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Contributed Paper

# Improved Micropropagation Protocol of an Endangered Medicinal Plant-*Picrorhiza Kurroa* Royle ex Benth. Promptly Through Auxin Treatments

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## ABSTRACT

An efficient and rapid protocol for mass propagation of *Picrorhiza kurroa* Royle ex Benth - an endangered medicinal plant- *in vitro* without using cytokinin has been developed. The nodal explants were obtained from in *vitro* raised seedlings grown in moist cotton raised from chill treated seeds (4°C) and *in vivo* matured plants in natural conditions respectively. Both types of genesis, direct and indirect, of shoot and root in this species using single type of hormones viz; auxins with MS basal medium is being reported for the first time. Among the various strengths of growth regulators tested best results were recorded when nodal explants from natural plants were cultured on MS basal media supplemented with 0.25 $mg^{-l}$ 2,4-D $mgl^{-l}$, 0.25 $mgl^{-l}$ BAP ,0.2 $mgl^{-l}$ NAA and 0.6 $mgl^{-l}$ NAA for profuse callusing, shoot induction, indirect and direct rapid shoot proliferation respectively. Combination treatments of auxins were also testified but the results were not satisfactory as that of NAA alone. The rooting was also optimized using auxins alone or/and in combinations of two. The root induction per explants was maximum in the medium containing MS basal medium supplemented with 0.4 $mgl^{-l}$ NAA. The rooted plantlets were hardened in polycups containing sterile soil, sand and vermiculite in equal ratio and were successfully acclimatized and established in soil with 81.5 % survival rate.

**Keywords:** chill treatment, cytokinin free, micropropagation and *Picrorhiza kurroa.*

## 1. INTRODUCTION

*Picrorhiza kurroa* Royle ex. Benth commonly known as Kour, a fast depleting medicinal value plant belonging to family Scrophulairaceae. It is endemic and grows in inner ranges of alpine Himalayas, from Kashmir to Sikkim, of Indian state at an altitude of 3,000-5,000 feet above sea level. It is the principle source of glycosides that is Picroside-I, Picroside-II and Kutkoside [1,2]. Other identified active constituents are

apocynin, drosin, and nine cucurbitacin glycosides [3,4]. The active constituents are obtained from the dried root and rhizome. It finds its place in traditional as well as in modern system of medicine as purgative, antiperiodic, brain tonic, stomachic, dyspepsia and fever [5]. Current research has focused on its hepato-protective, anticholestic antioxidant and immune modulating activity [6,7].

The natural resurgence of *Picrorhiza kurroa* is through rhizomes and seeds, however their cultivation rate is very poor. The poor cultivation coupled with over exploitation for pharmaceutical use has depleted the species from natural habitat. The Indian system of medicine is predominantly dependant upon the use of plant based raw materials in most of their preparations and formulations, there by, widening the gap between demand and supply and thus putting further pressure on the species. Infact *P. kurroa* is now listed as one of the endangered plant species of India [8]. Owing to these factors, the species is at the verge of extinction. It is essential for the conservation of *P. kurroa* to encourage *ex-situ* plantation which require large scale planting material. In view of the problems of conventional propagation and high demand of planting material the large scale multiplication of this species can only be met efficiently and economically in a sort span of time by *in vitro* propagation. Therefore, an efficient *in vitro* propagation system for producing this plant is required to further clarify its potential medicinal values and germplasm conservation. Plant regeneration from *P. kurroa* has earlier been reported using shoot tips [9]. However some of the serious limitations in the above mentioned protocols were inconsistent under temperate conditions i.e*., in vitro* responses of various culture media in various ecozones. Therefore the present study has been carried out to ascertain the most appropriate basal culture and growth hormones for efficient *in vitro* regeneration under temperate conditions. Our findings are at variance from the earlier report in tissue culture of this species [10].

