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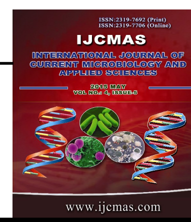

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Original Research Article

Rapid In vitro Micro Propagation of *Asparagus racemosus* Willd. from Nodal Explants

L. S. Patel[1*] and R. S. Patel[2]

[1]Jagdishprasad Jhabarmal Tibrewala University, Jhunjhunu, Rajasthan, India
[2]Maninagar Science College, Ahmedabad, Gujarat, India

**Corresponding author*

Keywords

Micro-propagation, Callus culture, Growth regulators, Root induction, Hardning

A B S T R A C T

Standardization of protocol for induction of callus and regeneration of plantlets was established through *in vitro* culture using Nodal explants of *Asparagus racemosus* Willd. The callus induction, multiple shoot regeneration and root induction was observed by using different concentration and combination of growth regulators. The highest percentage of callus induction was observed in MS medium supplemented with 0.1 mg/l NAA. The best response in terms of multiple shoot induction was observed on MS medium with IBA 1.0 mg/l + BAP 1.0 mg/l. When *in vitro* shoot lets were inoculated on to the half-strength MS basal media, rooting was observed with IBA 1.5 mg/l in nodal explants. Rooted shoots were transplanted in the green house for hardening and their survival rate was 75% in the field condition.

Introduction

Plant tissue culture techniques have become a powerful tool for studying and solving basic and applied problems in plant biotechnology. During the last thirty years, micro propagation and other *in vitro* techniques have become more widely used in commercial horticulture and agriculture for the mass propagation of crop plants (George, 1993). *In vitro* cell and tissue culture methodology is envisaged as a mean for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large-scale production, and for genetic manipulation studies.

Combinations of *in vitro* propagation techniques and cryopreservation may help in conservation of biodiversity of locally used medicinal plants.

In India, the Red Data Book has reports endangered species total 427 in which 124 species are endangered, 100 species are rare, 81 species are vulnerable, and 28 are considered extinct, 34 insufficiently known species. There are more than 300 species around the world (Gaur, 1999). The genus, *Asparagus* consists of herbs, shrubs and vines that are widespread all over the world

and represents highly valuable plant species having therapeutic and nutraceutical importance in addition to being consumed as food (Shasnay *et al.,* 2003). Medicinal plants play a vital role to preserve our health. *Asparagus racemosus* is one of the important medicinal plants extensively used by the traditional practioners in India for its medicinal value. The leaves and the tuberous roots of *Asparagus* are medically important in several diseases. *A. racemosus* is distributed throughout tropical and sub-tropical parts of India up to an altitude of 1500 m. (Velvan *et al.,* 2007). The healing qualities of Shatavari are useful in a wide array of ailments. Being a rasayana or rejuvenating herb, its restorative action is beneficial in women's complaints.

A. racemosus is mainly known for its phytoestrogenic properties. With an increasing realization that hormone replacement therapy with synthetic oestrogens is neither as safe nor as effective as previously envisaged, the interest in plant-derived oestrogens has increased tremendously making *A. racemosus* particularly important (Bopana and Saxena, 2007). Roots of *A. Racemosus* were found to possess antioxidant and anti-ADH activity (Kamat *et al.,* 2000; Wiboonpun *et al.,* 2004), anti-tumour and anticancer activity (Senna *et al.,* 1993; Shao *et al.,* 1996; Diwanay *et al.,* 2004), anti-ulcerogenic activity (Datta et al., 2002), anti-inflammatory activity (Mandal *et al.,* 1998) and antimicrobial activity (Mandal *et al.,* 2000).

From mature plants, used Nodal explants for micropropagation in many plant species like *Prosopis juliflora* (Nandwani and Ramawat, 1991), *Searsia dentate, Melia azedarach* (Hussain and Anis, 2009), *Oroxylum indicum* (Gokhale and Bansal, 2009). *Morus alba* (Balakrishnan *et al.,* 2009), *Crataeva adansonii*, *Spondias mangifera* (Tripathi & Kumari, 2010), *Acacia auriculiformis* (Girijashanker, 2011) *Dalbergia sissoo* (Ali *et al*., 2012).

