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Rapid Clonal Propagation of a Medicinal Plant - *Adhatoda Vasica* Nees. Using Tissue Culture Technique

M.A.K. Azad, M.N. Amin and F. Begum
Laboratory of Forest Products, Department of Forest Science,
Faculty of Agriculture, Utsunomiya University,
Utsunomiya 321-8505, Japan

Abstract: The proliferating axillary shoot cultures were established on MS medium supplemented with different concentrations of cytokinins and auxins, using nodal explants from the field grown mature plant of *A. vasica*. *In vitro* response of the explants to multiple shoot regeneration varied greatly with the position of the explanting branch on the donor plant. Highest frequencies of shoot formation and maximum number of shoots per explant were obtained on MS medium supplemented with 0.5 mg/l BA. The elongated shoots were rooted successfully on half strength of MS medium with 0.1-0.2 mg/l IBA or without any auxin. The complete plantlets thus regenerated *in vitro* were successfully transferred to the field.

Key words: Micro-propagation, nodal explant, *Adhatoda vasica*

Introduction

Herbal medicine is one of the most remarkable uses of plant based bio-diversity. As many as 75 to 90% of the world's rural people rely on herbal medicine for their primary health care. The success of any health care system depends on the availability of suitable drugs on a sustainable basis. Medicinal plants play a key role in world heath care systems (Bajaj and Williams, 1995). *Adhatoda vasica* Nees is an evergreen shrub, distributed from the Punjab in the North and Bengal and Assam in the South-East to the Cylon, Malaya and Singapore in the South. It is one of the most important medicinal plant of this region.

The plant is valued for containing bronchodilator alkaloids, mainly vasicine. All parts of the plant are used in herbal medicine and particularly the leaves are credited with insecticidal and parasiticidal properties. The root is useful in strangury, leucorrhoea, bronchitis, asthma, bilious vomiting, sore eyes, fever and gonorrhoea. It is a valuable antiseptic, antiperiodic and anthelmintic. The leaves are considered as a very efficacious remedy for all sorts of coughs and asthma, diarrhoea and dysentery. The leaves are also used for rheumatism. The flowers and the fruit are bitter, aromatic and antispasmodic. The fresh flowers are used in ophthalmia, lessen

strangury and jaundice (Kirtikar and Basu, 1994). Apart from its diverse medicinal and insecticidal uses the plant is also known for reclaiming degraded soil, artificial ripening of fruits and as a fodder for horses. The stem is used for production of a yellow dye and the wood for gun powder charcoal and beads (Singh ***et al.***, 1990). As the alkaloid content of plant varies with genotype therefore, it is recommended to propagate *A. **vasica*** plant using vegetative method (Dastur, 1985).

The plant is conventionally propagated by seeds and by stem cuttings. Chomchalow and Sahavacharin (1981) first attempted regeneration of *A. **vasica*** through tissue culture. Later Jaiswal ***et al.*** (1989) reported regeneration of *A. **vasica*** plantlets ***in vitro*** by culturing nodal explants on MS medium. However, the limited number of plantlets was produced in both cases. The present investigation was therefore, undertaken to establish protocol for large-scale regeneration of plantlets ***in vitro*** from the nodal explants of mature plant with a view to cloning high alkaloid containing genotypes.

Materials and Methods

Healthy, disease-free, tender twigs (4-5 cm) were collected from the mature plants and washed thoroughly under running tap water. Surface sterilization of the material included treatment with 1% Savlon for 10 min., washing in distilled water for 4-5 times, rinsing in 80% ethanol for 30 sec. and finally treatment with 0.1% $HgCl_2$ for different duration of time. To remove every trace of the sterilant, the material was then washed with sterile distilled water at least 4-5 changes of water. The shoot segments containing nodes (1–1.5 cm) were prepared from the surface sterilized material and were used as explants. The prepared nodal explants were cultured on different growth regulators supplemented agar gelled media. Induction of axillary growth and multiplication of shoots were examined using MS medium variously supplemented with BA and Kn alone or in combination with NAA and IBA. Elongated shoots were transferred to half-strength of hormone-free MS medium for root induction and development. The medium was fortified with 20-30 gm/l sucrose and gelled with 6 gm/l agar (BDH). The media pH was adjusted to 5.7±0.1 before autoclaving at 121°C under 1.1 kg/cm^2 pressure for 20 min. Cultures were maintained at 26±1°C with a light intensity of 2000-3000 lux provided during 16 h photoperiod.