## 2. MATERIALS AND METHODOLOGY

### 2.1 Raising of Seedling

The seeds of *Picrorhiza kurroa* were .procured from Regional Research Laboratory (RRL) field stations, Yarikah (District, Budgam) and Bonera (District, Pulwama) at J&K, India and were surface sterilized by number of consecutive chemical treatments started with 4-5 washing by double distilled water, treatment with fungicide (bebeston), and teepol for 5 minutes respectively. Under laminar air flow seeds were washed 3-4 times with sterilized distilled water and then immersed in 70% alcohol for 30 seconds, followed by treatment with 1% (w/v) aqueous mercuric chloride for 3 minutes. The sterilized seeds were imbibed for 15 days at different temperatures viz; $0^{o}$C, $2^{o}$C ,$4^{o}$C ,$6^{o}$C ,$8^{o}$C and $10^{o}$C. Treated seeds were kept in dark at $20\pm^{o}$C on moist absorbent cotton placed in the petri plates and there germination was monitored on regular intervals. Two week following seed germination under in *vitro* conditions ($20\pm1^{o}$C) nodal explants from germinated seeds and from matured plants in natural conditions were excised and inoculated in a group of ten in culture tubes (Borosil) containing 20 ml of semisolid MS, [11], medium supplemented with or without different concentrations of BAP (0.1 $mgl^{-1}$ , 0.25 $mgl^{-1}$ and 0.5 $mgl^{-1}$) and 2,4-D (0.1 $mgl^{-1}$, 0.25 $mgl^{-1}$ and 0.5 $mgl^{-1}$). The hormone free media were kept as control.

### 2.2 Sub-Culturing of Nodal Explants and callus with Induced Buds for Shoot Regeneration.

The nodal explant segments and callus with induced buds from 0.25 $mgl^{-1}$ BAP and

2,4-D treated media respectively were excised after 10-12 days and sub-cultured thrice in the same media for large regeneration under aseptic conditions.

### 2.3 *In Vitro* Shoot Enlargement and Multiplication.

*In vitro* raised and regenerated callus with induced shoot buds and nodal segments functions as a propagules for *in vitro* shoot growth and multiplication. Different concentrations of NAA (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 $mgl^{-1}$) alone were used for shoot formation and multiplication and for both types of organogenesis. NAA 0.6 $mgl^{-1}$ in combination with three concentrations (0.1, 0.25 and 0.5 $mgl^{-1}$) of IAA and IBA separately were also used for the purpose. At the end of three week sub-culturing the number of shoots were counted.

### 2.4 *In Vitro* Root Induction, Enlargement and Multiplication

The shoot tips measuring 05 - 1 cm in length were used in an experimental attempt to produce roots on the MS medium (control) and on MS medium supplemented with different concentrations of auxins viz., NAA (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 $mgl^{-1}$) individually and investigate their effects. Like shoot multiplication, NAA 0.4 $mgl^{-1}$ in combination with three concentrations (0.1, 0.25 and 0.5 $mgl^{-1}$) of IAA and IBA separately were also used for the purpose. The percentage of rooting, number of roots per shoot tip and total root length (cm) were recorded. The hardening and acclimatization of *in vitro* raised plantlets of *Picrorhiza kurroa* were done by a method adopted by Jabeen *et al.* [12] for *Inula racemosa.*

### 2.5 Cultural Conditions and Statistical Analysis

The MS medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Mumbai, India) was used in all experiments The pH of the medium was adjusted to 5.8 $\pm$ 0.03 prior to autoclaving for 20 minutes at 120°C. Ten seeds was inoculated per petri plate containing moist cotton for initiating the *in vitro* seedling and each explants *in vitro* raised were sub-cultured in a 25 x 150 mm glass tube containing 20 ml semisolid medium for shoot multiplication or rooting and its multiplication. The cultures were incubated at a temperature of 25 $\pm$ 3°C under 17 hour daily illumination with white fluorescent light. Ten replicates of each treatment were made and studied and the experiment was laid both on systematic and randomized pattern. The data was statistically analyzed using analysis of variance (ANOVA) and the least significant difference (LSD) values were calculated at P = 0.05 for comparing means of the treatments [13].