Standardization of protocols for *in vitro* multiplication of satawari have been reported by many authors (Pise *et al.,* 2011; Mehta and Subramanian, 2005; Evans and Trease*,* 2002; Sharan *et al.,* 2011; Singh *et al.,* 2013).

Materials and Methods

Explant source: Micropropagation is usually initiated from explants, which are small pieces collected from whole plant. Nodal explants of Shatawari were collected in Patan district home garden, Gujarat (N. G.), India. All the plant samples of shatawari was thoroughly washed with tap water followed by washed with distilled water were for remove any dirt or filthy particles present on the surface; Fungi and bacteria contamination that was controlled by the first step in the *in vitro* culture sterilization. The collected explants were thoroughly rinsed with tap water for 30 minutes for removal of the external dust and then surface sterilized with 1 % Bavistin (fungicide) for 30 minutes in ordered sodium hypochlorite (NaOcl) for 5 minutes, and then 0.1% $HgCl_2$ for 2-5 minutes in a Laminar Air flow Chamber under aseptic conditions, finally washed thoroughly with sterile for several times for removal the traces of HgCl2. Finally, the explants for the sterile petriplate have been chosen. For microbial infection, a widely used sodium hypochloride surface sterilization.

Culture medium and condition: The young meristem cutting explants were inoculated on to sterilized semisolid basal MS medium (Murashige and Skoog's, 1962) supplemented with different concentrations

and combinations of different plant growth regulators.

Callus induction: In callus induction, used auxins like NAA, IAA, IBA, 2,4D (2, 4-dichlorphenoxyacetic acid) and cytokines like BAP (6-benzylaminopurine) and KIN (6- furfurylaminopurine) as supplement to the basal medium individually as well as in different combinations. For the entire study the PGRs concentration was used in mg/l (milligram per liter) and finally morphogenic responses were recorded.

Shoot regeneration Medium: The developed calli were transferred to regeneration medium various concentrations of growth regulators alone and in combination also. The percentage of shooting, days required for shooting, shoots per explants and shoot length were estimated.

Rooting medium: The elongated shoots were transplanted on half strength MS medium having various concentration of IBA, IBA with BAP (0.1-2 mg/L) either alone or in combinations. The data were recorded in terms of percentage of rooting, number of days required for rooting and root length.

Environmental condition: The pH of the medium was adjusted to 5.8 before gelling with Agar and prior to autoclaving for 20 min at 120° C and at 15 lbs psi pressure. Molten medium of 20 ml was dispensed into the culture tube and plugged with non absorbent cotton wrapped in one layer of cheesecloth.

All the cultures were incubated in a growth room with a 16h photoperiod except callus culture and the temperature was maintained at 25 ± 30° C with 50-60% relative humidity in the culture room were manipulated according to experimental conditions to get optimal results. Each treatment consisted of 5 replicates and repeated thrice.

Acclimatization and transfer of plantlets to soil: *In vitro* regenerated plantlets were potted on plastic pots containing equal ratio of sterile sand, soil and farmyard manure (1:1:1), the plantlets were covered with transparent polythene bag to maintain nearly 80% humidity level for first ten days. After this the plantlets were shifted to green house at $25 + 2^{o}$C under less humidity and natural sunlight and finally, they were transplanted in the natural condition.

Statistical Analysis: Each experiment was run with 3 replicates and repeated five times. Quantified the all effect of different treatment and determined the level of significance by ANOVA (analysis of variance). The data was represented by mean ± standard error. The data were analyzed statistically using single way analysis of variance (ANOVA).

Results and Discussion

Callus induction

Callus induction was observed after inoculation of the explants on MS medium containing different concentrations of auxine and cytokines.

Most of the cultures in hormone media induced callus in the beginning but MS hormone free media showed no response of any kind in callus induction with all the explants.

The maximum % of callus formation in nodal explants to be found (93.33 ± 0.00) was observed in 0.1 mg/l NAA were the induced calli were Greenish Yellow in colour and structurally Compact with dry

weight 0.82 ± 0.04gm after 25 days (Fig.1) followed by (92.86 ± 0.00) was observed in 2.0 mg/l KN were the induced calli were Dark brown in colour and structurally friable with dry weight 0.56 ± 0.03gm (Fig.2) followed by (91.67 ± 0.00) was observed in 1.0 mg/l NAA + 0.5 mg/l BAP were the induced calli were brown in colour and structurally friable with 0.80 ± 0.04gm dry weight (Fig.3). The callusing response gradually decreases with increases the concentration of NAA, gradually increases with increases the concentration of KN. The highest callusing (0.82 ± 0.04 and 0.80 ± 0.04 gm dry weight) was obtained in 0.1 mg/l NAA and 1.0 mg/l NAA + 0.5 mg/l BAP respectively. The lowest callusing (0.16 ± 0.01 gm dry weight) was obtained in 1.75 mg/l 2, 4-D after 25 days (Table.1).

