STUDIES ON MICROPROPAGATION OF SEABUCKTHORN (*HIPPOPHAE RHAMNOIDES* L.)

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Objectives

- **Establishment of aseptic culture**
- **Improvement of multiple shoots formation in explants**
- **Improvement of rooting rate**

Methodology

- Plant materials: Active and dormant buds
- Sterilization of buds
- Eradication of phenolics accumulation
- Eradication of vitrification
- Promotion of shoots formation in explants
- Promotion of rooting in expalnts

Plant material-Seabuckthorn (*H. rhamnoides* ssp. *turkestanica*)

- **Indigenous and widely adapted (2500-3800 m asl) in Himalayas**
- **High yielding (5 kg fruits/plant) selection**
- **Rich in oil (5%).**

Cont..

Plant material for micropropagation

- Cuttings of seabuckthorn selection collected in October month from plus trees at HAREC, Kukumseri (2730 m asl) and buried in pit with wet sand at HAREC, Bajaura (1200 m asl).
- Cuttings treated with 500 ppm NAA and planted in January under green house conditions
- Active buds and dormant buds collected in March and October respectively from 2 year old plants

Chemicals for sterilization of buds

- 1500 ppm ascorbic acid and citric acid solution, each in double distilled water
- 0.1% Detergent (Tee- Pol) “
- 250 mg/100 ml Tetracycline “
- 70% Ethyl Alcohol “
- 0.1% Mercuric Chloride “

Eradication of phenolics

Treatment	Concentration/ Quantity
Ascorbic acid + Citric acid w/v in sterile DDW	1500 ppm each
Low temperature	4 ˚C
Pre culture of explant on plain Agar supplemented with sucrose and inositol	3% Sucrose, 100 ppm inositol

Eradication of vitrification & Formation of multiple shoots

- **MS medium**
- **½ MS “**
- **WPM “**

Hormone treatments

- **BAP 0.3: NAA 0.2 BAP 0.2: NAA 0.3 BAP 0.3: NAA 0.1 BAP 0.2: NAA 0.05BAP 1.0: IAA 0.5 BAP 0.5: IAA 1.0 BAP 0.05: IAA 2.0 BAP 2.0: IAA 0.05BAP 0.5: IBA 0.1 BAP 0.01: IBA 0.2 BAP 0.1: IBA 0.5**

(BAP: 6-Benzylaminopurine, NAA: Naphthalene acetic acid, IAA: Indole Acetic acid, IBA: Indole butyric acid)

Formation of roots in explants

- IBA 0.5 ppm on WPM
- IBA 1.0 ppm “ “
- IBA 1.5 ppm “ “
- IBA 2.0 ppm “ “
- IBA 2.5 ppm “ “

RESULTS AND DISCUSSION

Effects of various chemicals on sterilization of active and dormant buds

Active buds

Inoculation	0.1% Detergent	250 mg Tetracy	70% EtOH	0.1% $HgCl_2$	Contamination (%)	Survival (%)
50	30 min	----	1 min	4 min	36 (72%)	14 (28%)
50	30 min	15 min	1min	4 min	32 (64%)	18 (36%)
50	1 hr	1 hr	3 min	5 min	26(52%)	24 (48%)
50	1 hr	2 hr	3 min	5 min	23 (46%)	27 (54%)
50	1.5 hrs	2 hr	4 min	6 min	16 (32%)	34 (68%)
50	**2 hrs**	**2 hrs**	**4 min**	**6 min**	**2 (4%)**	**48 (96%)**

Dormant Buds

Inoculation	0.1% Detergent	250 mg Tetracy	70% EtOH	0.1% $HgCl_2$	Contamination (%)	Survival (%)
50	30 min	----	1 min	4 min	50 (100%)	0 (0%)
50	30	15 min	1min	4 min	50 (100%)	0 (0%)
50	1 hr	1 hr	3 min	5 min	50 (100%)	0 (0%)
50	2 hrs	2 hrs	5 min	7 min	45 (90%)	5 (10%)
50	2.5 hrs	4 hrs	10 min	12 min	30 (60%)	20 (40%)
50	4 hrs	8 hrs	13 min	16 min	15 (30%)	35 (70%)
50	**5 hrs**	**Over night**	**15 min**	**18 min**	**3 (6%)**	**47 (94%)**

Standardization of surface sterilization

Explant (Active buds & Dormant buds) treatments:

- **1500 ppm ascorbic acid and citric acid solution (one hour)**
- **Low temperature treatment (4-5 ˚C, 5-6 days)**
- **Surface sterilization with 0.1% Tee-pol detergent**

  (2 hours & 5 hours, respectively)
- **Tetracycline solution (250 mg/100 ml) (2 hours & Over night, respectively)**
- **EtOH 70% (4 minutes & 15 minutes, respectively)**
- **$HgCl_2$ 0.1% (6 minutes & 18 minutes, respectively)**

Effect of pH on phenolics accumulation in active & dormant buds (on MS medium)

- ✓ **At Media pH of 5.75, lowest level of phenolics accumulation**
- ✓ **Increase in phenolics accumulation with the increase in media pH**

pH	5.5	5.75	6.0	6.25	6.50	6.75	7.0	7.25
Apical buds	1.66	**1.25**	1.91	1.41	1.33	1.58	1.75	1.91
Dormant buds	1.83	**1.33**	1.58	1.66	1.81	1.75	1.91	2.36

Note: Based on visual observations on the scale of 0-3

Effect of pretreatment, low temperature, preculture and culture media on explant phenolics accumulation

