34±1.0	5±1.0	1.7±0.2
36	1.0	1.0	24±1.73	6.33±2.08	1.2±0.1

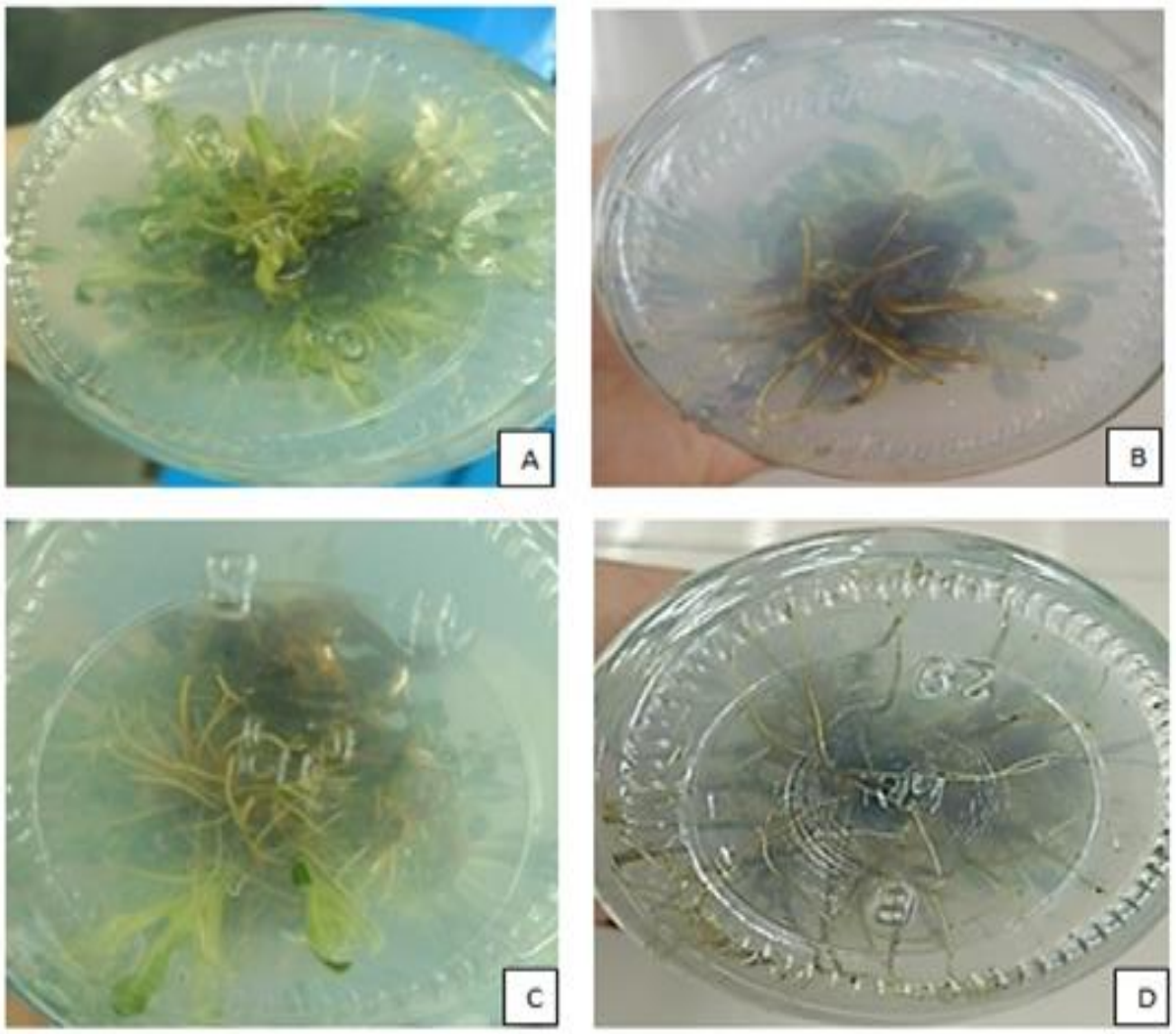

Fig 3a: Profuse rooting induced on MS medium containing 2,4-D (0.8 mgl^{-1}) and IAA (0.4 mgl^{-1}) (A) Root initiation in between of 3 week (B) growth of root after 3 week (C) after 4 week (D) after 6 week

Hardening and Acclimatization

After the plantlets formation in *in vitro* grown condition these were removed gently from the jam bottles, and transferred to the pots containing soil: sand: vermiculite (1:1:1) ratio in the greenhouse conditions having temperature 25°C and 65.0% relative humidity for hardening and acclimatization (Fig.3(B)).

