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IN VITRO PROPAGATION OF "CRITICALLY ENDANGERED" MEDICINAL PLANT-*ASPARAGUS RACEMOSUS*

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ABSTRACT

1. *Asparagus racemosus* is an herb used as a rasayana in Ayurveda. The root of *A racemosus* is popular for its various medicinal uses such as appetizer, stomachic, tonic aphrodisiac galactogogus etc. In this experiment we have found the shoot induction after 3 weeks when Nodal explants segments were cultured in basal medium supplemented with BAP, Kin and Ads and Shoots induction on MS basal medium supplemented with BAP + kin were transferred to MS basal medium containing BAP + Ads and BAP + Kin respectively to the Proliferation shoots. Stem segments cultured on MS basal medium supplemented with various concentrations of BAP, AdS and Kin induce shoot, But a combination of BAP + Kin and BAP + Ads showed the highest percentage of shoot proliferation after 7-14 days. The elongated shoots (3-4 cm length) derived from MS Basal medium supplemented with BAP & ADS were excised and Transferred to MS medium containing NAA + BAP + kin and BAP + NAA for rooting. But a combination of NAA + BAP + Kin showed highest percentage of root formation within7-10 days. Eighty percent of shoots produced well developed roots in 15-20 days when MS medium was supplemented with NAA (1.0mg/l)
2. Highest callus induction from nodal explants was found best in combination of 1.0 mg/L NAA + 1.0 mg/L 2,4-D + 0.5 mg/L BAP.

KEYWORDS : ***Asparagus Racemosus,*** **shoot induction, Callus Induction , Root induction, BAP, Kin , NAA , 2,4-D and Ads**

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INTRODUCTION

Asparagus racemosus Wild.

Is a multidimensional medicinal plant. Family *Liliaceae* English name Asparagus, Indian name shatmuli, Satavari, (Sanskrit) Satawar, satavari, (Hindi) , Its distribution Tropical and subtropical India. It is straggling or scan dent, much branched, spinous shrub. The plant is a climber growing to 1-2m in length. The leaves are like pine needles, small and uniform. The inflorescence has tinny white flowers, in small spikes. The roots are finger like and clustered [15]**.** It has thorny branches the ripen fruit is small, round and red. Seeds are black. Its phytoestrogen properties are extensively used in combating menopausal symptoms and increasing lactation [18]**.** It also has antioxidants[11] properties [17] and is widely used in Ayurveda for treating dyspepsia **.** The major active medicinal constituents are steroidal saponins shatavarins I-IV that is present in the roots of the plant [19]**.** According to Ayurveda, asparagus is much useful in case of reproductive disease. Plant is rich source of plant derived estrogens. It is cardio tonic, hypoglycemic, antioxytocis to uterine contraction, diuretic, antioxidant, insulin secreting an improving potency, many formulation based on Asparagus are in commerce used for bleeding disorders, gout, low sperm count[8]**.** The methanol extracts of roots of Asparagus wild reported to show considerable *in vitro* antibacterial activity against various common pathogens[13]**.**

There is nothing to hoist in speaking that *A. racemosus* is a doctor. It is suggested in nervous disorders, dyspepsia, diarrhea, dysentery, neuropathy, cough, certain infectious disease[8]. Root paste which stimulates milk secretion, is used as an invigorating tonic to lactating women. It is taken to treat high fever. Root tubers are fed to get relief from milking disorder of cattle, which is regarded as appetizer. They are also useful in dysentery, tumors, inflammations, disease of blood and eyes, throat complains, tuberculosis leprosy, epilepsy and high blindness. Roots are also used in disease of Kidney and liver, scalding urine, gleets. Fruits are eaten to treat pimple[6,16]. The whole plant is used for treatment of diarrhoea, Diabetes, and Rheumatism. Seed are also used for blood purification. It has been reported in the Indian and British Pharmacopoeias and in traditional systems of medicine such as Ayurveda, Unani, and Siddha. The multiple uses of this species have increased its commercial demand, resulting in over-exploitation. Because of destructive harvesting, the natural population of *A.racemosus* is rapidly disappearing, and it is recognized as vulnerable[4]**.** During the last two decades the technique of plant tissue culture has developed as a new and powerful tool for crop improvement[5]. The development of an efficient micropropagation protocol will play significant role in meeting the requirements for commercial cultivation, thereby conserving the species in its natural habitat.