## 3. RESULTS AND DISCUSSION

### 3.1 Seed germination

The germination of seeds of *P. Kurroa* seems very difficult under natural conditions and it requires cold temperature for germination. In the present work the germination was observed in all seeds, treated with different temperatures, in petriplates and maximum (93%) was observed in seeds at 4°C (Cold treatment; 5-10 days) on absorbent cotton provided with 16 hours photoperiod and occurs in 5-10 days *in vitro* as compared to six months under *in vivo* conditions (Figure 2A). Other treatments were showing less response as compared to 4°C (Figure 1). This suggests that lower temperature had some effect on the germination rate and could be altering the functions of cold-shock proteins as has been earlier reported by Jones [14] The cold shocking proteins is primarily being used by the plant species in artic and temperate region for tolerating the severe cold conditions [15]. Kashmir being the temperate region and this particular plant has been adopted to grow at lower temperature in and around 4°C as

depicted from the results.

**3.2 Shoot regeneration and multiplication**

Nodal explants were excised aseptically from the seedlings as well from natural plants condition and cultured on different phytoharmonal regimes (BAP and 2, 4-D). Former explant did not show any significant result as compared to the later one, the effect of which is depicted in Table 1. Callus formation was induced by all concentrations of 2, 4-D and direct regeneration was found in all concentrations of BAP during the study, although the rate was not high. The medium

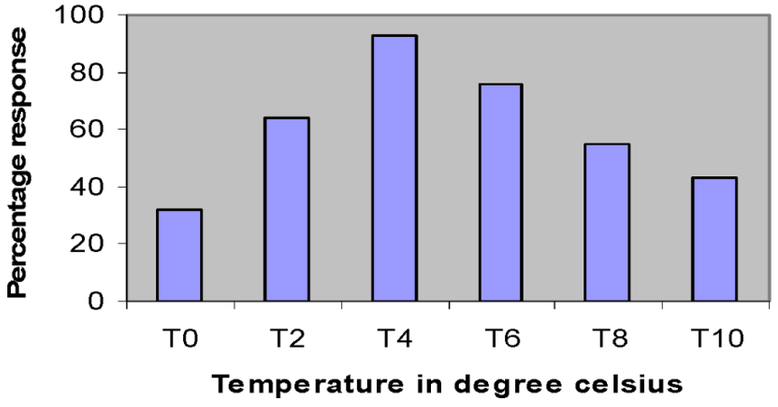


**Figure 1.** Effect of Temperature on Seedling Growth.

**Table 1.** Effect of BAP and 2, 4-D on shoot induction and regeneration.

| Medium | R | B | D | S | S1 | S2 | S3 | L |
|---|---|---|---|---|---|---|---|---|
| MS(Control) | 10 | $3.4^{c}\pm0.91$ | $12\pm2$ | $1.2^{b}\pm0.54$ | $3.4^{b}\pm0.55$ | $5.3^{b}\pm1.23$ | $9.3^{b}\pm1.17$ | $1.1^{a}\pm0.58$ |
| MS+0.1 BAP | 35 | $10.1^{b}\pm1.70$ | $11\pm3$ | $3.4^{b}\pm0.45$ | $5.0^{b}\pm1.18$ | $7.4^{b}\pm1.84$ | $10.0^{b}\pm1.64$ | $1.9^{a}\pm0.58$ |
| MS+0.25 BAP | 55 | $25.0^{a}\pm3.96$ | $10\pm2$ | $8.5^{a}\pm1.84$ | $10.3^{a}\pm2.93$ | $13.4^{a}\pm0.93$ | $16.4^{a}\pm1.58$ | $2.0^{a}\pm0.56$ |
| MS+0.5 BAP | 43 | $16.3^{b}\pm3.59$ | $13\pm3$ | $2.0^{b}\pm0.58$ | $3.2^{b}\pm0.76$ | $7.3^{b}\pm1.17$ | $9.4^{b}\pm0.95$ | $1.9^{a}\pm0.72$ |
| C.D=0.05 | | 6.21 | | 2.24 | 3.59 | 2.90 | 2.96 | 1.32 |
| MS+0.1 2,4-D | 10 | $2.6^{a}\pm0.71$ | - | + + | + + | + | + + | - |
| MS+0.25 2,4-D | 20 | $3.5^{a}\pm0.97$ | 20 | 1.5,+ | + + + + | + + + | + + + | - |
| MS+0.5 2,4-D | 10 | $3.4^{a}\pm0.89$ | - | + | + + | + + | + | - |
| C.D=0.05 | | 1.74 | | | | | | |