This preliminary study proved that nodal explants responded better at different concentration of NAA, BAP, KN and combination of NAA+BAP showed good response and statistically also more significant. Different concentration of 2, 4-D, NAA + KN and 2, 4-D + KN showed no good response which was not remarkable and statistically not significant. When IAA and IBA were used in place of NAA, BAP, KN and 2, 4-D, all explants died.

Yang established a very good tissue culture technique for micropopagation of Asparagus using different explants. However this protocol was lengthy and took long time. Using different explants as a source, several workers have developed protocol for *in vitro* culture of different Asparagus species (Stejner *et al.,* 2002, Nayak and Sen, 1998; Ghose and Sen 1994a, b 1996).

Shoot regeneration

Repeated subculture of explants was inoculated on fresh shoot proliferation medium with different concentration of hormone medium helped to achieve continuous production of healthy shoot.

In the 0.5mg/l BAP medium after 20 days, 85.71% explants produced 7.80±0.16 shoots per culture (Fig.4) and the average length of the shoot was 2.80 ± 0.12cm. In the 0.5mg/l NAA + 1.0mg/l BAP (Fig.5) and 1.0mg/l NAA + 2.0mg/l BAP (Fig.6) medium after 16 and 21 days respectively, 83.33% and 80.00% explants produced 9.00±0.14 and 8.40±0.30 shoots per culture and the average length of the shoot was 2.56 ± 0.02cm and 3.02 ± 0.09cm. In the 0.5mg/l IBA + 1.0mg/l BAP (Fig.7) and 1.0mg/l NAA + 2.0mg/l BAP (Fig.8) medium after 25 and 16 days respectively, 80% and 75% explants produced 5.00±0.44 and 4.00±0.31 shoots per culture and the average length of the shoot was 2.50 ± 0.07cm and 2.84 ± 0.07cm in nodal explants (Table.2). Similar response was also observed in the shoot multiplication from axillary buds of *A.racemosus* willd. in MS medium with BAP, NAA and IBA (Afroz *et al.,* 2010) On the other hand, in *A. adscendens,* the best shoot multiplication medium reported by Mehta and Subramanian (2005) is MS supplemented with NAA and Kn.

But as the BAP concentration was increased gradually, callus induction and a few multiple shoots were observed. Here root induction was completely blocked although NAA, KN, 2, 4- D and its different concentration. NAA and IBA gave good response with BAP in shoot proliferation form nodal explants after 25 days.

In vitro rooting and acclimatization

Rooting shoot lets obtained from repeated cultures were transferred to half strength MS media supplemented with different concentration of NAA, BAP, KN, 2,4-D, IAA and IBA supplemented individually and combination to the media for rooting.

Table.1 Effect of Cytokinins and auxins supplemented individually and in various combinations on callus induction and callus growth of *Asparagus racemosus* Willd. from NODAL explants

