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"RAPID *IN-VITRO* REGENERATION OF AN ENDANGERED MEDICINAL PLANT SARPAGANDHA *(RAUVOLFIA SERPENTINA* L.)"

Runa Rashmi* and Dr. M. P. Trivedi

Department of Botany and Biotechnology, Patna Science College, Patna University, Patna, Bihar-800001, India.

***Corresponding Author: Runa Rashmi**

Department of Botany and Biotechnology, Patna Science College, Patna University, Patna, Bihar-800001, India.

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ABSTRACT

Rauvolfia serpentina (L.) is commonly known as Sarpagandha is an important medicinal shrub of family Apocynaceae. It comprises of total 80 species. Explants of an alkaloid producing plant cultured *in vitro* and has been found to retain the capacity to synthesis alkaloids identical to that in the intact plant. Alkaloids of these plants have a great medicinal importance to treat cardiovascular diseases, hypertension, diabetes, malaria, menorrhagia, cancer, etc. The members are wild as well as cultivated with milky saps that contain secondary metabolites which have medicinal value. In view of these facts the study was conducted for micropropagation of *Rauvolfia serpentina.* MS media supplemented with different concentrations (0.5–10.0 mg/l) of NAA, 2, 4-D, BAP and KIN were used singly and in combination. Among all the growth hormones 2, 4-D was the best for callus induction (93% in stem and 97% in leaf) and in combination 2, 4-D and BAP (85% in stem and 95% in leaf). Day of callus induction started from 13th to 37th day. This variation is due to the differences in culture conditions and the age of explants. In single combination BAP was the best for shooting (77%) and in double combinations BAP (1.5mg/l) and NAA (0.5mg/l) were the best for shooting (80%). Higher induction of root (85%) was observed at NAA (2mg/l) and in double combinations BAP (1.5mg/l) and NAA (0.5mg/l) were the best for rooting (87%). Regenerated plants after hardenings were transferred to soil and they showed 77% survival. The protocol was optimized by manipulations of different plant hormones for enhanced multiplication. Protocol explained here provides a rapid plant regeneration system which could be used for the commercial purposes.

KEYWORDS: *Rauvolfia serpentina*, callus culture, micropropagation, plant growth regulators.

INTRODUCTION

Rauvolfia serpentina (L.) is commonly known as sarpagandha is an important medicinal shrub of family Apocynaceae (Nathan Kline, 1954). It comprises of total 80 species. Explants of an alkaloid producing plant cultured *in vitro* and has been found to retain the capacity to synthesis alkaloids identical to that in the intact plant (Yoshimatsu and Shimomura, 1991). Sometimes, high yield of secondary metabolites is observed in tissue grown as callus masses produced during differentiation (Benavides and Caso, 1993; Maheshwari *et al*., 2007). Beside this callus culture facilitates optimization of alkaloids production (Yamamoto and Yamada, 1986; Premjet *et al*., 2002; Anitha and Kumari, 2006) and subsequent isolation (Kirillova *et al*., 2001). Milky sap of this plant contains secondary metabolites or alkaloids. Alkaloids of this plant have a great medicinal importance to treat cardiovascular diseases (Anitha and Kumari, 2006), hypertension (Von Poser *et al*., 1990), arrhythmia (Kirillova *et al*., 2001), various psychiatric diseases (Bhatara *et al*., 1997; Kirtikar and Basu, 1993), breast cancer (Stanford *et al*., 1986) and human promyelocytic leukemia (Itoh *et al*., 2005).

Rauvolfia is threatened in India due to indiscriminate collection and overexploitation of natural resources for commercial purposes to meet the requirements of pharmaceutical industries, coupled with limited cultivation (Nayar and Sastry, 1987; Gupta, 1989). IUCN has kept this plant under endangered status. As propagation by means of seeds is very much difficult due to low germination percentage (Salma *et al*., 2008). Low seed germination is due to the presence of Cinnamic acid and derivatives in the seed (Mitra, 1976; Sahu, 979). *Rauvolfia* was *in vitro* cultured to make it available in large amount by various workers (Mitra and Kaul, 1964; Mathur *et al.*, 1993; Ghosh *et al*., 1998). In view of these facts the study was conducted for the development of an efficient protocol for *in vitro* multiplication of this plant by optimizing the growth regulators such as auxins and cytokinins.