Key to table: R=% Response; B= Average number of shoot buds per explant; D= Average number of days for shoot induction;S= Average number of shoots formed; S1= Average numer of shoots after Ist sub culture;S2= Average number of shoots after 2nd sub culture; S3= Average nuber of shoots after 3rd sub culture; L= Average number of shoot length; Plus sign(+) indicate the callusing pattern more plus means good and profuse callusing. (Values with same superscript letters are insignificant at $p>0.05$).

of MS+0.25 $mgl^{-1}$ 2,4-D and MS+0.25 $mgl^{-1}$ BAP stimulated callus induction rate in 20% explants (Figure 2B) and direct shoot regeneration rate in 55 % explants respectively (Table 1). Similar type of reports was found by Hatano *et al.* [16] for *Aconitum carmichaeli* Debx. and by Qadri *et al.* [17] for *Atropa acuminate* Royle. Contrary to Lal *et al.* [18] reporting *in vitro* plant multiplication from callus in 2, 4-D supplemented media however in the present study NAA proved to most effective. Indirect and direct adventitious shoot regeneration, multiplication as well as their elongation were evaluated on MS medium using various concentrations of NAA (0.2, 0.4, 0.6, 0.8 and 1.0 $mgl^{-1}$) alone or in combination with IAA and IBA (Tables 2 and 3) within 10 week culture. Earlier, Upadhayay *et al.* [10] reported *in vitro* propagation by using BAP, but in the present study cytokinin has not been proven effective. These variations in the response of *P. kurroa* could be due to ecotypic differences [19]. The average shoot number 18.4±1.10 per explant for indirect organogenesis was observed on 0.2 $mgl^{-1}$ NAA with 80 % response and 18.3±0.57 per explant for direct organogenesis was observed on 0.6 $mgl^{-1}$ NAA (Figure 2C) with 95% response. These results are very much in conformity with the observations of Qadri *et al.* [17]; Ahuja *et al.* [20] and Zarate *et al.* [21]. Callus regeneration was observed at the basal cut ends of each explant which is in agreement with Toth *et al* [22] in *A. belladonna*. The combination effect of IAA and IBA with NAA on nodal segments also promoted direct and indirect shoot multiplication and elongation. However for indirect regeneration

**Table 2.** Effect of NAA alone or in combination with IAA and IBA on indirect shoot proliferation and multiplication.

| Medium | %response | Avg. no.of Shoots/explant | Callus %tage | Shoot length |
|---|---|---|---|---|
| MS(control) | 10 | $3.3^{e}$±0.76 | 2 | $1.1^{c}$±0.44 |
| MS+ 0.2 NAA | 80 | $18.4^{cd}$±1.10 | 43 | $6.8^{b}$±0.72 |
| MS+ 0.4 NAA | 76 | $15.3^{bc}$±0.27 | 28 | $5.8^{a}$±0.89 |
| MS+ 0.6 NAA | 70 | $13.4^{a}$±0.57 | 39 | $5.8^{a}$±0.50 |
| MS+ 0.8 NAA | 80 | $13.6^{b}$±0.67 | 19 | $4.5^{a}$±0.76 |
| MS+ 1.0 NAA | 63 | $10.8^{d}$±0.95 | 14 | $4.3^{b}$+±0.62 |
| C.D=0.05 | | 1.70 | | 1.42 |
| Combination | | | | |
| MS+ 0.2 NAA+0.1 IAA | 14 | $4.2^{a}$±0.64 | 22 | $1.5^{a}$±0.34 |
| MS+ 0.2 NAA+0.25 IAA | 13 | $5.5^{a}$±1.35 | 31 | $1.9^{a}$±0.42 |
| MS+ 0.2NAA+0.5 IAA | 10 | $4.8^{a}$±1.73 | 23 | $2.8^{a}$±0.89 |
| MS+ 0.2NAA+0.1 IBA | 9 | $2.3^{a}$±0.83 | 24 | $2.8^{a}$±0.46 |
| MS+ 0.2NAA+0.25 IBA | 10 | $5.2^{a}$±0.62 | 25 | $1.1^{a}$±0.58 |
| MS+ 0.2NAA+0.5 IBA | 12 | $2.6^{a}$±1.26 | 34 | $2.7^{a}$±0.59 |
| C.D.=0.05 | | 2.3 | | 1.04 |

The units of lenght are in centimeters (cm), (Values with same superscript letters are insignificant at $p>0.05$).