Growth regulators (mg/l)	Concentration of growth regulators (mg/l)	% of callus formation	Intensity of callus	Nature of callus	Texture of callus	Dry weight of callus(g) after 25 days (Mean ±SE)
NAA	0.1	93.33	+++	Greenish Yellow	Compact	0.82 ± 0.04
	0.5	86.67	+++	Green	Friable	0.52 ± 0.03
	1.0	61.54	++	Green	Friable	0.50 ± 0.05
	1.5	53.33	++	Brown	Compact	0.46 ± 0.04
	1.75	0.00	-	-	-	0.00 ± 0.00
	2.0	0.00	-	-	-	0.00 ± 0.00
BAP	0.1	66.67	++	Light brownish	Friable	0.34 ± 0.03
	0.5	64.29	++	Dark brown	Friable	0.24 ± 0.02
	1.0	53.33	++	Light green	Friable	0.22 ± 0.02
	1.5	0.00	-	-	-	0.00 ± 0.00
	1.75	0.00	-	-	-	0.00 ± 0.00
	2.0	0.00	-	-	-	0.00 ± 0.00
KN	0.1	0.00	-	-		0.00 ± 0.00
	0.5	0.00	-	-		0.00 ± 0.00
	1.0	53.33	++	Dark brown	Compact	0.36 ± 0.02
	1.5	66.67	++	Green	Compact	0.40 ± 0.03
	1.75	71.43	++	Light brownish	Friable	0.50 ± 0.03
	2.0	92.86	+++	Dark brown	Friable	0.56 ± 0.03
2,4-D	0.1	66.67	++	Creemish brown	Friable	0.26 ± 0.02
	0.5	76.92	+++	Greenish yellow	compact	0.30 ± 0.01
	1.0	46.67	++	Brown	compact	0.26 ± 0.02
	1.5	73.33	++	Light brownish	Friable	0.24 ± 0.02
	1.75	66.67	++	Dark brown	compact	0.16 ± 0.01
	2.0	73.33	++	Brown	compact	0.20 ± 0.02
NAA+BAP	0.1+0.1	0.00	-	-	-	0.00 ± 0.00
	0.1+0.5	0.00	-	-	-	0.00 ± 0.00
	0.1+1.0	0.00	-	-	-	0.00 ± 0.00
	0.1+2.0	46.67	++	Light brownish	compact	0.36 ± 0.02
	0.5+0.1	6.00	+	Greenish yellow	compact	0.44 ± 0.02
	0.5+0.5	73.33	++	Dark brown	compact	0.48 ± 0.02
	0.5+1.0	60.00	++	Brown	Friable	0.58 ± 0.05
	0.5+2.0	76.92	+++	Creemish brown	Friable	0.60 ± 0.02
	1.0+1.0	64.29	++	Green	Friable	0.64 ± 0.04
	1.0+0.5	91.67	+++	Brown	Friable	0.80 ± 0.04
	1.0+1.0	66.67	++	Light brownish	compact	0.24 ± 0.03
	1.0+2.0	73.33	++	Dark brown	compact	0.18 ± 0.01
NAA+KN	0.1+0.1	40.00	+	Green	Friable	0.26 ± 0.02
	0.5+0.5	80.00	+++	Brown	compact	0.48 ± 0.02
	1.0+1.0	60.00	++	Light brownish	Friable	0.36 ± 0.04
2,4-D+KN	0.1+0.1	66.67	++	Creemish brown	compact	0.34 ± 0.04
	0.5+0.5	53.33	++	Dark brown	Friable	0.40 ± 0.03
	1.0+1.0	60.00	++	Brown	Friable	0.48 ± 0.06

* Significant at $p \leq 0.05$ level; (-) No response (+) Good (++) Very good (+++) Excellent; Mean and standard error of 5 replicates each.

Table.2 Effect of Cytokinins and auxins supplemented individually and in various combinations on multiple shoot formation of *Asparagus racemosus* Willd. from NODAL explants

