ISSN 2449-8866

Current Life Sciences

Research Article

Effect of hormone for *in vitro* propagation of *Asparagus racemosus* Wild.

Niroj Paudel[1]*, Mukti Ram Aryal[2], Rudra Hari Puri[2]

[1] Department of Applied Plant Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea

[2] Department of Botany, Tri-Chandra multiple Campus (Tribhuvan University), Nepal

*Corresponding author: Niroj Paudel; E-mail: nirojjirauna@gmail.com

Received: 17 July 2018; **Revised submission:** 27 August 2018; **Accepted:** 31 August 2018

DOI: http://dx.doi.org/10.5281/zenodo.1407185

ABSTRACT

Asparagus racemosus Wild. is an undershrub, climber which is extremely branched with woody stem and spines like reduced leaves. It founds in tropical and sub-tropical region of Nepal up to 1500 meter above sea level. *A. racemosus* Wild. is an endangered species widely used in modern drug development and Ayurveda as well. The subculture explant shows responses to the different concentration of hormone. The composition of MS + 0.5 mg/l BAP and MS + NAA 0.5 mg/l + kinetin 1 mg/l most significant for shoot multiplication. Over them, the composition MS = 0.01 mg/l NAA + 1.0 mg/l BAP along with MS + 0.5 mg/l NAA = 1.5 mg/l kinetin is effective for the development of shoot in *Asparagus racemosus*. The hormone play important role on the regeneration of shoot *in vitro* condition. The knowledge of hormonal requirement help to promote the development and growth of endangered plants for their rapid propagation.

Keywords: *Asparagus racemosus* Wild.; 6-benzyl amino purine (BAP); Growth and development; *In vitro* propagation; Medicinal plant; 1-napthalene acetic acid (NAA).

1. INTRODUCTION

Nepal has a wide range of climatic variation due to its geography and topography and almost all types of climate, season and soil from the world [1] that have already been identified [2]. The Ayurveda (homeopathic) health care system depends on the use of these highly valued native medicinal plants. Some medicinal plant from Kathmandu valley [3] from Argakahaci district [4]; recently reported from Biratnagar, eastern Nepal [5]. The first attempt to regenerate plant from tissue reported [6]. The culture medium [7, 8] developed for tobacco has served as the starting media for many plants.

Kohmura et al. [9] have developed an effective micro-propagating system involving induction of multiple bud clusters and somatic embryogenesis in *A. officinalis* L. CV "Hiroshima green", and other 14 genotypes. Schröder and Eimert [10] have studied the temporary immersion of *in vitro* grown internodal pieces of *A. officinalis* and their incubation in the dark in a liquid embryo induction medium. The explant can be very small pieces of plants, such as embryo, seed, shoot tip, meristem, root tip, callus, single cell and pollen grain [10].

Gupta et al. [11] tested seed germination of *A. racemosus* Wild. They observed a wide variation in germination percentages of the seeds ranging from 17 to 60 in different collection samples. Saensouk and Suddee [12] used young leaves and nodes of *A. racemosus* as explants and MS as the basal medium, NAA, and BA as the PGRs in their experiment. The percentage of callus on medium

combination with 0.5 mg/l NAA and 2 mg/l BA were 90 percent and 80 percent from leaves and nodes, respectively. Shooting was 100 percent from leaves cultured on medium added with 1 and 2 mg/l BA. Pontaroli and Camadro [13] studied the callus growth and plant regeneration from long-term callus cultures in two clones of *A. officinalis* cv. Argenteuil, to establish a suitable protocol for a prospective *in vitro* selection program.

Bopana and Saxena [14] have pointed out the importance of *A. racemosus* Wild. as an important medicinal plant of tropical and subtropical India. They have listed the medicinal usage of this species like treatment of neurodegenerative disorders and in alcohol abstinence-induced withdrawal symptoms.

Dutta [15] has mentioned the uses of *Asparagus racemosus* in treatment of various disease of liver, scalding, urine, gleets, and gonorrhea. Root power given as tonic for strength. Bopana and Saxena [16] mentioned that due to destructive harvesting, the natural population of *A. racemosus* is rapidly disappearing, and it recognized as 'vulnerable'. Hurgoiu and Blidar [17] studied the callus growth and organogenesis from the previously induced callii in *A. officinalis* (Jersey Knight F1).