**Table 3.** Effect of NAA alone or in combination with IAA and IBA on direct shoot proliferation and multiplication.

| Medium | %response | Avg. no.of Shoots/explant | Callus %tage | Shoot length |
|---|---|---|---|---|
| MS(control) | 10 | $3.3^{e}\pm0.76$ | 2 | $1.1^{c}\pm0.44$ |
| MS+ 0.2 NAA | 60 | $10.4^{cd}\pm1.10$ | 30 | $3.2^{b}\pm0.72$ |
| MS+ 0.4 NAA | 76 | $11.8^{bc}\pm0.27$ | 25 | $5.8^{a}\pm0.89$ |
| MS+ 0.6 NAA | 95 | $18.3^{a}\pm0.57$ | 40 | $5.6^{a}\pm0.50$ |
| MS+ 0.8 NAA | 80 | $13.2^{b}\pm0.67$ | 11 | $5.3^{a}\pm0.76$ |
| MS+ 1.0 NAA | 73 | $9.4^{d}\pm0.95$ | 4 | $3.3^{b}+\pm0.62$ |
| C.D=0.05 | | 1.60 | | 1.39 |
| Combination | | | | |
| MS+ 0.6 NAA+0.1 IAA | 10 | $3.2^{a}\pm0.64$ | 2 | $1.4^{a}\pm0.34$ |
| MS+ 0.6 NAA+0.25 IAA | 12 | $4.5^{a}\pm1.35$ | 3 | $2.0^{a}\pm0.42$ |
| MS+ 0.6 NAA+0.5 IAA | 9 | $4.8^{a}\pm1.73$ | 2 | $1.3^{a}\pm0.89$ |
| MS+ 0.6 NAA+0.1 IBA | 11 | $3.3^{a}\pm0.83$ | 2 | $1.8^{a}\pm0.46$ |
| MS+ 0.6 NAA+0.25 IBA | 10 | $4.2^{a}\pm0.62$ | 2 | $2.1^{a}\pm0.58$ |
| MS+ 0.6 NAA+0.5 IBA | 11 | $5.6^{a}\pm1.26$ | 3 | $2.1^{a}\pm0.59$ |
| C.D.=005 | | 2.29 | | 1.14 |

The units of lenght are in centimeters (cm), (Values with same superscript letters are insignificant at $p>0.05$).

average shoot numbers $4.2\pm0.64$ per explant was observed on NAA(0.2 $mgl^{-1}$) + IAA (0.1 $mgl^{-1}$) with 14% response (Table 2) and for direct regeneration the number was $5.6\pm1.26$ per explant on NAA(0.6 $mgl^{-1}$) + IBA (0.5 $mgl^{-1}$) with 11% response (Table 3). Such findings are strongly supported by those of Ahuja *et al* [20] and Jabeen *et al* [12].

### 3.3 Rooting *in vitro* and Hardening

Although *P. kurroa* showed spontaneous rooting, the addition of auxin in the rooting medium facilitated higher rooting quality than PGR-free medium in terms of root length and number (Table 4). Direct root initiation and elongation was observed after sub culturing of isolated, elongated shoots onto MS basal medium supplemented with different concentrations of NAA (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 $mgl^{-1}$) alone as well as in combination with IAA and IBA (0.1,0.25 and 0.5 $mgl^{-1}$ each) within 4 weeks of their culture period. The rooting response of shoots was 92% (Figure 2D) in MS+0.4 $mgl^{-1}$ NAA. The primary roots become visible after 7 days of culturing, however. Long thin multiple roots were achieved after 4 weeks. Similar results were reported for many species when NAA were being used and was clearly the best among the auxins tested alone or in combination with other auxins. Each explant developed an average of $17.2\pm0.53$ roots with root and shoot length $11.3\pm0.45$ and $20.5\pm0.57$ respectively in MS+ 0.4 $mgl^{-1}$ NAA media with an average response of 92% (Table 4). *In vitro* plantlets were complete with