Results

Establishment of *in vitro* culture

For the primary establishment of ***in vitro*** culture from field-grown plants surface sterilization of the explants was essential as microbial contamination attached to the surface of the explants. To over come contamination problem, surface sterilization of the explant was done with 0.1% $HgCl_2$ for 3, 5, 7, 10, 13, 16, 18 and 20 min. The explants collected from the mature plants of *A. **vasica*** were found to be very sensitive to $HgCl_2$ treatment. In the first attempt of surface

sterilization with 0.1% $HgCl_2$ for a treatment duration of 3-5 min resulted for contamination of 100% explants. Most of the cultured explants showed fungal contamination within 3 to 10 days of inoculation. Only 4-8% contamination-free cultures were obtained when the explants were treated for 7-10 min with 0.1% $HgCl_2$. These explants remained green and showed healthy growth and proliferation of axillary shoots. But when the explants were treated with $HgCl_2$ for 13-18 min, that caused death of 20-70% explants. On the other hand 100% explants died when the explants were treated with $HgCl_2$ for 20 min (Fig. 1).

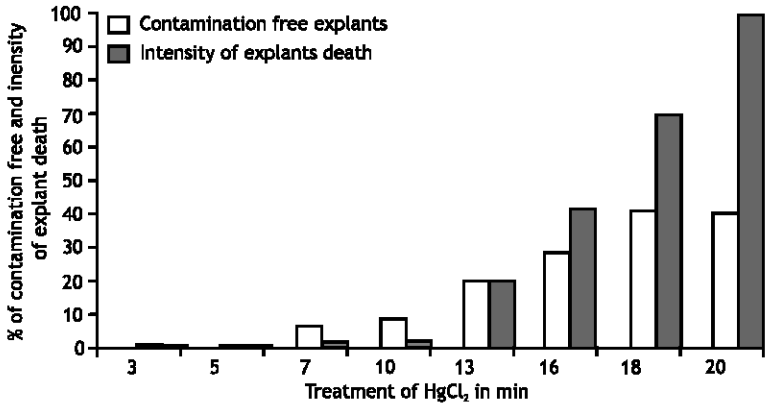

Fig. 1: Contamination free explants and intensity of explant death after treatment of $HgCl_2$

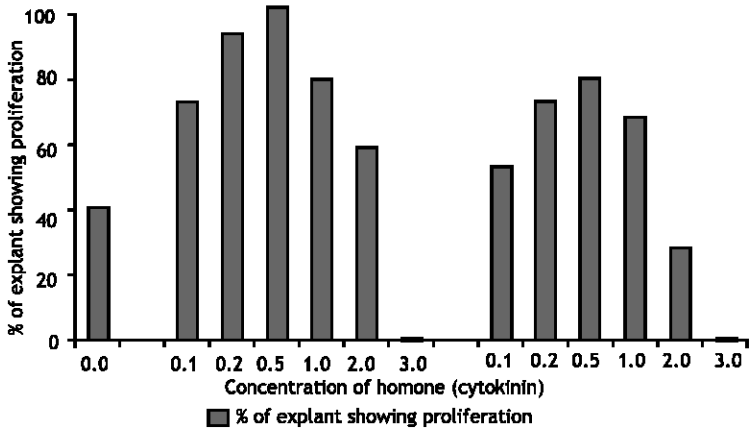

Fig. 2: Shoot proliferation rate of nodal explants of *A. vasica* on MS medium supplemented with different concentrations of BA and Kn