MATERIALS AND METHODS

1. Collection of explant

Explants stem (nodal) and leaf of *Rauvolfia serpentina* were collected from the medicinal plant garden of Patna Science College under Patna University Patna, India (Fig. 1). The plants were identified, confirmed and

authenticated by Dr. M. P. Trivedi, Associate Prof. in Botany of Patna Science College, Patna. After authentication of this plant, *in vitro* culture studies were carried out.

2. Surface Sterilization and Culture Media

Explants – leaf and stem, washed thoroughly with running tap water for 30 minutes and then dipped for 15 sec. in 70% ethanol after that submerged in a disinfectant calcium hypochlorite (0.5%) for 25 minutes. Tween 80 added to the above solution to improve contact between tissue and disinfectant. Explants removed from disinfectant and washed 5 times in sterile distilled water. Explants blotted on filter paper in 5 replicates in Laminar Air Flow before placing it on Murashige and Skoog (MS) media.

Standard procedure was followed for the preparation of media with slight variations (Gamborg *et al*.1975). The pH of the media was adjusted to 5.8 and heat resistant growth regulators (NAA, 2, 4-D, BAP and KIN) were added to the media prior to sterilization done at 15 lbs/in for 15 min at 121^{o}C. All media were solidified with 8g/l agar. After autoclaving further work done under Laminar Air Flow.

3. Callusing

For callus induction juvenile stem (nodal) and leaf about 5 mm in length were aseptically prepared and were implanted vertically on MS medium prepared with specific concentrations of hormones. Culture of stem and leaf explants were initially incubated under darkness in a culture chamber at 25°C for callus induction.

4. Shoot Regeneration on Callus

The callus was cut into small pieces when it was observed in entire explants. Each piece of callus was transferred to MS media having same growth hormones in similar composition and concentration as for callus induction. Subsequently, calli were incubated under a 16/8 h (light/dark) photoperiod with light fluorescent lighting at an intensity of 60 µE m^{-5} s^{1} on a constant temperature as of callusing (25^{o}C) for shooting. Sub culturing was done after every 15th day.

5. Root Regeneration and Acclimatization

For initiation of roots the 5-7 weeks old shoots 3.5 cm in length were cultured on MS media having same growth hormones in similar composition, concentration and incubation as for shooting. The complete rooted plantlets 75 days old were washed to free them of agar and dipped in 0.2% bavistin fungicide for 10 minutes to protect from fungal attack in near future. These plants were potted in small plastic pots containing sterilized soil. The plantlets were covered with polythene bags to maintain high humidity. These were acclimatized at 28^{o}C less than 16 hours, photoperiod and watered regularly. After 3-4 weeks the polythene bags were removed and established plantlets were transplanted in earthen pots in a green house with watering at 2-3 days intervals.]

RESULTS

All the experiments were carried in triplicates and the mean value was recorded (Fig. 2).

1. Callusing

1.1. Effects of different concentrations of auxins and cytokinins singly on callus induction-

MS media supplemented with different concentrations (0.5–10.0 mg/l) of 1-naphthaleneacetic acid (NAA) showed stimulatory effects on callus induction. Maximum callusing response (70% in stem and 74% in leaf) was recorded at 1.5 mg/l of NAA. At 0.5mg/l the callusing response was recorded less and it increased up to 2mg/l. At 2.5mg/l onward callusing response was reduced and found minimum at 5mg/l. At 10mg/l no callusing or growth was observed. It was observed that the higher concentration of NAA in media had an inhibitory effect on callus proliferation.

2, 4-Dichlorophenoxyacetic acid (2, 4-D) with different concentrations (0.5-10 mg/l) showed stimulatory effects on callus induction (Table-1 and Figure-3). Maximum callusing response (93% in stem and 97% in leaf) was noted at 2.5mg/l.