Chemical Pretreatment	L.Temp. 4°C (Hours)	Plain Agar (with Sucrose & Inositol)	WPM	MS
1500 ppm ascorbic acid + citric acid each solution (one hour)	0	(+)	Phenolics (++++)	Phenolics (+++++)
	24	0	(+++)	(++++)
	48	0	(++)	(+++)
	72	0	(++)	(+++)
	96	0	(++)	(+++)
	120	0	(++)	(+++)
	144	0	(+)	(++)

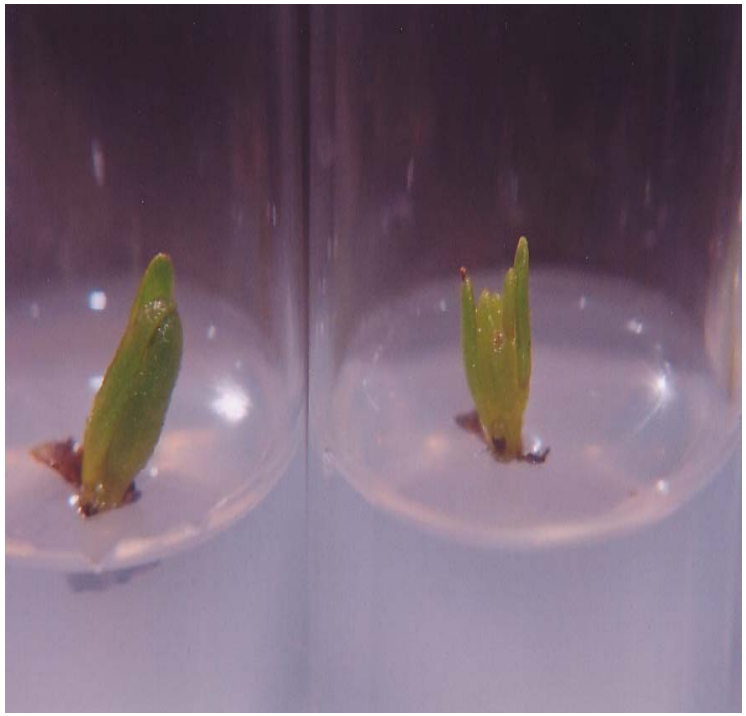
Note: Based on visual observations. (On a scale of 0 to +++++),

0 = No phenolics, +++++ = Maximum phenolics accumulation. (20 replications repeated twice).

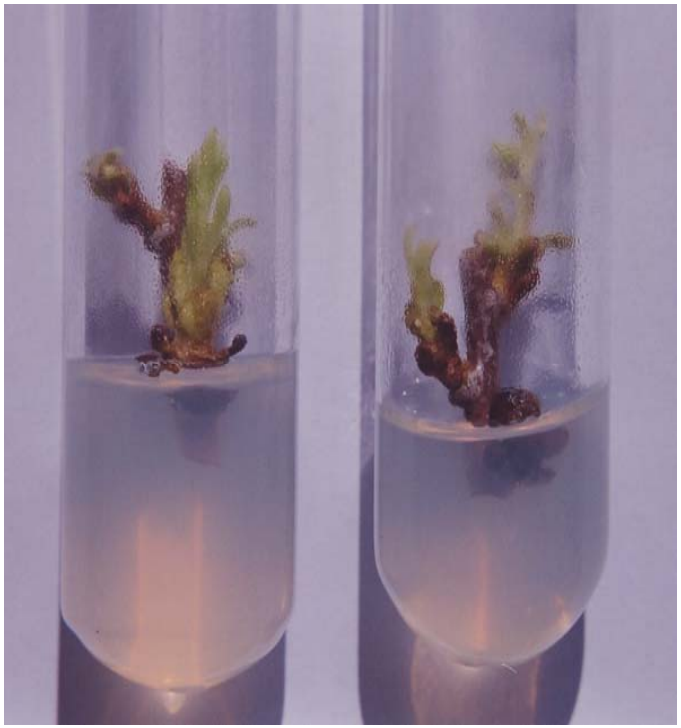
Establishment of aseptic culture

Treatment	Concentration/ Quantity	Duration
Ascorbic acid + Citric acid w/v in sterile DDW	1500 ppm each	1 hr
Low temperature	4 ˚C	1-6 days
Pre culture of explant on plain Agar supplemented with sucrose and inositol	3% Sucrose, 100 ppm inositol, pH 5.75	2 weeks.

Aseptic buds of seabuckthorn



Active buds



Dormant buds

Findings of establishment of aseptic culture

- ✓ Dormancy break in more than 90% dormant buds
- ✓ Contamination free cultures
- ✓ No phenolics accumulation
- ✓ No toxicity to the explant