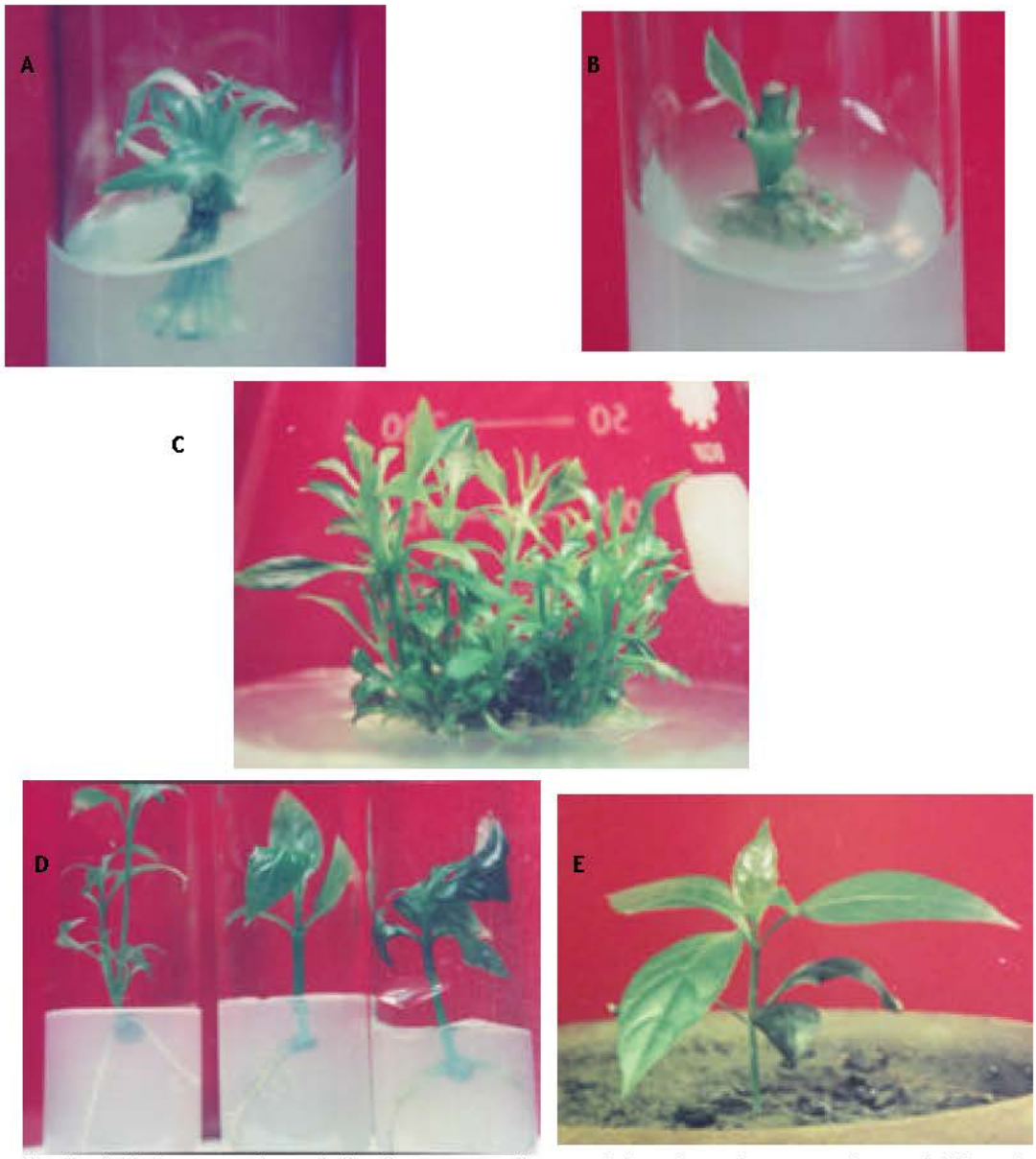

Fig. 3: A-E: Regeneration of platelet *in vitro* from nodal explant of mature plant of *Adhatoda vasica* Nees

A-B: Proliferation of axillary shoots from nodal explants cultured on MS medium supplemented with 0.5 mg/l BA (Fig. A) and 0.5 mg/l Kn (Fig. B) after six weeks of culture