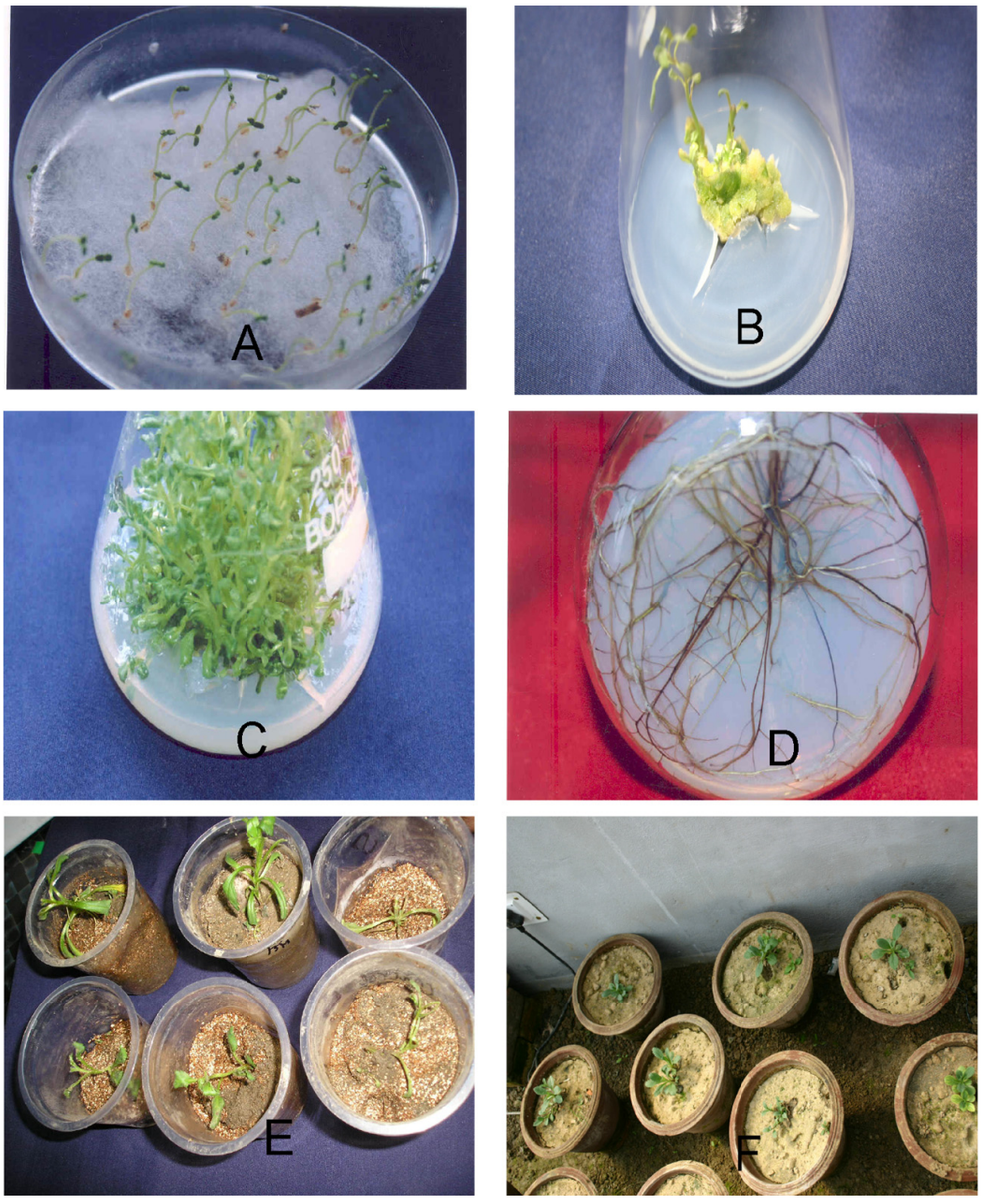


**Figure 2. A.** Germination from seeds; **B.** Shoot induction and regeneration from callus; **C.** Shoot proliferation in 0.4 $mg^{-1}$ NAA. **D.** Rooted plantlets; **E.** Hardening of plants; **Figure F.** Field transfer.