Kumar and Vijay [18] used MS medium and BA and K as cytokinin (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) and IAA, IBA and NAA as auxins (0.1, 0.5 and 1.0 mg/l) in combinations to see the various effects in *A. racemosus* nodes. They very little callus with IAA 3.0 mg/l whereas maximum callus as well as shoot proliferation at 2.0 mg/l NAA. They observed that BA up to 2.0 mg/l with any concentration of IAA either a very little or no callus at all. Pant and Joshi [19] have successfully multiplied *A. racemosus* using tissue culture technique. Plant growth regulators are important in plant tissue culture since they play vital roles in steam elongation, tropism and apical dominance. Auxins, cytokinin, gibberellins, ethylene and abscisic acid are the five general classes of hormones [20].

The objectives of this research are to find out a reliable and cost effective method of rapid propagation of the plant. *A. racemosus* as well as to understand its behavior under different phytohormone conditions.

2. MATERIALS AND METHODS

2.1. Plant material

The younger plant material (*Asparagus racemosus* Wild.) collected from botanical garden of Department of Botany of Tri Chandra campus, Kathmandu.

2.2. Surface sterilization of *ex-plant*

Shoots washed under running tap water for about one hour with few drops of liquid detergent. After washing with detergent the explants thoroughly rinsed with distilled water for 4-5 times to remove any traces of detergent remaining in explants. After these treatments, explants taken inside the laminar airflow for further sterilization. Explants were surface sterilized with freshly prepared 0.1% w/v aqueous solution of Sodium hypo chloride for 10 minutes. Then again, explants thoroughly rinsed 3-4 times with sterilized distilled water to remove any traces of Sodium hypo chloride.

2.3. Sterilization of glassware's

All the glassware dipped in the detergent water for overnight. They washed thoroughly with tap water using bottlebrush then autoclave.

2.4. Sterilization of media and equipment

All the media, utensils (forceps, blade holders, blades if reused, needles, brush etc.) and sterile water used inside the laminar hood were sterilized at 15 lb/sq. inch pressure and 121^{o}C for 20 minutes in a portable autoclave.

2.5. Preparation of *ex-plants*

For the explants, after mentioned surface sterilization processed used. In addition, they used as the explants for *in vitro* experiments. In case of shoot tip, culture approximately 0.5-2.0 cm long parts of these organs excised inside the laminar hood using a sterile blade.

2.6. Media preparation

A medium is the formulation of different inorganic salts and organic compounds necessary for the nutrition of plant or plant part under *in vitro* culture condition. There are various types of culture media recommended for various types of cultures and plants. This is necessary to identify a suitable media for better results (outcomes). This can done either by a better understanding of the nutritional requirements of cultured cells and tissues or by the results of previous similar experiments conducted by various workers on similar plants or plant parts. In general, the tissue culture medium must contain the 16 essential elements for plant growth.

2.7. Preparation of stock solutions

Different stock solutions containing macro elements (A), microelements (B), iron source (C), vitamins (D) and iodine sources (KI).

The strength of "Stock A" and "Stock C" containing macro elements raised to 10x while making the stock solutions for convenience. All the compounds of macro salts dissolved in distilled water and stored in the brown bottle inside the refrigerator. The iron source compounds needed slight warming to dissolve completely in the distilled water.

Similarly, the strengths of the micronutrients and vitamins raised to 100x in the stock solutions. The KI solution was prepared separately.

For the preparation of stock solutions, all the chemicals were weighed separately using a digital electronic balance. The individual chemicals dissolved one by one and a required final volume (500 ml) made by adding required amount of distilled water. All the stocks were stored in clean brown bottles inside the refrigerator.

The myoinositol added freshly during the media preparation time.

2.8. Preparation of MS medium

For the preparation of one liter of MS medium, following procedure followed. By using either a measuring cylinder or a pipette 100 ml of stock A (x10), 1 ml of stock B (x100), 1 ml of KI (x100) solution, 10 ml of stock C (x10) and 1 ml of stock D (x100) were mixed in a conical flask of capacity 1000 ml. An appropriate amount of myoinositol (0.1 g) weighed and added freshly. Similarly, 30 g of sucrose added and stirred with the help of a glass rod. When the sucrose completely dissolved, the pH measured using a pH meter. The pH of the medium adjusted to 5.8 ±0.1 with 0.1 M HCl or NaOH. After adjusting the pH, the medium heated over an electric heater. When the media was nearly boiling took out of the heater and added pre-weighed 8.00 g of agar with continuous stirring.