C: Proliferation and development of multiple shoots from nodal segment

D: Formation of roots from the regenerated shoots cultured on auxin-free medium

E: Growth of transplant on the soil after twelve weeks of transfer under *ex vitro* condition

Table 1: Effects of different concentrations and combinations of BA with NAA and IBA on axillary shoot proliferation from *in vitro* grown nodal explant. There were 15-20 explants in each treatment and data ($\bar{x}$± SE) were collected after 6 weeks of culture

Growth regulators (mg/l)	% of explant showing proliferation	No. of total shoots per culture	No. of usable shoots per culture	Average length of the longest shoots (cm)	* Callus formation
BA + NAA					
0.2 + 0.1	65.0	4.0 ± 0.53	3.1 ± 0.45	3.3 ± 0.37	
+ 0.2	31.0	1.6 ± 0.46	1.0 ± 0.35	1.0 ± 0.25	++
+ 0.5	-	-	-	-	+++
+ 1.0	-	-	-	-	+++
0.5 + 0.1	93.0	10.5 ± 0.21	9.1 ± 0.37	4.0 ± 0.26	+
+ 0.2	71.0	3.8 ± 0.63	2.3 ± 0.16	3.2 ± 0.19	++
+ 0.5	37.0	2.3 ± 0.39	1.0 ±0.21	1.0 ± 0.12	+++
+ 1.0	-	-	-	-	+++
1.0 + 0.1	75.0	5.2 ± .33	3.8 ± 0.29	3.5 ± 0.38	+
+ 0.2	62.0	3.0 ± 0.19	2.5 ± 0.18	2.5 ± 0.27	++
+ 0.5	56.0	2.5 ± 0.21	2.1 ± 0.71	1.6 ± 0.63	+++
+ 1.0	-	-	-	-	+++
2.0 + 0.1	50.0	2.3 ± 0.45	2.0 ± 0.51	1.6 ± 0.32	++
+ 0.2	44.0	2.1 ± 0.24	1.5 ± 0.21	1.0 ± 0.20	+++
+ 0.5	19.0	2.0 ± 0.16		-	+++
+ 1.0	-	-	-	-	+++
BA + IBA					
0.2 + 0.1	59.0	3.0 ± 0.43	2.3 ± 0.34	2.1 ± 0.32	
+ 0.2	27.0	1.3 ± 0.24	1.1 ± 0.21	1.0 ± 0.12	+
+ 0.5	-	-	-	-	++
+ 1.0	-	-	-	-	+++
0.5 + 0.1	79.0	4.0 ± 0.21	3.1 ± 0.41	3.4 ± 0.17	
+ 0.2	60.0	3.5 ± 0.15	1.7 ± 0.56	2.6 ± 0.45	+
+ 0.5	35.0	1.8 ± 0.42	1.0 ± 0.36	0.5 ± 0.22	++
+ 1.0	-	-	-	-	+++
1.0 + 0.1	63.0	3.0 ± 0.34	2.1 ± 0.13	2.5 ± 0.64	+
+ 0.2	50.0	2.8 ± 0.57	1.5 ± 0.54	2.4 ± 0.55	+
+ 0.5	44.0	2.0 ± 0.63	1.0 ± 0.64	1.5 ± 0.21	++
+ 1.0	-	-	-	-	+++
2.0 + 0.1	41.0	2.0 ± 0.42	2.1 ± 0.32	1.3 ± 0.17	+
+ 0.2	35.0	1.5 ± 0.21	1.2 ± 0.23	1.0 ± 0.13	+
+ 0.5	16.0	1.2 ± 0.16	-	-	+++
+ 1.0	-	-	-	-	+++

* Callus rating value = (+) poor; (++) moderate; (+++) massive callus formation

Table 2 : *In vitro* rooting response of *A. vasica* micro cuttings without and with auxin at 25 °C under 16-h photoperiod. Each treatment consisted of 20 cuttings and data were collected after 6 weeks