No callus formation was observed on stem and leaf explants inoculated on MS media supplemented with 0.5 mg/l to 10 mg/l of Kinetin (KIN).

With 6- benzylaminopurine (BAP) maximum callusing response (60% in stem and 64% in leaf) was noted at 2.5mg/l. Lower concentrations of BAP (0.5mg/l to 1.5mg/l) were unable to induce callusing and higher concentrations of BAP (10 mg/l) in media had an inhibitory effect on callus induction.

1.2 Effects of different concentrations and combinations of growth hormones on leaf and stem callus induction.

2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 6-benzylaminopurine (BAP*)* with different concentrations (0.5-10mg/l) showed stimulatory effects on callus induction (Table-2). Maximum callusing response (85% in stem and 95% in leaf) was recorded at BAP 1mg/l and *2, 4-D* 2mg/l. At 3mg/l of BAP and 2, 4-D 1 mg/l swelling of callus was observed. At 5mg/l to 10mg/l of BAP and 2, 4-D no callusing or growth was observed.

MS media supplemented with different concentrations (0.5–10.0 mg/l) of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP*)* showed stimulatory effects on callus induction. Maximum callusing response (75% in stem and 77% in leaf) was recorded at 0.5mg/l and 1 mg/l for BAP and NAA respectively.

Different concentrations (0. 5–10.0 mg/l) of 1-naphthaleneacetic acid (NAA) and KIN showed stimulatory effects on callus induction. Maximum callusing response (63% in stem and 73% in leaf) was recorded at KIN 1mg/l and NAA 2mg/l. At 2.5mg/l to

10mg/l of KIN and NAA no callusing or growth was observed.

2 *In Vitro* Shoot Regeneration

All the experiments were carried in triplicates and the mean value was recorded.

2.1. Effects of different concentrations of auxins and cytokinins singly on shoot regeneration

MS media supplemented with different concentrations (2–5.0 mg/l) of 6-benzylaminopurine (BAP) maximum shooting response (77%) was noted at 2.5mg/l (Table-3). Lower concentrations of BAP (2mg/l) was having low value to induce shooting and higher concentrations of BAP (up to 5 mg/l) in media had lower effect on shooting. Days of shoot generation started from 21th to 37th days.

No shoot formation was observed of callus on MS media supplemented with 0.5 mg/l to 10 mg/l of 1-naphthaleneacetic acid (NAA), and 2, 4-Dichlorophenoxyacetic acid (2, 4-D).

1.2. Effects of different concentrations and combinations of growth hormones on shoot regeneration-

2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 6-benzylaminopurine (BAP) with different concentration (0.1-2.5 mg/l) showed stimulatory effects on shoot regeneration (Table-4). Maximum shooting response (65%) was recorded at BAP 1.5 mg/l and 2, 4-D 1.5 mg/l. Day of shoot generation started from 25th to 39th day.

MS media supplemented with different concentrations (0.1–3.0 mg/l) of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) showed stimulatory effects on shoot regeneration (Table-5, Figure-4). Maximum shooting response (80%) was recorded at BAP 1.5 mg/l and NAA 0.5 mg/l. Days of shoot generation started from 19th to 39th day.

3. *IN VITRO* ROOT REGENERATION

All the experiments were carried in triplicates and the mean value was recorded.

3.1. Effects of different concentrations of auxins and cytokinins singly on root regeneration-

MS media supplemented with different concentrations (2–5.0 mg/l) of 1-naphthaleneacetic acid (NAA) maximum rooting response (85%), (Table-6). Lower concentrations of BAP (0.5- 1mg/l) was having low value to induce rooting and higher concentration of BAP (up to 5 mg/l) in media had lower effect on rooting. Day of root generation started from 09th to 17th day No root formation was observed on shoot inoculated on MS media supplemented with 0.5 mg/l to 10 mg/l of 6-benzylaminopurine (BAP), and 2, 4-Dichlorophenoxyacetic acid (2, 4-D).