Now, the media was again heated, boiled and poure in glass jam bottles. One liter of medium poured in 20 jam bottles. The mouths (openings) of the bottles were covered. All the media sterilized at 15 lb/sq. inch pressure and 121^{o}C for 20 minutes in a portable autoclave.

The required amounts of PGR/s in the media added before autoclaving from their stocks using sterile pipettes.

2.9. Preparation of PGRs stock solutions

For the preparation of hormone solutions of all the auxins and cytokinins, 10 mg of each of the PGRs dissolved in few drops of their respective solvents (ethanol, KOH and NaOH) in separate test tube. These solutions made 100 ml by adding distilled water. These taken as the stock solution of 100 mg/l and are preserved in brown bottles in the freezer. The amount in ml of the stock added to one liter of the final medium makes the same amount of PGR in mg/l (i.e. 1.0 ml of NAA stock if added to one liter of medium makes 1.0 mg/l NAA).

For the preparation of different concentrations of different hormones from the stock solutions for various purposes, the following formula used:

$$S_1V_1 = S_2V_2$$

Where: S_1= strength of stock i.e., 1000 ppm., V1= volume of the stock to be taken, S_2 = strength of the hormone required (ppm), V_2 = Total volume required.

2.10. Inoculation of *ex-plants*

All the media and necessary utensils were, again sterilized under the UV light for 45 minutes before using. The laminar airflow hood washed/

sprayed with alcohol before turning the UV light on to minimize the risk of contamination. The explants after a series of surface sterilization process mentioned were ready for inoculation. The explants of about 0.5-2.0 cm were prepared and inoculated in the medium under the laminar airflow hood. The transfer was done close to a burner with a pair of sterile forceps. Similar method used in all cultures and sub cultures.

2.11. Culture conditions

All the culture bottles containing specific media after inoculation of the explants kept in a culture room. The temperature of the culture room maintained at 25±2^{0}C by running an air conditioner. The room was illuminated for 16-hours (everyday photoperiod) with the light intensity of 3000 lux using cool white fluorescent tubes.

2.12. Acclimatization

The *in vitro* rooted plantlets removed, washed carefully and planted in the coco peat. All the rooted plantlets (both *in vitro* and *in vivo*) transferred to sand soil mixture (1:1). All the plantings were regularly observed, and watered at an interval of 2-5 days depending upon the moisture on the bed. The humidity inside the dome maintained up to 90% for the first week. Gradually the humidity decreased by allowing more air to circulate in the successive weeks. Finally, established plants either transferred to the garden or distributed to the interested growers.

3. RESULTS

3.1. Culture of shoot tip of an *ex-plant*

For the inducing of multiple shoots, shoot tips of early young shoot of *A. racemosus* Wild. was inoculated on the MS basal media, supplement with various pairs of concentration of growth hormones (NAA, BAP, kinetin). 5 weeks of first inoculation, first sub culture done slightly changing the initial pairs of hormone concentrations, and it was remained for about 6 week (Figs. 1 and 2).