Growth regulators (mg/l)	Concentration of growth regulators (mg/l)	Number of days required for shoots	% of shoot formation response	Number of shoots (Mean ± SE)	Shoot length (Mean ±SE)
BAP	0.1	16	83.33	5.40 ± 0.22	2.48 ± 0.19
	0.5	20	85.71	7.80 ± 0.16	2.80 ± 0.12
	1.0	17	60.00	3.20 ± 0.26	2.24 ± 0.12
	1.5	00	00.00	0.00 ± 0.00	0.00 ± 0.00
	0.75	00	00.00	0.00 ± 0.00	0.00 ± 0.00
	2.0	00	00.00	0.00 ± 0.00	0.00 ± 0.00
NAA+BAP	0.1+0.1	00	00.00	0.00 ± 0.00	0.00 ± 0.00
	0.1+0.5	00	00.00	0.00 ± 0.00	0.00 ± 0.00
	0.1+1.0	16	66.67	5.00 ± 0.31	1.54 ± 0.06
	0.1+2.0	13	50.00	3.80 ± 0.16	2.74 ± 0.10
	0.5+0.1	09	50.00	4.00 ± 0.31	3.46 ± 0.08
	0.5+0.5	12	71.43	3.00 ± 0.31	1.56 ± 0.05
	0.5+1.0	16	83.33	9.00 ± 0.14	2.56 ± 0.02
	0.5+2.0	13	60.00	1.80 ± 0.16	2.50 ± 0.07
	1.0+0.1	14	57.14	3.20 ± 0.26	1.28 ± 0.03
	1.0+0.5	12	75.00	2.40 ± 0.22	2.20 ± 0.04
	1.0+1.0	17	66.67	3.00 ± 0.31	1.72 ± 0.03
	1.0+2.0	21	80.00	8.40 ± 0.30	3.02 ± 0.09
IBA +BAP	0.1+0.1	00	00.00	0.00 ± 0.00	0.00 ± 0.00
	0.1+0.5	16	66.67	1.80 ± 0.26	1.54 ± 0.06
	0.1+1.0	13	75.00	3.80 ± 0.29	2.74 ± 0.09
	0.1+2.0	14	60.00	3.80 ± 0.26	2.88 ± 0.03
	0.5+0.1	12	33.33	2.60 ± 0.22	1.56 ± 0.05
	0.5+0.5	16	83.33	2.80 ± 0.16	2.56 ± 0.02
	0.5+1.0	25	80.00	5.00 ± 0.44	2.50 ± 0.07
	0.5+2.0	16	75.00	3.00 ± 0.31	1.28 ± 0.03
	1.0+0.1	00	00.00	0.00 ± 0.00	0.00 ± 0.00
	1.0+0.5	00	00.00	0.00 ± 0.00	0.00 ± 0.00
	1.0+1.0	12	71.43	3.60 ± 0.22	3.24 ± 0.04
	1.0+2.0	16	75.00	4.00 ± 0.31	2.84 ± 0.07

* Significant at $p \leq 0.05$ level

Mean and standard error of 5 replicates each.

Table.3 Effect of Cytokinins and auxins supplemented individually and in various combinations on in vitro grown shoots of *Asparagus racemosus* Willd. from NODAL explants

Growth regulators (mg/l)	Concentration of growth regulators (mg/l)	% of Root formation response	Number of Roots (Mean ± SE)	Root length (Mean ±SE)
IBA	0.1	75.0	2.20 ± 0.167	2.30 ± 0.032
	0.5	66.7	2.80 ± 0.167	3.12 ± 0.038
	1.0	60.0	3.00 ± 0.244	3.00 ± 0.080
	1.5	87.5	3.60 ± 0.178	4.00 ± 0.024
	1.75	00.0	0.00 ± 0.000	0.00 ± 0.000
	2.0	00.0	0.00 ± 0.000	0.00 ± 0.000
IBA+BAP	0.1+0.1	00.0	0.00 ± 0.000	0.00 ± 0.000
	0.1+0.5	00.0	0.00 ± 0.000	0.00 ± 0.000
	0.1+1.0	00.0	0.00 ± 0.000	0.00 ± 0.000
	0.1+2.0	00.0	0.00 ± 0.000	0.00 ± 0.000
	0.5+0.1	00.0	0.00 ± 0.000	0.00 ± 0.000
	0.5+0.5	75.0	2.20 ± 0.167	2.28 ± 0.052
	0.5+1.0	66.7	2.40 ± 0.228	2.32 ± 0.068
	0.5+2.0	50.0	2.80 ± 0.167	2.80 ± 0.032
	1.0+0.1	83.3	3.60 ± 0.228	3.62 ± 0.036
	1.0+0.5	75.0	1.40 ± 0.228	2.72 ± 0.030
	1.0+1.0	60.0	2.20 ± 0.296	2.66 ± 0.059
	1.0+2.0	50.0	1.60 ± 0.228	2.18 ± 0.033

* Significant at $p \leq 0.05$ level
Mean and standard error of 5 replicates each.