***3.2.* Effects of different concentrations and combinations of growth hormones on Root regeneration-**

2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 6-benzylaminopurine (BAP) with different concentrations (0.1-2.5 mg/l) showed stimulatory effects on root regeneration (Table-7). Maximum rooting response (64%) was recorded at BAP 1.5 mg/l and 2, 4-D 1.5 mg/l. Days of root generation started from 11th to 19th day.

MS media supplemented with different concentrations (0.1–3.0 mg/l) of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) showed stimulatory effects on root regeneration (Table -8 and Figure-5). Maximum rooting (87%) was recorded at BAP 1.5 mg/l and NAA 0.5 mg/l. Days of root generation started from 09th to 18th day.

The most essential requirement for the successful transplantation is to maintain the plants under a very high humidity (85%). To achieve it, fan cooling system was used and temperature was maintained at 28^{o}C for the first 15days. They were covered with clear plastic(Figure-6). Later some small holes were poked in the plastic for air circulation. In the next step they were transferred to the shade house (75%) with overhead sprinkler system for irrigation for next 15 days (Figure-7). Finally they were transferred to the open area for 13 days before transferring them to the field.

Figure legends

Figure1**:** Whole plant of *Rauvolfia serpentina*

Figure 2: Explants in triplicates on MS media with growth hormones for callus induction.

Figure 3: Callus induction of MS fortified with 2, 4-D (2.5 mg/l).

Figure 4: Shoot regeneration of *Rauvolfia serpentina* on MS fortified with BAP (1.5 mg/l) and NAA (0.5 mg/l).

Figure 5: Root regeneration of *Rauvolfia serpentina* on MS fortified with BAP (1.5 mg/l) and NAA (0.5 mg/l).

Figure 6: Hardening- Plants covered with plastic.

Figure 7: Hardening- Plant ready to transfer into soil.

Figure 1: Whole plant of *Rauvolfia serpentina*

Figure 2: Explants in triplicates on MS media with growth hormones for callus induction in *Rauvolfia serpentine*

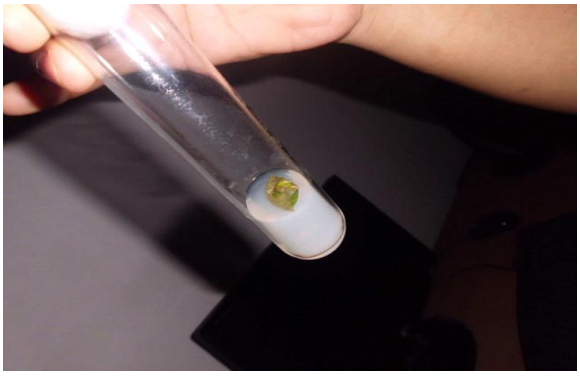

Figure 3: Callus induction of MS fortified with 2, 4-D (2.5 mg/l).

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Figure 5: Root regeneration of *Rauvolfia serpentina* on MS fortified with BAP (1.5 mg/l) and NAA (0.5 mg/l).

Figure 6: Hardening- Plants covered with plastic.

Figure 7: Hardening- Plant ready to transfer into soil.

Table Captions

Table -1. Callus induction on stem (nodal) and leaf explants on MS medium under the influence of different concentrations of 2, 4-D mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Table 2. Callus induction on stem (nodal) and leaf explants on MS medium supplemented with different concentrations and combinations of BAP and 2,4-D mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Table- 3. Shoot regeneration on MS medium under the influence of different concentrations of BAP mg/l (2, 2.5, 3, 4 and 5).

Table -4. Shoot regeneration on MS medium supplemented with different concentrations and combinations of BAP and 2, 4-D mg/l (0.1 to 2.5).

Table -5. Shoot regeneration on MS medium supplemented with different concentrations and combinations of BAP and NAA mg/l (0.1, 1, 1.5, 2, 2.5 and 3).

Table -6. Root regeneration from shoot on MS medium under the influence of different concentrations of NAA mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Table -7. Root regeneration from shoot on MS medium supplemented with different concentrations and combinations of BAP and 2, 4-D mg/l (0.1 to 2.5).