MATERIALS AND METHODS

Preparation of Stock Solution of Plant Growth Regulator

The stock solutions of various chemicals were prepared in double distilled water and stored in sterile reagent bottles in a refrigerator after filtering the contents, Benzylaminopurine (BAP), Kinetin (Kin) and Adenine Sulfate (AdS) were dissolved first in 1N NaOH solution and then, the required amount of double distilled water was added to make the required concentration of stock solution. Fresh solutions of organic salts and vitamins were used in the culture media.

Preparation of culture media

Double distilled water was used for the preparation of medium. The amounts of macro and micro nutrients, organic salts, vitamins, growth regulators and sucrose were added to the double distilled water. The final volume was made in a graduated cylinder/breaker by adding double distilled water. The pH of the solution was adjusted to 5.7-5.8 using either 0.1N HCl or 0.1N NaOH. For solidification of the medium, agar powder (Tissue culture grade, agar-agar type) @ 0.8% w/v was added to warm solution and then, boiled for proper dissolving and melting of agar powder. Then the medium was poured in glass vessel (i.e. culture tube or culture bottle). After that the

vessel was covered by lid or with aluminium foil. Basal nutrient media used during this investigation are given in table (1) was BM1-BM7 and RM1-RM5 modification of Murashige and Skoog medium (1962) was used in present project.

Procedure

The required quantities of stock solutions 50 ml of stock 1, 5ml of stock 2, 10ml of stock 3, 10ml of stock 4, 10ml of stock 5 was added. The pH of the solution was adjusted 5.8 by adding NaOH and HCl drop by drop. Final volume of medium was made to 1 litter by adding distilled water. Adding of 8% agar, which was dissolved in warm water followed by constant stirring. The medium thus prepared was dispensed into different culture tubes or culture bottles each having 20 to 30 ml of the media. The medium was autoclaved at 15 lbs pressure and 121°C temperature for 20 minutes. The medium was finally allowed to cool and solidify.

ISOLATION OF EXPLANT

Source of explants

1. The explant parts such as nodal segments shoot tip, and *In vivo* germinated seeds were taken from plants of *Asparagus racemosus Wild,* growing in open environmental conditions was carried out in Plant Tissue culture laboratory , Department of Biotechnology, School of life sciences ,SINGHANIA UNIVERSITY (Rajasthan) during February - March seasons and from *in vitro* germinated seeds.
2. Generally In tissue Culture, Meristematic ends of the plants tissues are used like the stem tip, auxiliary bud tip and root tip because these tissues have high rates of cell division.

INOCULATION

Before inoculation, inside the laminar air flow chamber the ultraviolet lamp was put on carefully to avoid contamination for at least 20 minutes, the working table of laminar air flow chamber was wiped thoroughly with 70% ethanol before use. The material required for inoculation was steam sterilized. The hands were cleaned with 70% ethanol. Then, the individual explants were inoculated in individual culture tubes or culture bottles having solidified culture medium. Forceps and scalpels were flame sterilized before each inoculation. The explants were then, cultured on MS medium supplemented with different concentration of BAP, Kn, AdS and NAA.

Inoculums for various experiment following explants has been used

- Apical/lateral young shoots from *in vitro* germinate seeds.
- Apical/lateral young shoots from *in vivo* field growing plant.
- Nodal segment (one node below apical bud).
- Callus tissue, derived from young shoot segments.
- Both freshly induced and old subculture callus tissue had been employed for morphogenetic studies.
- Three to four pieces of the inoculums were put in each culture vessels. Inoculation was performed in completely aseptic conditions.

Inoculation of nodal explants for shooting

Young shoot segments (nodal explants), 1-2 nodes below the apical bud were taken from *in vitro* and *in vivo* explants, their cut ends inserted in semisolid nutrient medium, for shooting induction

Inoculation of stem segments for callusing

To induce callus in shoot segments, young green shoot (1-1.5 cm) length obtain from shoots, *in vitro* growing conditions in aseptic cultures, were placed upon callus inducing.

Table 1
Different Combination of BAP, Kn and Ads used for Shoot proliferation.

S,No.	MS-Medium (Lit)	BAP (ml/L)	Kn (ml/L)	AdS (mg/L)
BM I	1	1.5	-	-
BM2	1	-	1.5	_
BM3	1	1	1	-
BM4	1	1.5	-	100
BM5	1	0.75		
BM6	1	-	0.75	-
BM7	1	0.5	0.5	-

Table 2
Different Combination of BAP, Kn and NAA used for Root proliferation.