Type of auxin	Conc. Of auxin (mg/l)	% of cutting rooted	No of roots per rooted Cutting	Average lenght of the roots (cm)	Days to emergence of roots	Callus formation at the cutting base
		60.0	1.2±0.25	2.1±0.13	7	*
NIL		80.0	2.5±0.41	2.5±0.27	9	*
		90.0	3.0±0.23	3.0±0.32	11	*
		95.0	3.2±0.35	3.5±0.45	13	*
		100.0	3.5±0.64	4.0±0.39	15	*
	0.1	100.0	2.3±0.53	2.6±0.63	10-15	-
IBA	0.2	100.0	3.3±0.31	3.2±0.21	8-10	-
	0.5	80.0	2.6±0.22	2.5±0.18	7-12	+
	1.0	-	-	-	-	+++
	0.1	100.0	2.1±0.13	1.9±0.23	8-12	-
NAA	0.2	90.0	2.8±0.33	2.5±0.31	7-10	+
	0.5	70.0	1.8±0.15	1.3±0.21	10-12	+++
	1.0	-	-	-	-	+++
	0.1	60.0	1.3±0.14	1.7±0.42	10-12	-
IAA	0.2	45.0	1.8±0.25	2.0±0.34	8-12	-
	0.5	30.0	1.5±0.17	1.5±0.25	8-10	++
	1.0	-	-	-	-	+++

(-) Indicate no rooting, (*) indicates no callusing, (+) indicate slight callusing, (++) indicate considerable callusing and (+++) profuse callusing

Axillary Shoot Proliferation

The nodal segments of field-grown, mature plant origin that survived in establishment stage were cultured on MS medium supplemented with BA and Kn at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0 and 3.0 mg/l for selecting optimum cytokinin concentration for the maximum shoot production. Data on different growth parameters from different treatments were recorded after 6 weeks of culture initiation following one transfer to the new medium. Data not has shown in this paper. Type and concentration of the cytokinins used remarkably influenced regeneration of axillary shoot from nodal segments. The cytokinin BA, at most of the concentrations, was comparatively more effective in proliferating axillary shoot whilst Kn was considerably less effective (Fig. 2). With the increase of cytokinin concentration from 0.1–0.5 mg/l percentage of explants showing proliferation and number of shoots per culture increased gradually. Further increase in cytokinin concentration to 1.0–2.0 mg/l did not improve any of the parameters but reduced the proliferation of shoots and at the highest level (3.0 mg/l) of BA the cultured explants failed to proliferate any shoot. The explants produced the highest number of 7.4±0.53 usable shoots (Fig. 3A) on the medium with 0.5 mg/l BA, followed by 4.9±0.13 usable shoots on the medium with 0.2 mg/l BA and lowest of 1.2 ± 0.08 usable shoots on the medium with 0.1 mg/l Kn

(Fig. 3B). The media containing 0.1, 0.2, 0.5, 1.0 and 2.0 mg/l of BA treatments induced proliferation of the axillary shoots in 73.0, 93.0, 100, 80 and 59.0% explants. Through this experiment it was realized that BA was the most effective cytokinin and the most preferred concentration of BA was 0.5 mg/l. At this concentration the other cytokinin, Kn produced remarkably lower number of usable shoots.

Effects of BA in Combination with NAA and IBA

In this experiment nodal explants from ***in vitro*** grown shoots were cultured on MS medium supplemented with different concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of BA in all possible combinations with 4 different concentrations (0.1, 0.2, 0.5 and 1.0 mg/l) of NAA and IBA for selecting best BA-NAA and BA-IBA combinations. The relative amounts and ratios of BA and NAA present in the medium remarkably influenced proliferation of axillary shoot from nodal segments (Table 1). In most of the cases presence of NAA decreased the rate of normal shoot development and simultaneously increased the rate of callus formation at the cut margins of the explants. However, normal axillary shoot formation occurred only at 0.1–0.2 mg/l of NAA with a higher proportion (0.5-1.0 mg/l) of BA. Among different treatments, combination of 0.5 mg/l BA and 0.1 mg/l NAA was found to be the best formulation where maximum frequency of axillary shoots proliferating culture (93.0%) and highest number of shoots per culture (10.5±0.21) and the number of usable shoots per culture (9.1±0.37) were observed (Fig. 3C). It was followed by the combination of 1.0 mg/l BA and 0.1 mg/l NAA. At these combinations of BA and NAA the proliferated axillary shoots were healthier than those proliferated at 0.5-1.0 mg/l BA alone. At rest of the combinations of BA and NAA callus began to form at cut ends of explants within 2 weeks of culture. Small (+) amount of callus had formed on 0.2 mg/l NAA with all levels of BA, whereas more callus (+++) produced when 0.5-1.0 mg/l NAA added with all levels of BA. Addition of 1.0 mg/l NAA to the medium containing all levels of BA failed to induce formation of any axillary shoot.