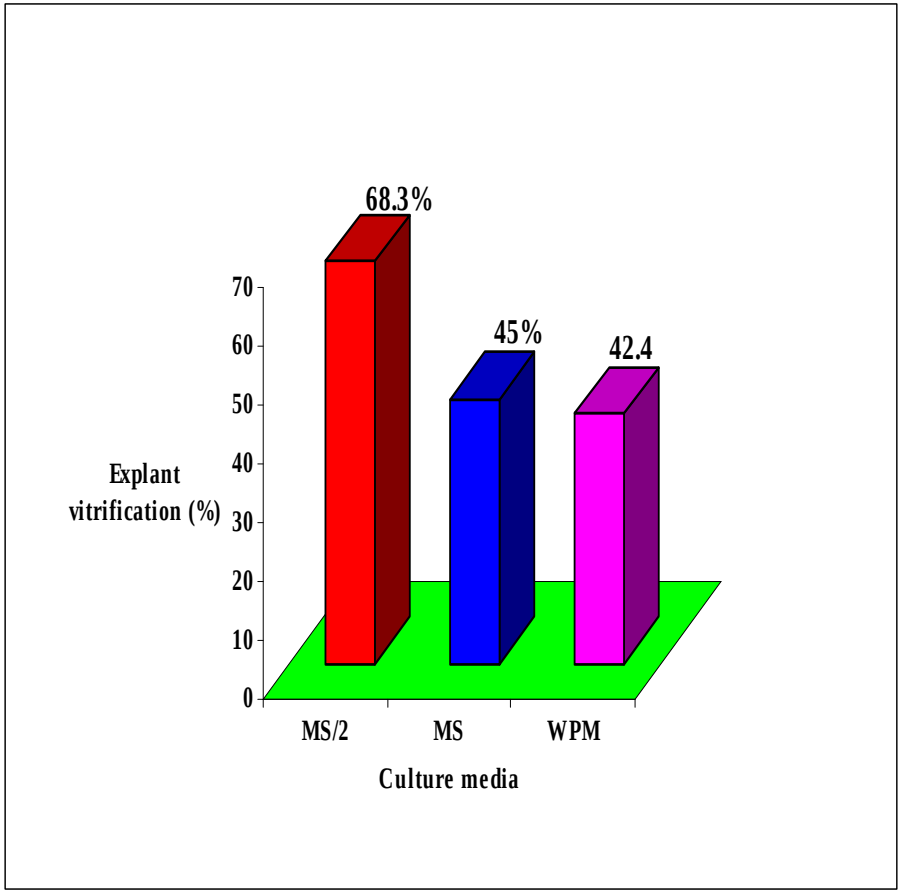
Effect of different culture media on % explant vitrification

Explant vitrification

- MS -45%
- ½ MS- 68.3%
- WPM-42.4%

✓ WPM had lowest explant vitrification

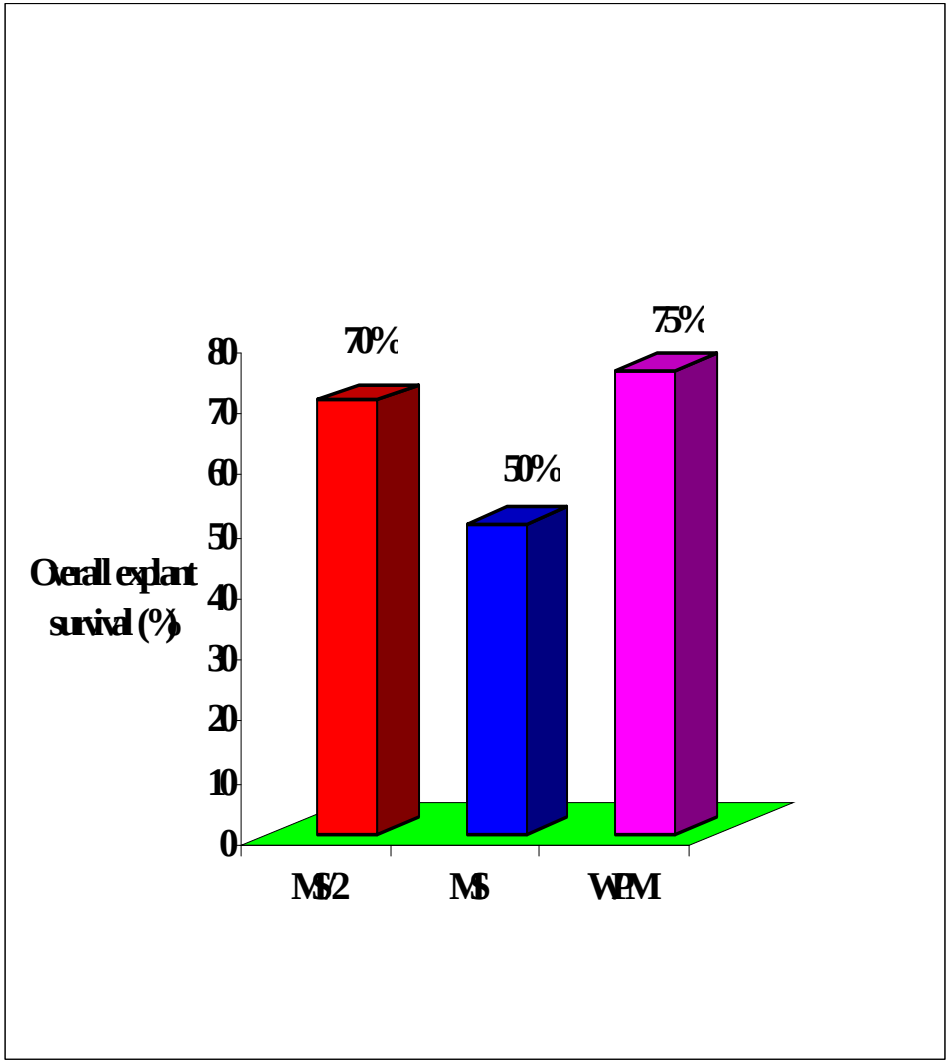
Note: Vitrification level ceased in 2nd passage.