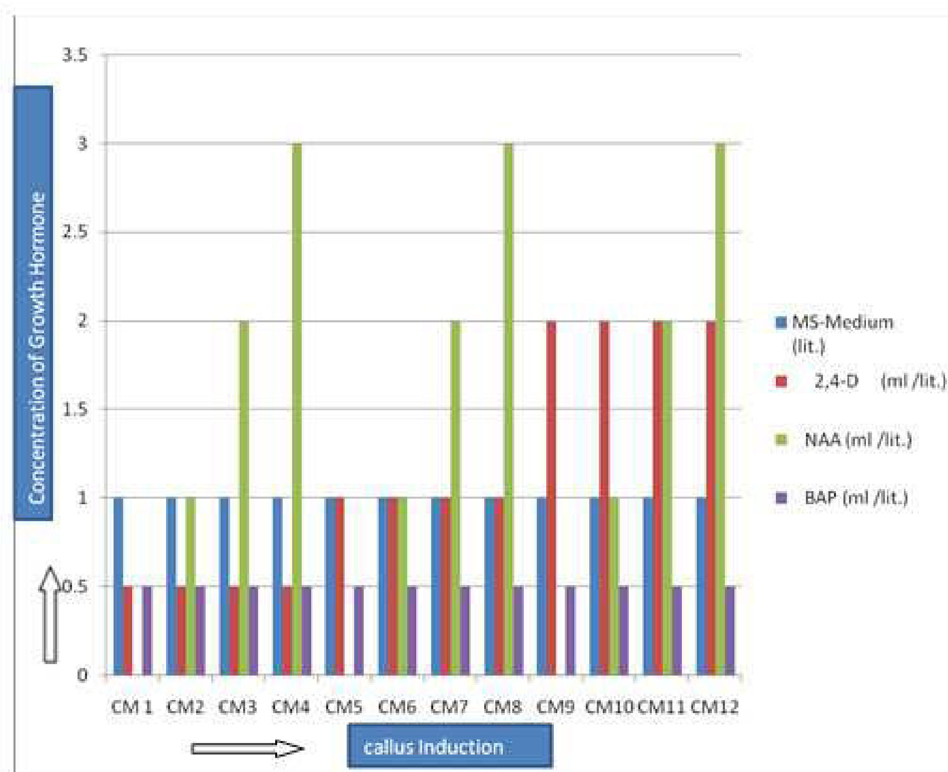
S.No.	MS-Medium (L)	BAP (m1/L)	Kin (ml/L)	NAA (m1/L)
RM 1	1	0.5	-	1
RM2	1	1	1	0.5
RM3	1	-	1.5	1
RM4	1	2	1.5	-
RM5	1	0.5	1.5	-

Callus induction
Callus was induced in the food jars as from the first week of inoculation. After 28 days, the explants together with their primary calli were subcultured in media of same composition in culture bottles.

Table 3
Different Combination of 2,4-D, NAA and BAP used for Callus Induction.

S.No	MS-Medium (lit.)	2,4-D (ml /lit.)	NAA (ml /lit.)	BAP (ml /lit.)
CM 1	1	0.5	0	0.5
CM2	1	0.5	1	0.5
CM3	1	0.5	2	0.5
CM4	1	0.5	3	0.5
CM5	1	1	0	0.5
CM6	1	1	1	0.5
CM7	1	1	2	0.5
CM8	1	1	3	0.5
CM9	1	2	0	0.5
CM10	1	2	1	0.5
CM11	1	2	2	0.5
CM12	1	2	3	0.5

Graph1
Different Combination of 2,4-D, NAA and BAP used for Callus Induction.



INCUBATION OF CULTURE
After inoculation cultures were kept in the incubation room where the temperature and light period was maintained at 25 ± 2^0C and 16 hrs. photoperiod with approximate 1500 lux intensity of light respectively. Source of the light was from the four florescent tube lights each of four feet and 40 W (Philips make) a total of 22 culture bottles for each treatment has been kept on the culture racks. After three week period callus developed in the medium or shoots/roots develop from explants.