shoot as well as root after four weeks in culture. The complete plantlets, thus formed hardened in polycups (Figure 2E) containing sterile soil, sand and vermiculite (1:1:1) and transferred to pots where 81.5% plants survived successfully (Figure 2F).

**Table 4.** Effect of NAA alone or in combination with IAA and IBA on root proliferation and multiplication.

| Medium | %response | Days to root induction | No.of root/ explant | Root length | Shoot length | Callusing |
|---|---|---|---|---|---|---|
| MS(control) | - | - | - | - | $3.3^{c}\pm0.53$ | + + |
| MS+ 0.2 NAA | 83 | 22±3 | $13.5^{b}\pm1.2$ | $9.5^{a}\pm1.22$ | $16.2^{b}\pm0.59$ | + |
| MS+ 0.4 NAA | 92 | 18±2 | $17.2^{a}\pm0.53$ | $11.3^{a}\pm0.45$ | $20.5^{a}\pm0.57$ | + |
| MS+ 0.6 NAA | 80 | 13±2 | $14.4^{b}\pm0.96$ | $4.5^{c}\pm0.93$ | $21.4^{a}\pm0.57$ | + |
| MS+ 0.8 NAA | 30 | 15±3 | $12.6^{b}\pm1.27$ | $5.3^{c}\pm0.68$ | $9.2^{c}\pm0.52$ | + + |
| MS+ 1.0 NAA | 20 | 25±3 | $7.2^{c}\pm0.55$ | $1.8^{d}\pm1.6$ | $4.6^{d}\pm0.58$ | + |
| C.D=0.05 | | | 1.81 | 2.01 | 1.17 | |
| Combination | | | | | | |
| MS+ 0.4 NAA+0.1 IAA | 72 | 23±3 | 11.3b±0.75 | 7.5a±1.02 | 13.3b±0.57 | + |
| MS+ 0.4 NAA+0.25 IAA | 63 | 23±4 | 13.4a±0.87 | 2.3b±0.88 | 12.5b±0.67 | + + |
| MS+ 0.4 NAA+0.5 IAA | 70 | 24±3 | 14.1a±0.53 | 4.5b±1.22 | 12.1b±0.47 | + |
| MS+ 0.4 NAA+0.1 IBA | 65 | 22±2 | 11.4b±0.78 | 3.6b±0.84 | 14.2a±0.49 | + |
| MS+ 0.4 NAA+0.25 IBA | 70 | 21±2 | 12.5ab±1.10 | 8.2a±0.49 | 15.5a±0.85 | - |
| MS+ 0.4 NAA+0.5 IBA | 70 | 22±3 | 13.6a±1.32 | 3.6b±0.58 | 16.2a±0.49 | - |
| C.D.=0.05 | | | 1.80 | 1.69 | 1.25 | |

The units of lenght are in centimeters (cm), (Values with same superscript letters are insignificant at $p>0.05$).

## 4. CONCLUSIONS

*In vitro* requirement is an efficient means of *ex situ* conservation of plant diversity and it assists in sustainable maintenance of the present day dwindling germplasm on long term basis, especially for medicinal plant**.** The study established the *in vitro* propagation system of *P. kurroa* and high proliferation rate was achieved with uniform and vigorous growth in the field. . In comparison to the results of Upadhyay [10] this method achieved a proliferation rate of approximately, 15-19 folds (based on the nodes per explant) or 3-4 folds (based on shoots per explant) per month. This high proliferation rate could only be achieved using micro propagation rather than the traditional methods because; at present the rate of propagation in nature is far less than the rate of exploration. The protocol described here could be used for large scale propagation of this valuable, endangered medicinal plant. The suitability of this efficient regeneration protocol for genetic transformation will be investigated for biochemical analysis.

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