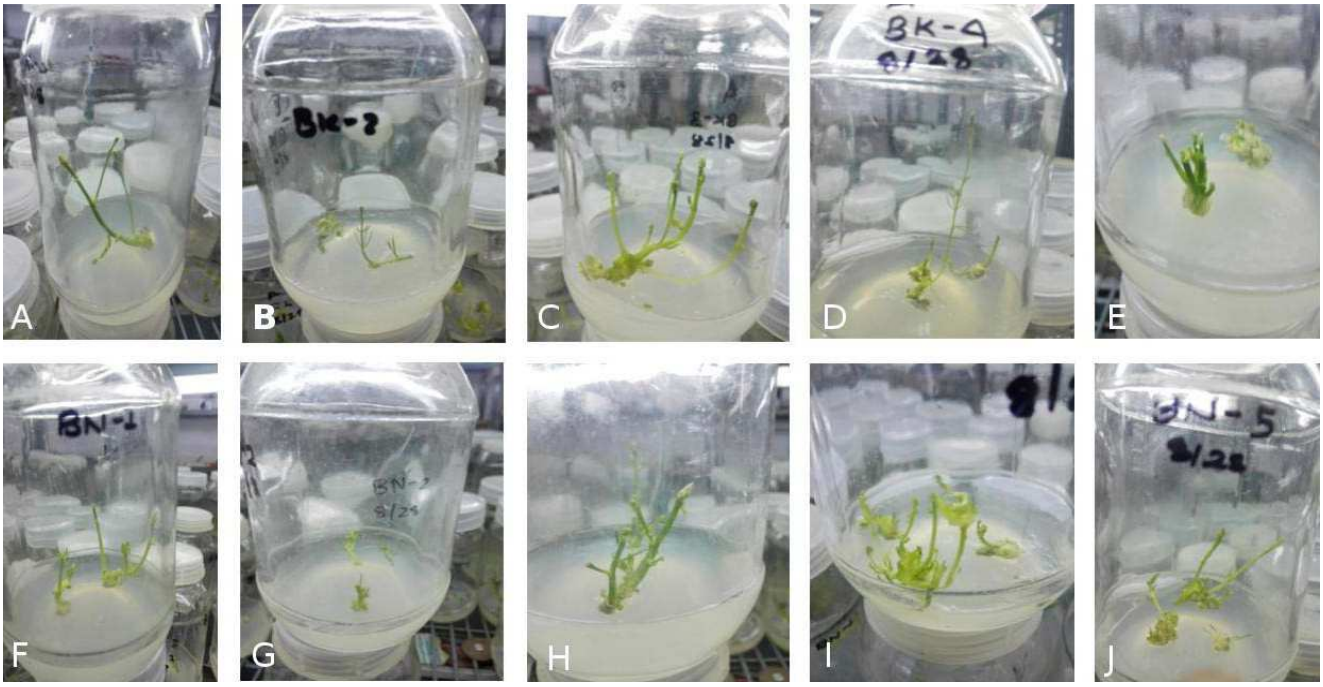

Figure 1. Four-week-old plant in MS media: **A.** NAA 0.1 mg/l + kinetin 0.1 mg/l, **B.** 0.1 mg/l NAA + 0.5 mg/l kinetin, **C**. NAA 0.5 mg/l + Kinetin 1.0 mg/l, **D.** NAA 0.5 mg/l + kinetin 1.5 mg/l, **E.** NAA 0.5 mg/l + kinetin 2.5 mg/l, **F.** NAA 1 mg/l + BAP 1 mg/l, **G.** NAA 0.1 mg/l + BAP 1.0 mg/l, **H.** NAA 0.01 mg/l + BAP 1 mg/l, **I.** NAA 0.1 mg/l + BAP 0.5 mg/l, **J.** NAA 0.01 mg/l + BAP 0.5 mg/l.