RESULTS

Callus Formation
1. The details of the combinations used are presented in Table 4.
2. Maximum Induction of callus was found with combination of growth hormones i.e. 1.0 mg/L NAA + 1.0 mg/L 2,4-D + 0.5 mg/L BAP (Figure 15). In all the tubes fast growing green compact callus appeared within 25 to 30 days of inoculation. But the MS media containing only 1.0mg/L 2, 4-D and 0.5mg/L BAP (Figure 16).give Yellow coloured callus

Therefore after 2-3 subcultures in the induction medium, Calli were maintained in MS media supplemented with 1.0 mg/L NAA + 1.2 mg/L 2,4-D and 0.5 mg/L BAP. Under the above conditions the time required for the induction was about 25 to 30 days.

Table 4
Growth response of nodal explants of Asparagus racemosus.

Growth hormone (mg/L) 2,4-D	+NAA	+BAP	No. of tubes inoculated	---- Growth response of nodal explants ---- No. of tubes contaminated	% Induction	Colour	Average callus wt (g) after30 days	Average callua wt (g) after 60 days
0.5	0.0	0.5	30	0	20	Brown	0.24 ± 0.08	0.396± 0.08
0.5	1.0	0.5	30	0	40	Greenish yellow	0.34 ± 0.05	0.583± 0.13
0.5	2.0	0.5	30	3	30	Yellow	0.31 ± 0.12	0.554± 0.06
0.5	3.0	0.5	30	0	30	Brown	0.36 ± 0.03	0.583± 0.08
1.0	0.0	0.5	35	2	40	Green	0.29 ± 0.13	0.41± 0.04
1.0	1.0	0.5	35	1	100	Green	0.47 ± 0.07	0.830± 0.06
1.0	2.0	0.5	35	1	70	Greenish yellow	0.24 ± 0.09	0.425± 0.11
1.0	3.0	0.5	35	0	40	Brownish yellow	0.27 ± 0.06	0.373± 0.07
2.0	0.0	0.5	30	0	30	Green	0.28 ± 0.05	0.501± 0.14
2.0	1.0	0.5	30	3	30	Green	0.31 ± 0.03	0.583± 0.08
2.0	2.0	0.5	30	0	20	Brown	0.36 ± 0.07	0.487± 0.07
2.0	3.0	0.5	30	0	10	Brown	0.31 ± 0.08	0.431± 0.12

The Effect of plant growth regulators on differentiation of callus

There was formation of green nodular calli after 2-3 weeks when Nodal explants segments were cultured basal medium supplemented with BAP (2ml/1), Kin (1ml/1) and NAA (1ml/1).

Figure 1-6
Multiple bud, root and shoot induction both from the node as well as callus cultures with NAA and BAP in combinations (NAA 0.1+BAP 0.5, NAA 0.1+BAP 1.0, NAA 0.5+BAP 0.5, NAA 0.5+BAP 1.0, NAA 1.0+BAP 0.1 and NAA 1.0+BAP 2.0 respectively).

Shoot Initiation

Nodal explants were cultured on MS medium supplemented with different concentrations of BAP, Kinetin and Ads. The nodal segments get swollen after 3 days and the small originating shoot segment were observed as shown in Table 5(Graph.2**)**.When the hormone concentration is given as 1.5ml BAP(fig.10) ,1.5ml Kn(fig.7) and 1.5mlBAP and 100mg Ads in 1l MS medium then 60% response was

observed and it is 80%(max.) When the hormone concentration is given as 1ml BAP and 1ml Kn in 1l MS medium (fig.9). It was observed 40% when the hormone concentration is given as respectively 0.75ml BAP (fig.8) and 0.75ml Kn(fig.11) in 1l MS medium. When the hormone concentration is given as respectively 0.5ml BAP and 0.5ml Kn in 1l MS medium then 20% response was observed (fig.12).Good response was observed in fig.13 and fig.14 when transferred to same medium. The best combination for callus induction from nodal explants was found in fig.15 and 40% induction was found in fig.16 when added 1.0mg/l 2,4-D and 0.5mg/l BAP. Then the explants showed shoot initiation after three weeks.

Table5
The explants cultured on MS medium supplemented with different concentration of BAP, Kin and Ads.