Effects of different culture media on explant survival (%)

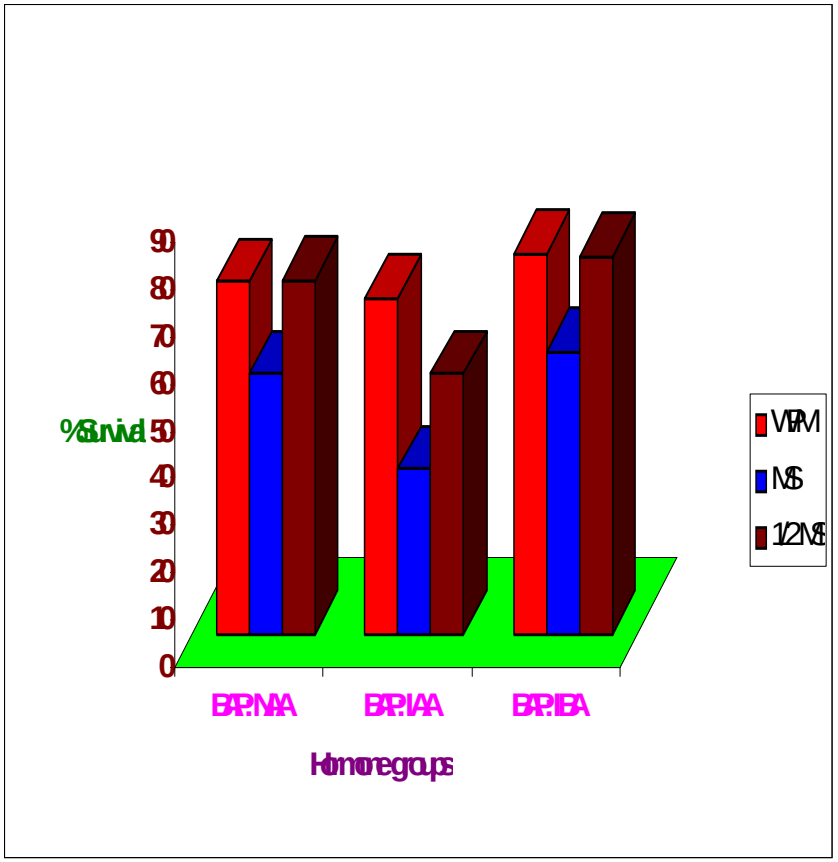
- MS: 50%
- ½ MS: 70%
- WPM: 75%

✓ WPM



Effect of Media –hormone interactions on explant survival (%)

Hormone Group	WPM	MS	½ MS
BAP: NAA	74	55	75
BAP: IAA	71	35	55
BAP: IBA	81	60	80

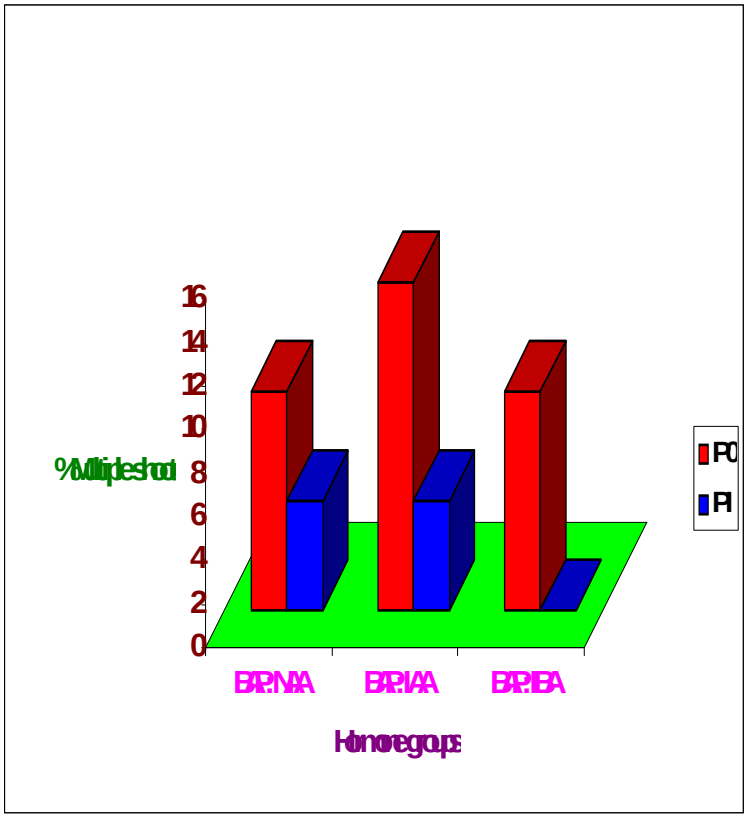


Multiple shoots formation in explants

- **Explants cultured on**
  - **MS, ½MS and WPM media with**
  - **pH 5.75-5.80, 1.2% agar, 16/8 Hrs photo period, 18ºC**
  - **BAP + NAA; BAP + IAA; BAP + IBA in different combinations.**