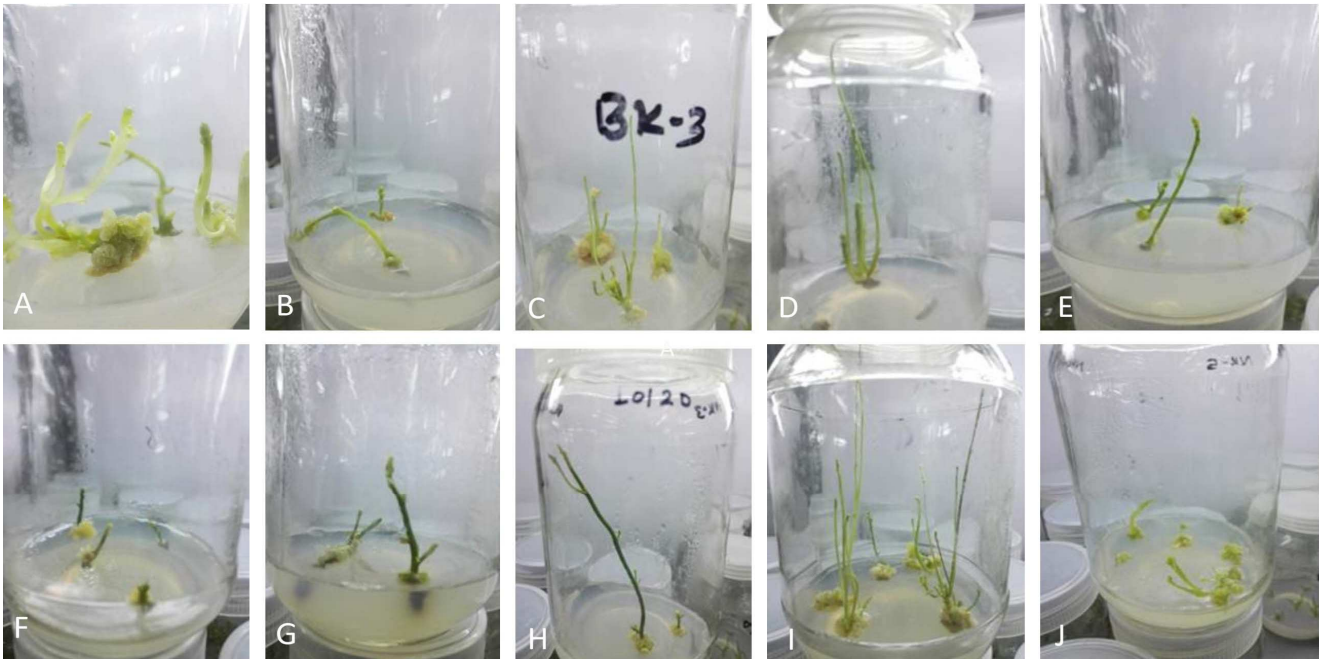

Figure 2. Five-week-old plant in MS media: **A**. NAA 1.0 mg/l + kinetin 0.1 mg/l, **B**. NAA 1.0 mg/l + kinetin 0.5 mg/l, **C**. NAA 0.5 mg/l + kinetin 1 mg/l, **D.** NAA 0.5 mg/l + kinetin 1.5 mg/l, **E**. NAA 0.5 mg/l + kinetin 2.5 mg/l, **F**. NAA 0.1 mg/l + BAP 0.1 mg/l, G. NAA 0.1 mg/l + BAP 1.0 mg/l, **H.** NAA 0.01 mg/l + BAP 1 mg/l, **I.** NAA 0.1 mg/l + BAP 0.5 mg/l, **J.** NAA 0.01 mg/l + BAP 0.5 mg/l.

3.2. Effect of different hormone concentrations (Tables 1-4)

Table 1. Effect of kinetin and NAA (5 weeks of inoculation).

S.N.	Growth hormones		Growth response	Height of shoots	Number of shoots	Observation
	NAA	Kinetin				
1	0.1	0.1	Shoots + callus	5-6 cm	3	Well growth of shoot, callus at the base of the shoot
2	0.1	0.5	Shoots + callus	4-5 cm	3	Callus appears at the base of the shoot. Normal growth of shoot
3	0.5	1	Shoots + roots	5-6 cm	5	Well growth of shoot. Multiplication of the shoot, root develops
4	0.5	1.5	Shoots	6-7.5 cm	4	Well growth of shoots
5	0.5	2.5	Stunted shoots	2-3 cm	5	Growth of shoots stops after 2-3 weeks. Small mass of callus seen

Table 2. Effect of NAA and BAP (5 weeks of inoculation).

S.N.	Growth hormones		Growth response	Height of shoots	Number of shoots	Observation
	NAA	BAP				
1	1	1.0	Shoots + callus	3-4 cm	4	Well growth of the shoot, small mass of callus at the base of shoot
2	0.1	1.0	Shoots + callus	2-3 cm	3	Mass of callus at the end of shoot tip
3	0.01	1.0	Shoots	3-4 cm	5	Well growth and multiplication of the shoot
4	0.1	0.5	Shoots + root	3-4 cm	6	Shoot multiplication and well growth of the shoot + root development
5	0.01	0.5	Shoots	4-5 cm	5	Well growth of the shoot

Table 3. Effect of NAA and kinetin (6 weeks of subculture).

S.N.	Growth hormones		Growth response	Height of shoots	Number of shoots	Observation
	NAA	Kinetin				
1	1	0.1	Callus	-	6	No any significance change in the shoot development, small mass of callus at the end
2	1	0.5	Shoots + root	1-2cm	2	Growth rate of shoot is low, small hairy root appears
3	0.5	1	Well grow of Shoots + roots	5-6 cm	10	Well growth of shoot + root
4	0.5	1.5	Shoots only	6-7.5 cm	5	Well growth and multiplication of the shoots. Growth of roots
5	0.5	2.5	Stunted shoots only	2-3 cm	4	Growth of shoot stops suddenly

Table 4. Effect of NAA and BAP (6 weeks of subculture).