Results on the influence of various concentrations and combinations of BA and IBA in axillary shoot proliferation from nodal explants are given in Table 1. Like BA and NAA, BA and IBA combinations also affected normal growth and proliferation of axillary shoots. The maximum of 79.0% explants produced normal shoots when they were cultured with 0.5 mg/l BA and 0.1 mg/l IBA. Other combinations of BA and IBA in the initial media greatly altered the growth behaviour of the cultured explants that proliferated axillary shoots. Among the different formulations of BA and IBA used in this experiment, the medium fortified with 0.5 mg/l BA and 0.1 mg/l IBA produced the highest number of usable shoots (3.1±0.41 per culture). While the media supplemented with 1.0 mg/l IBA + all levels of BA failed to proliferate axillary shoots from the cultured explant.

In most of the media formulations callus began to form at the cut ends of the explant within 15-20 days of culture depending on the BA and IBA ratios. Media amended with 1.0 mg/l IBA + 0.2-2.0 mg/l BA consistently produced large amount of callus (+++) than those amended with other concentrations of the growth regulators tested. Small (+) amount of callus formed in cultures grown in presence of 0.1-0.2 mg/l IBA with all levels of BA.

In vitro Rooting

The process of ***in vitro*** root initiation, development and elongation normally requires the medium that contains auxins. But some plants produced root ***in vitro*** even in the absence of auxin. To prove that whether *A. **vasica*** micro cuttings could be rooted without auxin, they were cultured on half-strength of MS medium with or without any auxin supplement. In this experiment in auxin free medium no rooting was found from the base of any micro cutting before 6 days of culture. However, after 6 days of incubation, only 60.0% rooting was obtained. Rooting frequency increased gradually with the passing of incubation time and it reached to 100% after 15 days of culture on rooting medium. The maximum number (3.5±0.64) of roots per micro cutting and average length of roots (4.0±0.39 cm) were higher than those at auxin-supplemented medium (Table 2). There was also no basal callusing from any micro cutting base even after 30 days of incubation. On the other hand, regenerated micro-shoots were cultured on half-strength of MS medium containing 0.1, 0.2, 0.5 and 1.0 mg/l of IBA, NAA and IAA for rooting. The results of the three types of auxin on percentage of root formation, their lengths and the number of roots per shoot as observed six weeks after culture are shown in Table 2. Among the three types of auxin, IBA was found to be comparatively more effective than NAA and IAA at all concentrations. Among various concentrations tested 0.1-0.2 mg/l regardless of the kind of auxin, proved to be the best in eliciting the highest frequency of root formation. On the other hand, 1.0 mg/l of IBA, NAA and IAA did not induce any shoot but produced only callus at the cut margin of the micro-shoots. When rooting behaviour of *A. **vasica*** micro cuttings on auxin-free medium was compared with that on auxin-supplemented medium it was noticed that roots produced on the latter medium were either thin and weak at low concentrations of auxin or thick and malformed at high concentrations of auxin and in most cases rooting associated with basal callus formation. Therefore, it was conceived from the rooting experiments that *A. **vasica*** micro cuttings could be rooted better using auxin-free medium only by manipulating the incubation time (Fig. 3D).