Fig.1 and **(1A)** Callus in MS medium with 0.1 mg/l NAA in Nodal explants

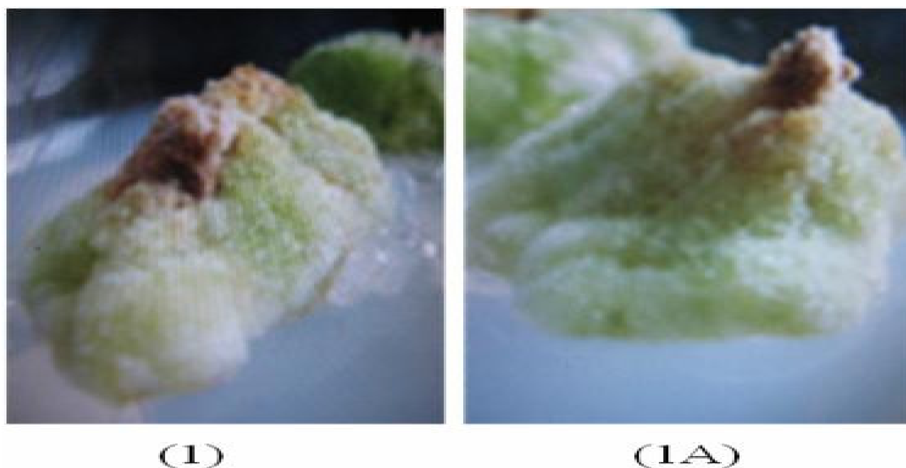


Fig.2 and **(2A)** Callus in MS medium with 2 mg/l KN in Nodal explants

Fig.3, (3A) and **(3B)** Callus in MS medium with 0.1mg/l NAA+0.5mg/l BAP in Nodal explants

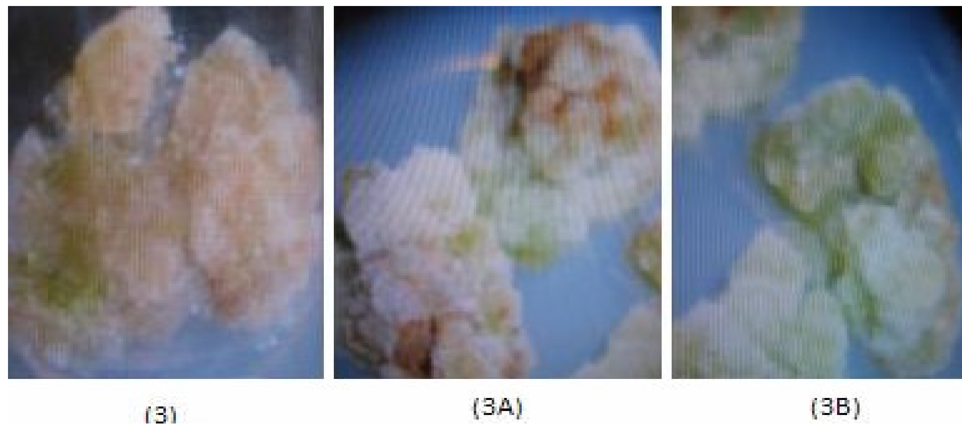


Fig.4 Multiple shoots in MS medium with 0.5mg/l BAP; **Fig.5** Multiple shoots in MS medium with 0.5mg/l NAA + 1.0mg/l BAP; **Fig.6** Multiple shoots in MS medium with 1.0mg/l NAA + 2.0mg/l BAP; **Fig.7** Multiple shoots in MS medium with 0.5mg/l IBA+ 1.0mg/l BAP; **Fig.8** Multiple shoots in MS medium with 1.0mg/l IBA+ 2.0mg/l BAP From nodal explants