Effect of hormone on multiple shoot development (%) on MS medium

Hormone	P 0	P 1
BAP: NAA	10	5
BAP: IAA	15	5
BAP: IBA	10	0

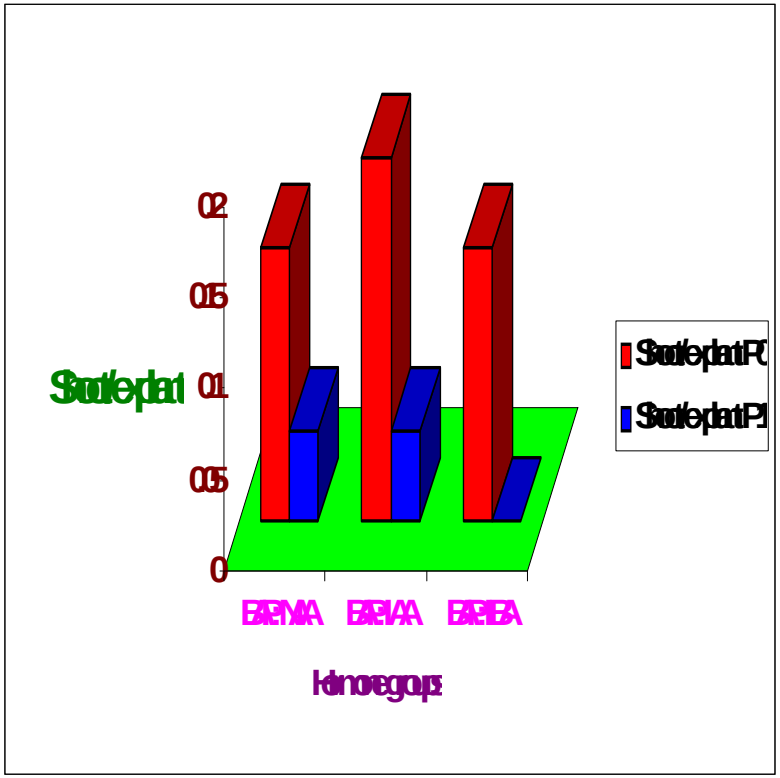


Note: P= Passage (subculturing)

✓**Low shoots formation and declines with passage**

Effect of Hormone on shoots/explant development on MS medium

Hormone group	P 0	P 1
BAP: NAA	0.15	0.05
BAP: IAA	**0.20**	0.05
BAP: IBA	0.15	0



✓**Low shoots formation and declines with passage**

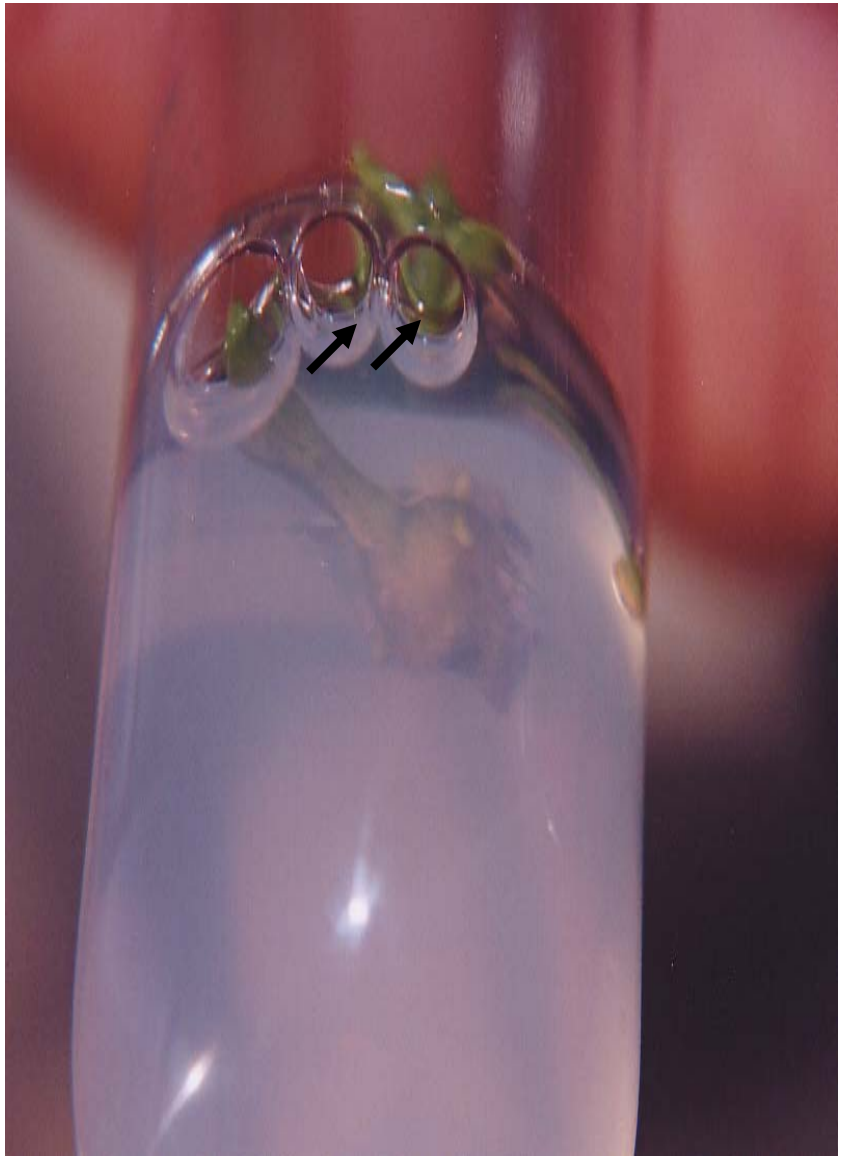
Effect of Hormone on % shoots & shoots/explant development on ½ MS Medium