S.No	MS-Medium (Lit)	BAP (ml/L)	Kn (ml/L)	AdS (mg/L)	No. of explants Used	Survival Rate	% age Response
BM1	1	1.5	-	-	10	8	***
BM2	1	-	1.5	-	10	8	***
BM3	1	1	1	-	12	10	****
BM4	1	1.5	-	100	12	10	***
BM5	1	0.75	-	-	8	4	**
BM6	1	-	0.75.	-	8	4	**
BM7	1	0.5	0.5	-	10	3	*

*%age of response Survival rate / No of explants used * 100 (* = 20%), (** = 40%), (*** = 60%), (**** = 80%).*

Graph 2
The explants cultured on MS medium supplemented with different concentration of BAP, Kin and Ads.

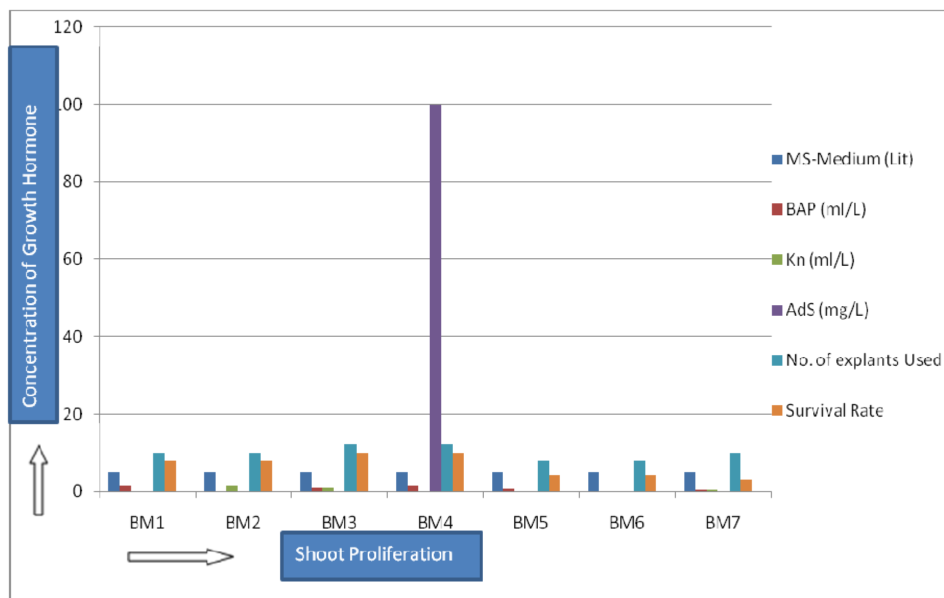


Figure 7
Shoot induction from nodal explants of Asparagus on MS Medium supplemented with Kin (1.5ml/L) after 3 weeks.

Figure 8
Shoot induction from nodal explants of Asparagus on MS Medium supplemented with BAP (0.75ml/L) after 3 weeks.

Figure 9
Shoot induction from nodal explants of Asparagus on MS Medium supplemented with BAP (1ml/L) + Kin (1ml/L) after 3 weeks.

Figure 10
Shoot induction from nodal explants of Asparagus on MS Medium supplemented with BAP (1.5ml/L) after 3 weeks.

Figure 11
Shoot induction from nodal explants of Asparagus on MS Medium supplemented with Kin (0.75ml/L) after 3 weeks.

Figre12
Shoot induction from nodal explants of Asparagus on MS Medium supplemented with BAP (0.5ml/L) + Kin (0.5ml/L) after 3 weeks.

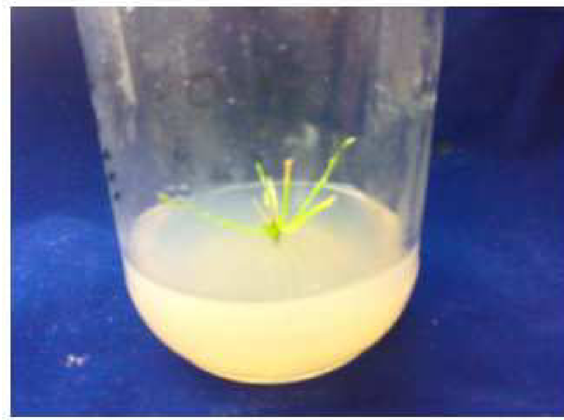


Figure 13
Shoot induction from nodal explants of Asparagus induced on MS Medium supplemented with Kin (0.75ml/L) and transfer to Kin (0.75ml/L) after 40 days.