Table -8. Root regeneration from shoot on MS medium supplemented with different concentrations and combinations of BAP and NAA mg/l (0.1, 1, 1.5, 2, 2.5 and 3).

Table -1. Callus induction on stem (nodal) and leaf explants on MS medium under the influence of different concentrations of 2, 4-D mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media 2,4-D mg/l	*Rauvolfia serpentina*					
	Stem			Leaf		
	% of callus induction	degree of callusing	day of callus induction	% of callus induction	degree of callusing	day of callus induction
0.5	-	-	-	-	-	-
1	-	-	-	-	-	-
1.5	-	-	-	-	-	-
2	76	+++	21	80	+++	20
2.5	93	++++	20	97	++++	19
3	25	+	31	21	+	29
4	19	+	33	23	+	27
5	15	+	35	17	+	31
10	No callusing	-	-	No callusing	-	-

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table 2. Callus induction on stem (nodal) and leaf explants on MS medium supplemented with different concentrations and combinations of BAP and 2,4-D mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media mg/l		*Rauvolfia serpentina*					
		Leaf			Stem		
		% of callus induction	degree of callusing	day of callus induction	% of callus induction	degree of callusing	day of callus induction
MS		-	-	-	-	-	-
BAP	2,4-D						
0.1	2	67	++	23	47	+	25
0.5	2	73	+++	20	69	++	21
1	1.5	21	+	31	20	+	33
1	2	95	++++	19	85	+++	19
1.5	1.5	23	+	31	20	+	33
1.5	2	42	+	25	45	+	27
1.5	2.5	39	+	26	35	+	29
2.5	1	25	+	30	23	+	31
2.5	2	17	+	33	21	+	33
3	1	swelling	-	-	swelling	-	-

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table- 3. Shoot regeneration on MS medium under the influence of different concentrations of BAP mg/l (2, 2.5, 3, 4 and 5).

Composition of media mg/l	*Rauvolfia serpentina*		
BAP	% of Shooting	degree of shooting	day of shoot induction
2	69	++	31
2.5	77	+++	29
3	61	++	33
4	35	+	35
5	19	+	37

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table -4. Shoot regeneration on MS medium supplemented with different concentrations and combinations of BAP and 2, 4-D mg/l (0.1 to 2.5).

Composition of media mg/l		*Rauvolfia serpentina*		
BAP	**2,4-D**	**% of Shooting**	**degree of shooting**	**day of shoot induction**
0.1	2	25	+	35
0.5	2	37	+	31
1	1.5	51	++	27
1	2	43	+	29
1.5	1.5	65	++	25
1.5	2	49	+	28
1.5	2.5	47	+	29
2.5	1	61	++	26
2.5	2	31	+	33

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table -5. Shoot regeneration on MS medium supplemented with different concentrations and combinations of BAP and NAA mg/l (0.1, 1, 1.5, 2, 2.5 and 3).

Composition of media mg/l		*Rauvolfia serpentina*		
BAP	**NAA**	**% of Shooting**	**degree of shooting**	**day of shoot induction**
0.5	0.5	71	+++	23
1	0.1	65	++	27
1.5	0.5	80	++++	21
2	0.1	75	+++	25
0.1	1	37	+	31
0.5	1	21	+	37
1	1	37	+	31
1.5	1	69	++	25
2	1	57	++	33
1	2	25	+	35
3	2	21	+	39

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table -6: Root regeneration from shoot on MS medium under the influence of different concentrations of NAA mg/l (0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10).

Composition of media mg/l	*Rauvolfia serpentina*		
NAA	**% of Rooting**	**degree of Rooting**	**day of Root induction**
0.5	55	++	13
1	69	++	12
1.5	83	++++	11
2	85	++++	12
2.5	79	+++	13
3	73	+++	14
4	65	++	14
5	41	+	15

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table -7: Root regeneration from shoot on MS medium supplemented with different concentrations and combinations of BAP and 2, 4-D mg/l (0.1 to 2.5).