Acclimatization

Rooted micro cuttings (plantlets) were initially planted in poly-bag containing garden soil and compost (1:1) or garden soil, sand and compost (2:1:2). After 7-10 days of indoor acclimatization, the plantlets from poly bags were transplanted directly to the field or to the larger pots (Fig. 3E). Plantlets were initially established in poly bags with a view to easy handling during transplantation

to the field. It was observed that the prevailing atmospheric conditions (mostly humidity and temperature) of transplanting season greatly influenced the initial survival of potted plantlets. In this regard, the month of October-November and February-March with moderate temperature and low humidity were found to be most suitable than any other months of the year. Similarly plantlets having 2-3 cm roots at their active elongation period survived better than those transferred with much elongated and branched root systems. It was observed that establishment of the transplants under ***ex vitro*** conditions was 60% on garden soil and compost potting mix and 80% on garden soil, sand and compost potting mix.

Discussion

For axillary shoot proliferation, nodal explants of mature field-grown plant were found to be better than those of ***in vitro*** grown seedlings. Many authors reported that explants from mature field-grown plant were better than seedling explants. It was found in cases of ***Holarrhena anttidysenterica*** (Datta and Datta, 1984), ***Asclepias curassavica*** (Paramanik and Datta, 1986) and ***Prosopis juliflora*** (Nandwani and Ramawat, 1991). In the present investigation the explants from mature-field grown plants produced significantly higher number of shoot in 100% culture.

BA and Kn at a concentration range 0.1-3.0 mg/l were tested for assessing the optimum concentrations of the cytokinins for early sprouting and maximum proliferation of axillary shoots. BA was found to be more effective than Kn on the proliferation and development of *A. **vasica*** shoots. Superiority of BA over other cytokinins in producing ***in vitro*** shoots has also been confirmed in other plants like ***Rosmarinus officinalis*** (Misra and Chaturvedi, 1984), ***Camellia sinensis*** (Arulpragasam and Latiff, 1986) and ***Atropa belladonna*** (Benjamin ***et al.***, 1987). Wareing and Phillips (1981) showed that synthetic cytokinin such as BA, was more active than naturally occurring cytokinin in shoot proliferation. Although BA at 0.2 and 0.5 mg/l produced more of less equal number of usable shoots but the total number of shoots proliferated at 0.5 mg/l was significantly higher, indicating that lower cytokinin concentration favoured shoot elongation while higher concentration favoured shoot proliferation.

For proliferation of shoots from nodal explants BA-NAA combination was also found to produce better result than other combinations and the explants produced only axillary shoots but no roots. On the shoot proliferation medium, a comparatively higher concentration of BA (0.5-1.0 mg/l) along with a lower concentration of NAA (0.1-0.2 mg/l) showed the best result. This is in agreement with the results of ***Camellia sinensis*** (Phukan and Mitra, 1984), ***Capsicum annuum*** (Agrawal ***et al.***, 1988) and ***Eucalyptus globosus*** (Islam ***et al.***, 1994). Contrary to the present result many plant species like ***Piper nigrum*** (Geetha ***et al.***, 1990) and ***Coffea arabica*** (Raghuramulu ***et al.***, 1989) produced multiple shoots from nodal explant on MS medium supplemented with Kn and NAA or IAA. This difference might be attributed to the genotypic difference of the plant materials used.

For inducing adventitious roots, micro-shoots prepared from ***in vitro*** proliferated shoots were cultured on agar gelled half-strength MS medium with of without any auxin supplement. In this experiment without auxin 100% micro-shoots produced root after 15 days and there was no callus formation at the cutting base of shoots. Similar observation was found of ***Helianthus annuus*** (Nataraja and Ganapathi, 1989). Percentages of root induction, number of roots per shoot were greatly influenced by the concentration and type of the auxins. Among three types of auxin used, IBA was found to be the best for root induction. This finding are in agreement with those observed in ***Cephaelis ipecacuanha*** (Jha and Jha, 1989), ***Ruscus hypophyllus*** (Wakhlu and Barna, 1989), ***Rehum emodi*** (Lal and Ahuja, 1989) and so other plant species.

Following the protocol described here selected genotypes of *A.* ***vasica*** can be clonally propagated with a shoot multiplication rate of 10 fold per 4-week, which is quite higher than the multiplication rates of earlier attempts (Chomchalow and Schavacharin, 1981 and Jaiswal ***et al.***, 1989).

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