Figure 14
Shoot induction from nodal explants of Asparagus induced on MS Medium supplemented with BAP (0.75ml/L) and transfer to BAP (0.75ml/L) after 40 days.

Callus Formation
Effect of plant growth regulators on differentiation of callus
There was formation of green nodular calli after 2-3 weeks when Nodal explants segments were cultured on basal medium supplemented with BAP (2ml/1), Kin (1ml/1) and NAA (1ml/1).

Figure 15
Callus in MS media with 1.0 mg/lit. NAA+1.0 mg/lit.2,4-D and 0.5mg/L BAP.

Figure 16
Yellow coloured callus developed in MS media containing only 1.0mg/L 2, 4-D and 0.5mg/L BAP.

Root Proliferation

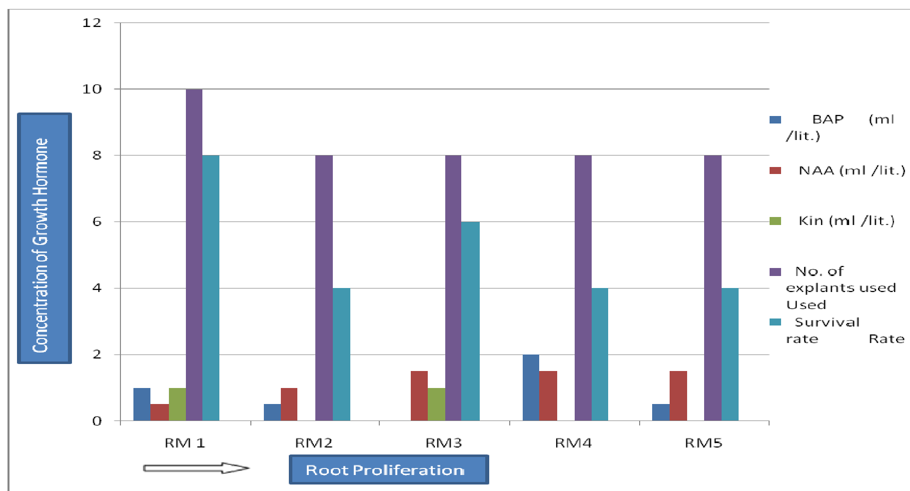
Root proliferation has been performed in *Asparagus racemosus* with the help of different concentration of BAP (1ml/lit.), kin (I ml /lit) and NAA (0.5 ml /lit.).The explants cultured on the media get swollen after 2-3 days and the small parts of roots were seen visually.

Table 6
In vitro cultures of Asparagus racemosus on MS medium supplemented with BAP, Kin and NAA.

S.No	BAP (ml /lit.)	NAA (ml /lit.) 0 0 0 0 0	Kin (ml /lit.)	No. of explants used	Survival rate	%age of response
RM 1	1	0.5	1	10	8	****
RM2	0.5	1	-	8	4	***
RM3	-	1.5	1	8	6	***
RM4	2	1.5		8	4	***
RM5	0.5	1.5	-	8	4	***

%age of response = Survival rate / No of explant used * 100
(* = 20%), (* * = 40%), (* * * = 60%), (** * * = 80%).

Graph 3
In vitro cultures of Asparagus racemosus on MS medium supplemented with BAP, Kin and NAA.



DISCUSSION

Culture Establishment (Stage-1)

Asparagus racemosus has a variety of usages. The first step on initiating in vitro culture is to successfully control the fungal as well as bacterial contaminations. The general surface sterilization procedure i.e. mercuric chloride for 5 min. failed to control the microbial infection in the explants. Hence, two procedures of surface sterilization were tried in the present study. The treatment of nodal explants with mercuric chloride for 5 min followed by sodium hypo chloride for 5 min. (T-2) was found to be the best in respect of explants survival (90%) and reduced explants contamination over the other treatments. Sodium hypochloride has bactericidal action and is generally used for surface sterilization and also helps in the controlling the microbial infection in the case of shoot proliferation.