S.N.	Growth hormones		Growth response	Height of shoots	Number of shoots	Observation
	NAA	BAP				
1	0.1	0.1	Shoots + callus	2-3 cm	3	Extensive mass of callus along with developing new shoots
2	0.1	1.0	Shoots + callus	3-4 cm	4	Shoot growth + callus
3	0.01	1.0	Shoots + callus	5-6 cm	4	Well growth of shoot and mass of callus at base of shoots
4	0.1	0.5	Shoots + roots	8-9 cm	13	Growth and development of shoot along with minute roots
5	0.01	0.5	Shoots only	3-4 cm	7	Growth of shoot, no callus

3.3 Acclimatization

After one months of hardening, these plantlets carried out for sand rooting. Sand rooting was done with the help of root hormone i.e. auxin. The survival rate of these plantlets were satisfactory i.e. out of 58 plantlets 31 (53.45%) plantlets were survived when these plants were transferred to the poly bag the survival rate was outstanding (Table 5, Fig. 3). Almost all the plantlets with root survived in polybag.

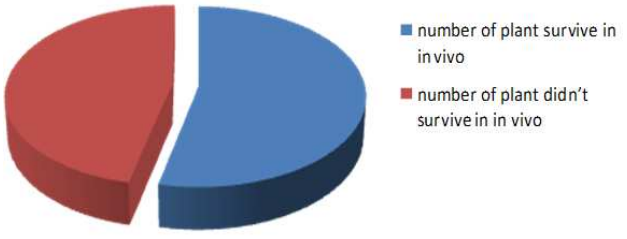

Figure 3. Pie chart showing the plants survive in *in vivo* condition.

Table 5. Plants showing response in *in vivo* condition (Green House).

Total no of plants	Plant survived *in vivo*	Plant can't survived *in vivo*
58	31	27
%	53.45%	46.55%

4. DISCUSSION

In vitro propagation of *A. racemosus* Wild. through the shoot tip of the plant successfully done in different hormone concentration of auxin and cytokinin. In this experiment, the growth of shoot was found to be normal than the growth of root.

Shrivastana et al. [20] working on *Citrus vulgaris* observed the requirement of BAP in shoot formation. However, in present experiment the rapid growth of shoots and multiplication of shoot observed in MS + NAA (0.1 mg/l) + BAP (0.5 mg/l).

Kumar and Vijay [18] reported successful establishment of plant raised through shoot tip

culture of *A. racemosus* Wild. for rapid clonal propagation. Shoot tip ex plant culture on the MS + kinetin (3.0 to 5.0 mg/l) reported multiple proliferations of shoots from the explant. In this experiment, multiple proliferations of shoots with growth of root observed in MS + NAA (0.5 mg/l) + kinetin (1.5 mg/l). The growth of root is due to the presence of auxin and kinetin-containing medium shows the growth of regenerated shoots.

Ranjitkar and Saiju [21] obtained micro shoot on BAP (3 mg/l) + NAA (0.1 mg/l) by shoot tip culture of *Rauwoflia serpentine* and subculture of these shoot tip on lower concentration of BAP (1 mg/l) + NAA (0.1 mg/l) results in shoot proliferation. Similar result obtained in this experiment; multiple numbers of small shoots obtained in MS + NAA (0.01 mg/l) + BAP (1 mg/l).

Ranjitkar et. al. [22] growth of shoots on MS + BAP (0.5 mg/l) + NAA (0.01 mg/l), and growth of multiple number of shoots in MS + BAP (1 mg/l) + NAA (0.01 mg/l), while culturing of shoot tip of *Swertia ciliata.* Similar result obtained in present experiment in same hormone concentration of NAA and BAP.