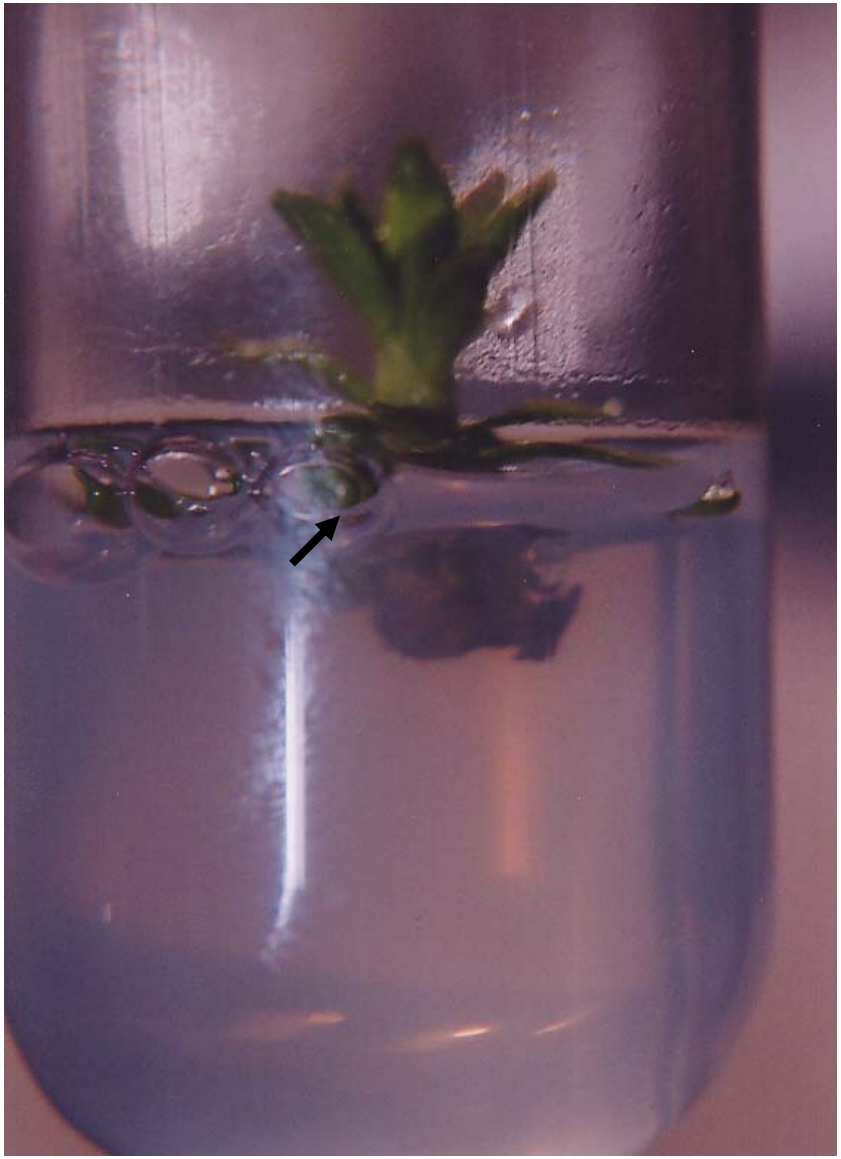
Hormone group	P 0	P 1	P 2	P 3
BAP: NAA	25	**32.5**	20	16.4
BAP:IAA	10	7.5	**15**	11.7
BAP: IBA	**45**	25	17.5	12.9

Hormone group	P 0	P 1	P 2	P 3
BAP: NAA	0.3	**0.53**	0.23	0.18
BAP:IAA	0.1	0.1	**0.28**	0.18
BAP: IBA	**0.75**	0.53	0.18	0.13