Shoots Proliferation (Stage-2)

When the nodal explants inoculated on MS medium supplemented with different concentrations of BAP, Ads and Kinetin in different combinations, the best shoot induction was achieved when the cultures were transferred to MS medium supplemented with BAP (1 ml/lit.) + kinetin (1 ml/lit.) and BAP (1.5ml/lit.) + AdS (100 mg/lit.)[9]. In other experiment study reported in *Asparagus racemosus in vitro* shoot proliferation was obtained by culturing single node segments in Murashige and Skoog's (MS) medium supplemented with 3.69 µM 2- isopentyl adenine and 3% sucrose with a multiplication rate of 3.5[4]**.**

Shoot proliferation (Stage-3)

The shoot proliferation medium comprises of different concentration of BAP and Ads in different combinations. The best proliferation with well differentiated micro-shoots was achieved when the small nodal segments were transferred to MS medium supplemented with BAP (1.5 ml/L) and Ads (100mg/lit.).The treatments recorded 15-20 % proliferation after 1 week of first sub-culture. The shoot proliferation in tissue culture is largely due to the action of BAP. Optimum dose of BAP enhances the multiple shoot proliferation as well as length. The best response in terms of multiple shoot formation was observed on MS medium supplemented with BAP 1.0mg/L + IBA 0.5mg/L; NAA (3.0mg/L) was found to be effective in production of roots. The variety isd –16 showed better response than variety isd - 28 towards shoot multiplication[12] . In other experiment study reported high rate of multiple shoots was obtained from nodal explants of *Asparagus racemosus* Roxb. On MS supplemented with 0.27 µM NAA, 0.46 µM Kn and 0.6 agar[14]**.**

Callus Formation and Proliferation (Stage-4)

The callus formation obtained when nodal explants inoculated on MS medium supplemented with different concentrations of BAP (2ml/1) and Kinetin (1.5ml/l) in different combinations. The best callus formation was achieved. Begum etal found 3-5mg/L of 2,4-D produced highest percentage of callus induction in Bangladeshi sugarcane varieties (Viz Nagarbari, L.jaba,isd – 16,isd-20 and clone I.123)[2]. In other experiment study reported in *Asparagus racemosus* The highest efficiency of callus formation was observed in the medium containing different concentration of 2, 4-D and kinetin[1].

Root Proliferation (Stage -5)

The root proliferation medium comprises of different concentration of BAP, Kin and NAA. The best proliferation with well initiated roots was achieved when the cultures were transferred to MS medium supplemented with BAP (I.0ml/lit), kinetin (1.0ml/lit.) and NAA (0.5ml/lit). The treatments recorded 10-15 % proliferation after 1-2 weeks. In other experiment study reported in *Asparagus racemosus* MS with 1.48µ M IBA, 3.90 µ M ancymidol and 3% sucrose[14]. *In vitro* culture of different *Asparagus* species incubation for three weeks led to a vigorous root growth recorded on MS with 1.48µ M IBA and 3.90µ M ancymidol. Shoots on ancymidol supplemented medium appeared healthy, thick and rigid compared to those obtained from MS supplemented with

auxins and cytokinins[20]. The present work lends support from the previous work done in the micropropagation of *Asparagus racemosus.* The highest shoot multiplication (4.4/shoot/explants) was achieved on six weeks on MS supplemented with BAP (1 ml/lit.), kinetin (1ml/lit.) and NAA (0.5ml/lit), Shows better performance than BAP (0.5ml/lit.), and NAA (1ml/lit). When the propagated shoots were transferred to a rooting medium (full, half, quarter strength) MS, supplemented with NAA concentrations, the highest rooting percentage was achieved in full strength MS media supplemented with NAA (1 ml/lit).

CONCLUSION

Nodal segments and apical buds of 20-30 days old *Asparagus racemosus* wild Plants were inoculated on MS medium supplemented with BAP (1ml/L) + Kinetin (1ml/L) and BAP (1.5ml/L) + Ads (100mg/L) and then transferred to same medium was found best shoot induction. Proliferated shoots were separated and transferred to NAA (0.5mg/l) + BAP (1mg/l) + kin(1mg/l) medium was found to best roots formation. Best regeneration of shoot was achieved when they were cultured on MS medium supplemented with BAP 1.0mg/L and IBA 0.5mg/L. Among the different media tested with 3mg/L NAA and 5% sucrose supplemented media proved best production of roots[7] and in other studies the best combination for callus induction from nodal explants was found to be 1.0 mg/L NAA + 1.0 mg/L 2,4-D + 0.5 mg/L BAP[10.] Therefore callus induction and regeneration of shoots and roots offers a definite scope for further improvement of this adapted genotype through gene manipulation using other biotechnological techniques.

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