Fig 3b: (A) Uprooted tissue culture raised plantlets (B) Potted tissue culture-raised plantlets

Discussion

In order to obtain sustained benefits of this important germplasm, *in vitro* growth and development have been undertaken at our laboratory. As micropropagation has been regarded as one of prime importance in plant propagation aseptically thus, direct regeneration has been attempted for this particular plant where shoot tips (explants) of varying sizes were inoculated. *In vitro* propagation of this explants of *P. kurroa* shall in turn help in establishing regeneration strategies of this plant species, which is facing difficulty of survival due to excessive harvest from the nature however, regeneration by conventional vegetative methods is in practice which requires as such large portion of germ plasm for seeding itself. Micropropagation technology is a major advantage for uniform maintenance of germplasm of elite nature in laboratory, green house as well as in field to conserve and make optimum harvest of the potential of this particular germplasm, which is presently under threatened categories of plants. This study of direct regeneration shall also pave the way for *ex situ* conservation of *P. kurroa.* The study conducted by us for direct regeneration showed highest shooting and rooting at 0.5 mg/l BAP, 1.0mg/l Kn and 0.8mg/l 2,4-D, 0.4mg/l IAA concentration vice versa. Micropropagation technology for *P. Kurroa* used by [22] for proliferation of shoots on MS medium supplemented with 2 mg/L IBA and 3 mg/L Kn. Nodal segment is used as an explant to regenerate plantlets [23]. [24] revealed that GA3 down regulate the biosynthesis of picrosides. Data of maximum root generation frequency recorded at 2, 4-D (0.5 mgl^{-1}) and NAA (0.4 mgl^{-1}) with an average percentage of 86.33±9.53 with an average number of roots 8.0±0.0 in indirect regeneration of *P. kurroa* established in cultured vessel containing MS medium [25]. Previous studies reported that, MS medium supplemented with Kn(3 mgl^{-1}), IBA (2 mgl^{-1}) shows good shooting response [26]. MS medium with IBA (2 mgl^{-1}), Kn(3 mgl^{-1}), sucrose 3% with agar (0.8%) was the medium with better results for shooting about 86.3% [27]. Some result indicates that Kn(2 mgl^{-1}) with IBA(0.5 mgl^{-1}) shows best shooting whereas best rooting result found when MS medium supplemented with IBA (1.0 mgl^{-1}).Through direct proliferation shows 99.94% response [28]. Rooting result of 100% when medium supplemented with 0.22 mgl^{-1} IBA [29]. Gamborg's B5 medium supplemented with 3 mgl^{-1} Kn and IBA (1 mgl^{-1}) induce multiple shooting with 94% frequency [30]. Multiple proliferation of shoots from the explants of *P. kurroa* when MS medium supplemented with Kn (3.0 to 5.0 mgl^{-1}) and IAA (1.0 mgl^{-1}) showed of shoots [31]. Our observations established a protocol for direct regeneration from shoot tip and directly regenerated plantlets are adjusted for hardening. Further such studies are quite important as they provide rapid and large-scale propagation protocols if not completely then partially.

Conclusion

The present study reveals the precise usages of established plant growth regulators for desired growth and development from auxiliary shoot tip taken as an explant. For the regeneration of plantlets this method is sufficient for successful conservation efforts up to fair extent of time period in culture conditions for *Picrorhiza kurroa*. Well developed *in vitro* methods are being used for the conservation and protection of over exploited plants due to various medicinal properties as well as bioactive constituents possessed by other plant resources. Our current plan of studies established a fairly worthy protocols to assist mass multiplication of this plant by providing suitably hardened germ plasm to the marginal farmers of Himalayan terrains of desired altitude with package of practice for cultivation in a small area for further development of seeding elite material and optimum harvest as well as to cater the need of pharmaceutical industries with authentic supply of raw materials.

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