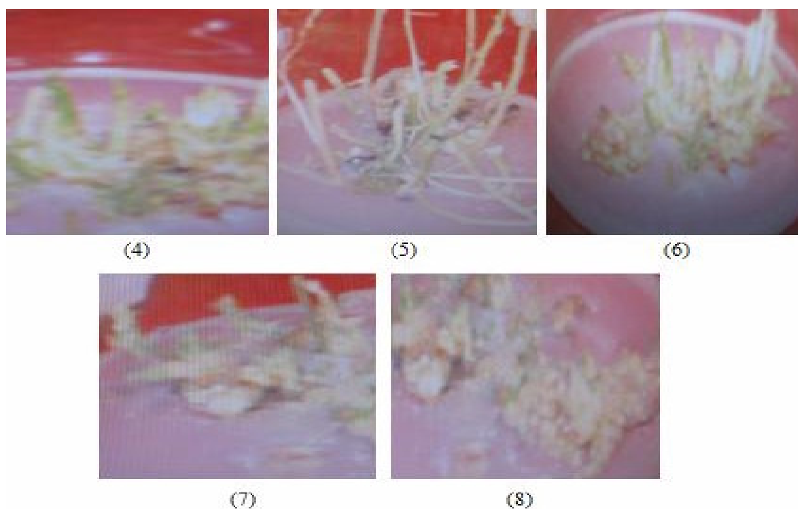
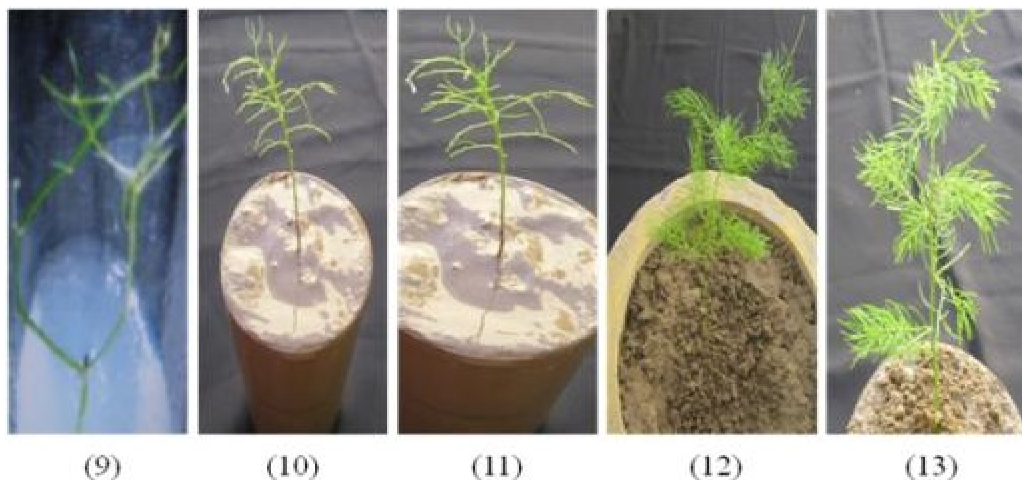


Fig.9 Multiple shoots from nodal explants; **Fig.10 -13** Acclimatized shoot in garden soil



Better root induction 87.5% and 83.3% development 3.6 ± 0.17 and 3.6 ± 0.22 roots per shoot with an average length of 4.00± 0.02cm and 3.62 0 ± 0.03cm was observed on MS media supplemented with 1.5mg/l IBA and 1.0mg/l IBA+ 0.1mg/l BAP respectively in nodal explants (Fig.9) all are statistically more significant (table.3).

In our experiment, other auxins like IAA and IBA did not show any kind of callus induction and shoot proliferation but root induction was gave good response in combinations of IBA and IBA with BAP concentration in nodal explants.

Benmoussa *et al.,* 1997 & Mehta and Subramanian, 2005 induced roots on media containing IBA in *A. densiflorous, A.adscendens and A.racemosus* shoots respectively whereas Gosh and sen, 1996 reported good rooting on MS media containing IAA in *A. verticillatus.* The number of roots induced was recorded to increase to with an increase in concentration but the root thickness gradually decrease with an increase in concentration of IBA and IBA with BAP.

The Regenerated plantlets have been not directly transplanted to field because of high mortality rate as they grown under optimal *in vitro* conditions like high humidity, low temperature and aseptic conditions on a nutrient medium with ample sugar for heterotrophic growth.

In the present study *In vitro* raised plantlets were first potted on plastic pots containing equal ratio of sterile sand, soil and farmyard manure (1:1:1), the plantlets were covered with transparent polythene bag to maintain nearly 80% humidity level for first ten-days. After this the plantlets were shifted to green house at 25 + 2^0 C under less humidity and natural sunlight and finally, they were transplanted in the natural condition. Rooted shoots were transplanted to the plastic pots showed 83% of survival. After this the plantlets were transplanted to green house at 25 + 2^0 C under less humidity finally, they were transplanted in the natural condition with 75% survival (Fig.10-13).

It is difficult to release a new variety of Asparagus by the plant tissue culture. Moreover also it takes long times to release a stable variety. Thus tissue culture technique can plays an important role in this regard for supply of disease free quality planting material in a year round basis and true to true types of the mother plant.

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