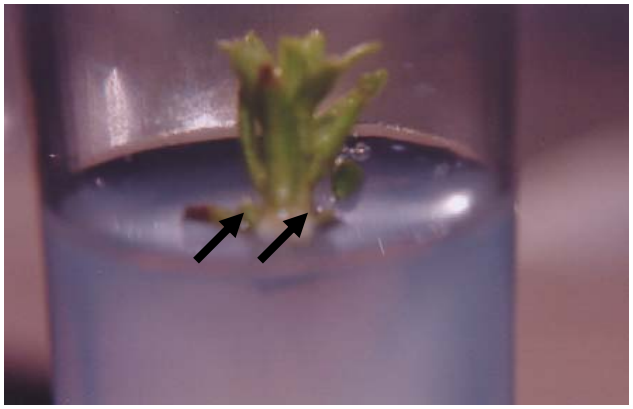
✓**BAP: IBA at P0**

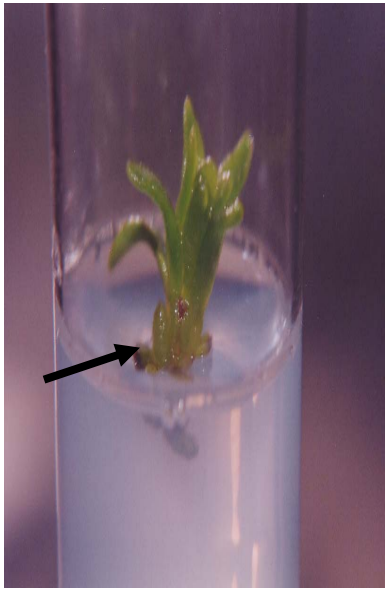
Multiple shoot initiation on ½ MS

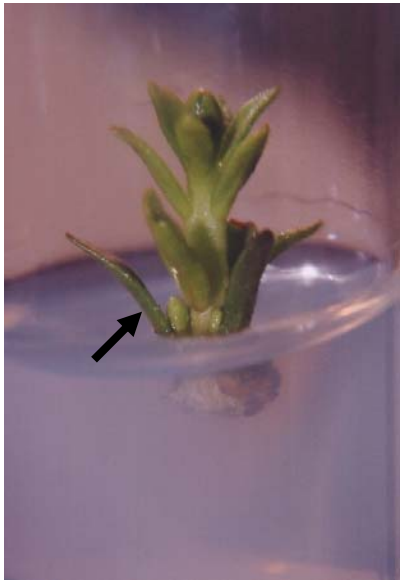


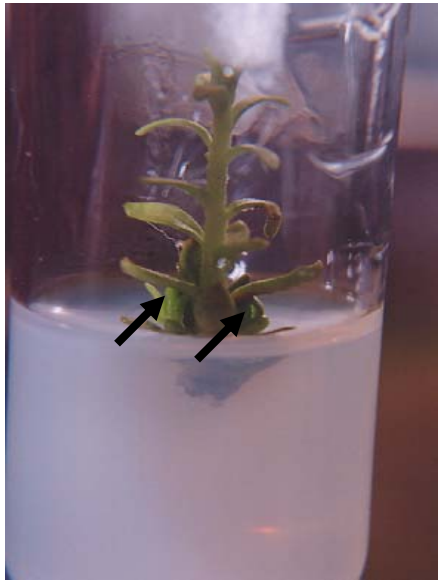


Spectrum of multiple shoots development on ½ MS