In combination of auxin and cytokinins maximum numbers of shoots were obtained in the hormone concentration of MS + NAA (0.5 mg/l) + kinetin (1.5 mg/l). On increasing the concentration of NAA and decreasing the concentration of kinetin, shoot didn't give any response, however callus developed at the end of the shoot i.e. in MS + NAA (1 mg/l) and kinetin (0.1 mg/l). higher concentration auxin accumulation might have affected in proper growth of the shoots because high concentration of auxin increase the production of ethylene; production and ethylene accumulation in the cultural media and may inhibit the growth and development of plant in *in vitro* culture. Conversely, ethylene may affect the transport and metabolism of the cytokinins, similar result with Rajkarnikar et al. [21] while doing shoot tip culture of *A. racemosus*. Paudel et al. [23] established hormonal effect on shoot regeneration for *Lycopersicon esculentum* L. in 11-12 week. Pant and Joshi [19] observe that BAP played good roles in shoot and bud inductions, whereas combinations of NAA and BAP at various levels were found to be effective in almost all cases, same result was obtained in present study. Also Aryal et al. [24] uses the hormonal treatment for the meristem culture in *Amomum subulatum* for elimination of Chhirkey and Foorkey.

Presence of kinetin along with the relatively lower concentration of NAA results in the growth and development of shoot, multiplication of shoots and at the end of the shoot, mass of callus obtained. In all the combination of auxin and cytokinin, none of the concentrations combine found to be statically significant to each other in inducing the shoot, multiplication of the shoot, callus development similar to that of Shrivastava and Rajani [20]. However in two pair of concentrations of auxin and cytokinin i.e. [NAA (0.5 mg/l) + kinetin (1 mg/l) and NAA (0.5 mg/l) + kinetin (1.5 mg/l)], among all the concentration combination only these two combinations were slightly statically significant in inducing multiple shoot. From this experiment, NAA at lower concentration along with high concentration of kinetin found to be greatly favored the shoot multiplication. This finding was similar to that work of the Pant et al. [19] with their experiment in the *A. racemosus.* Equal hormone concentration of NAA (0.1 mg/l) and kinetin (0.1 mg/l) favors the induction, growth and development of shoot, similar result obtained in NAA and BAP each of concentration of 1 mg/l. However, in case of very low concentration of auxin and NAA and high concentration of kinetin result is slightly different. The development of shoot at high concentration is not normal, growth of the shoot stunted after 2-3 weeks of inoculation.

From this experiment it was found that the most of the concentration combinations of NAA along with BAP were negatively significant in case of shoot elongation i.e. they retarded the growth of shoots but with KN the result was slightly better when KN or both were at higher levels. Although in some cases vitrifications were observed, higher Kn concentration generally gave shoot elongation whereas BAP mostly showed vitrification.

5. CONCLUSION

Due to lack of proper conservation strategies, technique, policies, awareness program, poverty, habitat destruction, illegal transport, over exploitation these plants are in state of extinction. In such a way *A. racemosus* Wild. is also in state of extinction.

From the overall study, it concluded that the plant tissue culture (i.e. *in vitro* propagation) technique is the most suitable technique for conservation of medicinal plants. It is easy method for production of large number of plant species within short period of time and in small place, without losing their properties and without damaging their original plant. Once suitable protocol is established, the cost will greatly reduce. From this experiment, we can conclude that MS + NAA 0.5 mg/l + kinetin 1.5 mg/l and MS + NAA 0.1 mg/l + BAP 0.5 mg/l is good for multiplication and development of shoots. In addition, can be concluded that micropropagation is the easiest, fastest and reliable method for the multiplication of this plant and other plants. With this technique, we can conserve the biodiversity for the present and future generation.

AUTHORS' CONTRIBUTION

NP: Manuscript preparation and experimental design. MRA: Experiment design, guidance, and RHP: Experiment design and data collection. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENT

The authors are grateful for Department of Botany Trichandra Multiple Campus (TU) for laboratory facilities.