Composition of media mg/l		*Rauvolfia serpentina*		
BAP	**2,4-D**	**% of Rooting**	**degree of Rooting**	**day of Root induction**
0.1	2	23	+	19
0.5	2	35	+	17
1	1.5	49	+	12
1	2	41	+	15
1.5	1.5	64	++	11
1.5	2	47	+	13
1.5	2.5	45	+	15
2.5	1	61	++	11
2.5	2	35	+	17

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = poor, ++ = good, +++ = excellent.

Table -8. Root regeneration from shoot on MS medium supplemented with different concentrations and combinations of BAP and NAA mg/l (0.1, 1, 1.5, 2, 2.5 and 3).

Composition of media mg/l		*Rauvolfia serpentine*		
BAP	**NAA**	**% of Rooting**	**degree of Rooting**	**day of Root induction**
1	0.1	-	-	-
1.5	0.5	87	++++	10
2	0.1	65	++	15
0.1	1	75	+++	11
0.5	1	75	+++	12
1	1	71	+++	13
1.5	1	73	+++	13
2	1	75	+++	11
1	2	55	++	15
3	2	35	+	18

(-) indicates no regeneration and (+) indicates status of callus induction.
+ = Poor, ++ = good, +++ = excellent.

DISCUSSION

1 Callusing

Standard procedure was followed for the preparation of media (Gamborg, 1968). In the present study, two explants leaf and nodal stem were used in which leaf explants were found the best for callus induction than stem, which is in accordance with the earlier findings (Mathur *et al.*, 1993). MS media without any growth hormone was unable to induce callus (Shah *et al*., 2003). Among all the growth hormones, 2, 4-D was the best for callus induction Mitra and Kaul, 1964).

In the present work Kin alone could not induce callus (Murashige *et. al*., 1974). In further experiments Kinetin (Kin) was supplemented to the MS media in combination with auxins (2, 4-D and NAA). It was observed that Kin had enhanced callus growth in the presence of auxins.

MS media fortified with 2, 4-D and BAP was found the best for callus induction (Roja *et al*., 1996). Day of callus induction started from 17th to 37th day (Schrawat et al., 2002). This variation observed in the present investigation may be attributed due to the difference in culture conditions and the age of explants.

2. Shoot generation

The callus was sub cultured in all BAP containing media differentiated into multiple shoots giving out an average of 5 shoots per piece of callus in MS +BAP (2.5mg/l). The media with lower concentration of NAA further gave multiple shoots as well as roots (Sarker *et al.*, 1996). Inductions of callus and plant regeneration are the most reliable tools to multiply the plants in a large scale (Sarker *et al.*, 1996). In combination of NAA and BAP normally lower NAA and higher BAP concentration favoured the production of shoot (Sudha and Seeni, 1996). MS media with BAP and NAA is most suitable for shooting in our case (Ahmad et al., 2002)..

In our findings Shoot culture was initiated on MS medium containing BAP (0.5 mg/l) with NAA (0.5 mg/L). Maximum shoot proliferation was achieved in medium containing BAP (1.5 mg/L) with NAA(0.5 mg/L) (80%). Among all the growth hormones, in single combination BAP (2.5 mg/l) was the best for shoot induction (77% from callus). Day of shoot generation started from 19th to 39th day.

3.3. Root generation

Root generation was satisfactory with BAP and NAA. NAA was the best in all other hormones like 2,4-D and BAP for rooting in our result (Sharma *et al*., 1999).

In our findings, root cultures were initiated on MS medium containing BAP (1 mg/l) with NAA (0.1 mg/L). Maximum root proliferation was achieved in media containing BAP (1.5 mg/L) with NAA (0.5 mg/L) (87% from shoot). Day of root generation started from 11th to 17th day.

4. Hardening

Plantlets after 81 days old in secondary hardening were ready for field transplantation. Regenerated plants after hardenings were transferred to soil and they showed 77% survival. The regenerated plants were morphologically similar to control plants.

Therefore, in the present investigation, a protocol for micro- propagation of locally adapted population is successfully established which may be used for commercial application as a substitute to natural propagation through conventional methods.

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