REFERENCES

1. Malla SB, Shakya, PR. Vegetation and medicinal plants of Nepal. Nepal National Commission for UNESCO, Regional Seminar on the Ecology of Tropical Highlands. HMG and UNESCO. 1968: 1-10.
2. Shrestha UK, Shrestha KK. Some physio-chemical parameters of *Asparagus racemosus* from Nepal. In: Bulletin of the Department of Plant Resources, 2008.
3. Paudel N, Aryal MR, Das BD, Adhikari DC, Rai PD, Shrestha R. Some medicinal plant from Kathmandu Valley, Central Nepal. Int J Sci Rep. 2018: 4(4): 78-81.
4. Paudel N, Paudel LP, Ghimire U, Das BD. Archichlamydae and Sympetalae flora of Arghakanchi District, Western Nepal. Int J Life Sci Res. 2017: 5(3): 73-81.
5. Paudel N, Adhikari DC, Das BD. Some medicinal plants uses in ethnical group from Biratnagar, Eastern, Nepal. Am Sci Res J Engin Technol Sci. 2018; 41(1): 233-239.
6. Haberlandt G. Cultur versuche mit isolierten pflanzenzellen sitz. Ber Mat Nat ki Kais Akad Wiss, Wien. 1902; 111: 69-92.
7. Skoog F, Millar CO. Chemical regulation of growth and organ formation in plant tissue culture in in vitro. Syrap Sac Exp Biot. 1975; 11: 119-131.
8. Murashige T. Plant propagation by tissue culture: practice with unrealised potential. In: Ammirato PV, Evans DA, Sharp WR, Bajaj YPS, eds. Handbook of plant cell culture. McGraw Hill Publishing, New York. 1990.
9. Kohmura H, Chokyu S, Harada T. Application of a new micropropagation system involving induction of bud clusters and somatic embryogenesis in *Asparagus*. Acta Horticult. 1996; 415: 119-128.
10. Schröder MB, Eimert K. Somatic embryogenesis in *Asparagus.* In: Quality enhancement of plant production through tissue culture (COST 843). Finland, 2000: 42-43.
11. Gupta S, Kumar A, Sharma SN. Improvement of seed germination in *Asparagus racemosus* Wild. J Herbs Spices Med Plants. 2002; 9(1): 3-9.
12. Saensouk P, Suddee N. No title. KKU Res J (Thailand). 2004: 31-39.
13. Pontarolli C, Camadro EL. Plant regeneration after long-term callus culture in clones of *Asparagus officinalis* L. Biocell. 2005; 29(3): 313-317.
14. Bopana N, Saxena S. *Asparagus racemosus*: ethnopharmacological evaluation and conservation needs. J Ethnopharmacol. 2007; 110(1): 1-15.
15. Dutta IC. Non timber forest product of Nepal. Hillside Press Ltd, Kathmandu Nepal. 2007: 44-45.
16. Bopana N, Saxena S. *In vitro* propagation of a high value medicinal plant: *Asparagus racemosus* Wild. In Vitro Cell Develop Biol. 2008; 44(6): 525-532.
17. Hurgoiu F, Blidar CF. Study regarding to *Asparagus officinalis* L. calus reaction, subcultured on aseptic medium with various growth regulators. Anal Univ Oradea Fasc Biol. 2008; XV.

18. Kumar A, Vijay N. *In vitro* plantlet regeneration in *Asparagus racemosus* through shoot bud differentiation on nodal segments. Science 2.0. 2009.
19. Pant, Krishna K, Joshi SD. Direct multiple shoot induction from the seed explants of wild Nepalese *Asparagus racemosus* Wild. Plant Arch. 2009; 9(1): 443-446.
20. Shrivastava N, Rajani M. Multiple shoot regeneration and tissue culture studies on *Bacopa monnieri* (L.) Pennell. Plant Cell Rep. 1999; 18(11): 919-923.
21. Rajkarnikar KM, Sainju HK, Bhatta GD. *In vitro* culture of *Rauwolfia serpentine* L. Benth. Ex. Kurz. Proc Nepal Japan Joint Symp. 2000: 232-234.
22. Ranjitkar KM, Bhatt GD, Adhikari MK. Micro propagation of *Swertia ciliata*. In: Scientific Publication Bull Dept. Plant Res. No. 24. Natural Product Research Laboratory, Thapathali, Kathmandu, Nepal. 2004: 10-12.
23. Paudel N, Paudel LP, Rai PD. Callus induction and shoot regeneration of *Lycopersicon esculentum* L. Int J Sci Res. 2017; 6(8): 1346-1348.
24. Aryal MR, Paudel N, Ranjit M. Elimination of Chhirkey and Foorkey viruses from meristem culture of large cardamom (*Amomum subulatum* Roxb.). Eur Online J Nat Social Sci. 2018; 7(2): 424- 442.