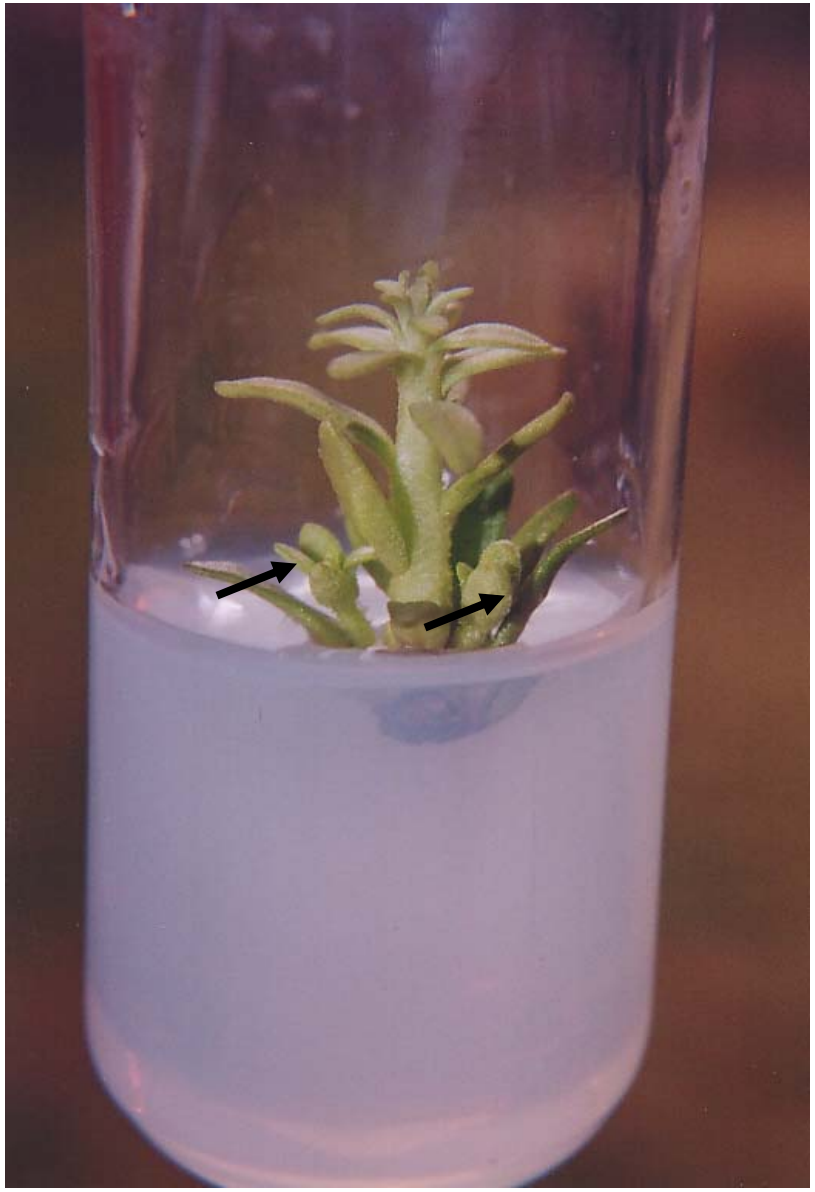


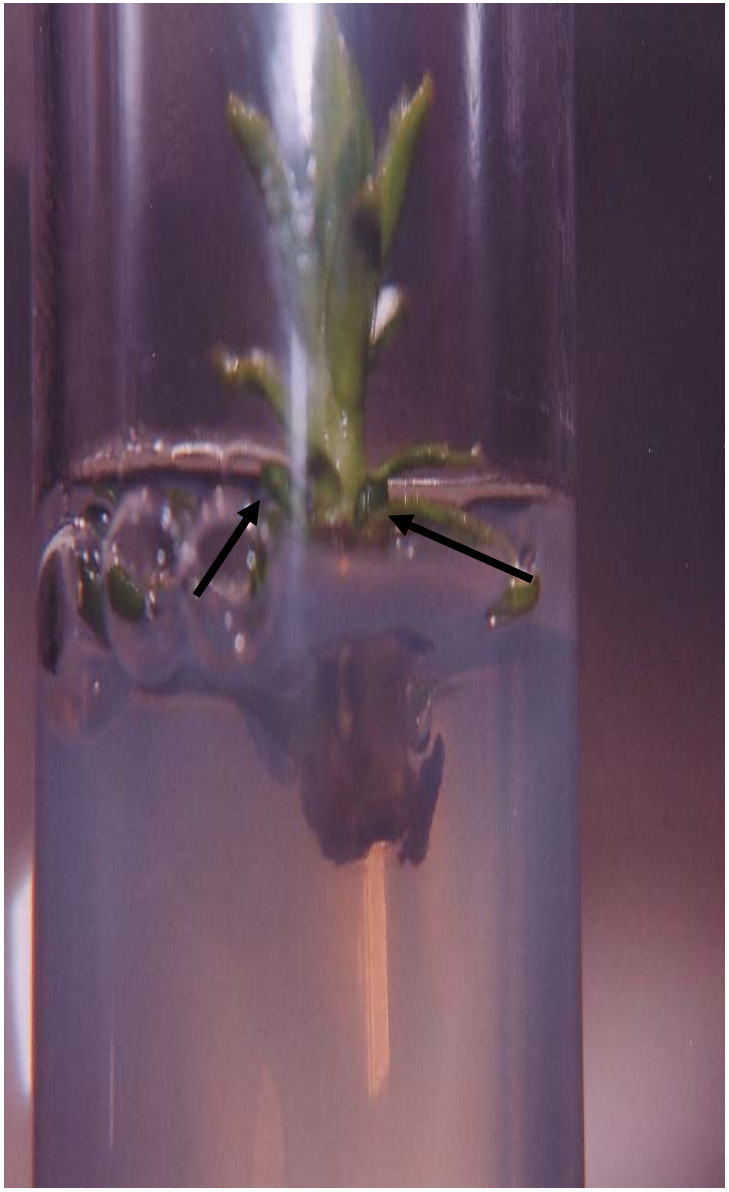




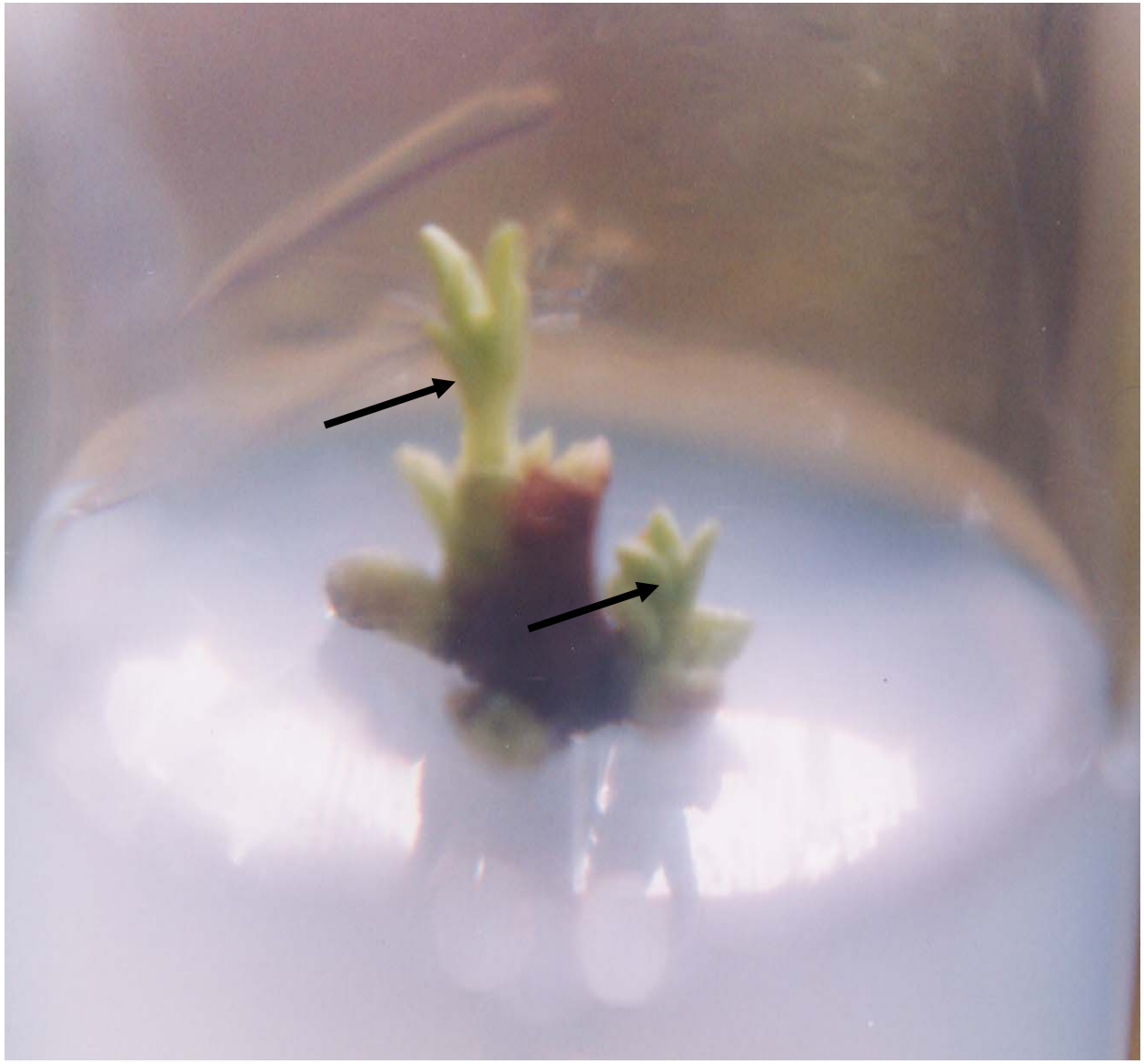
Multiple shoots induced with BAP 0.3 ppm+ NAA 0.2 ppm

Elongation of multiple shoots on ½ MS





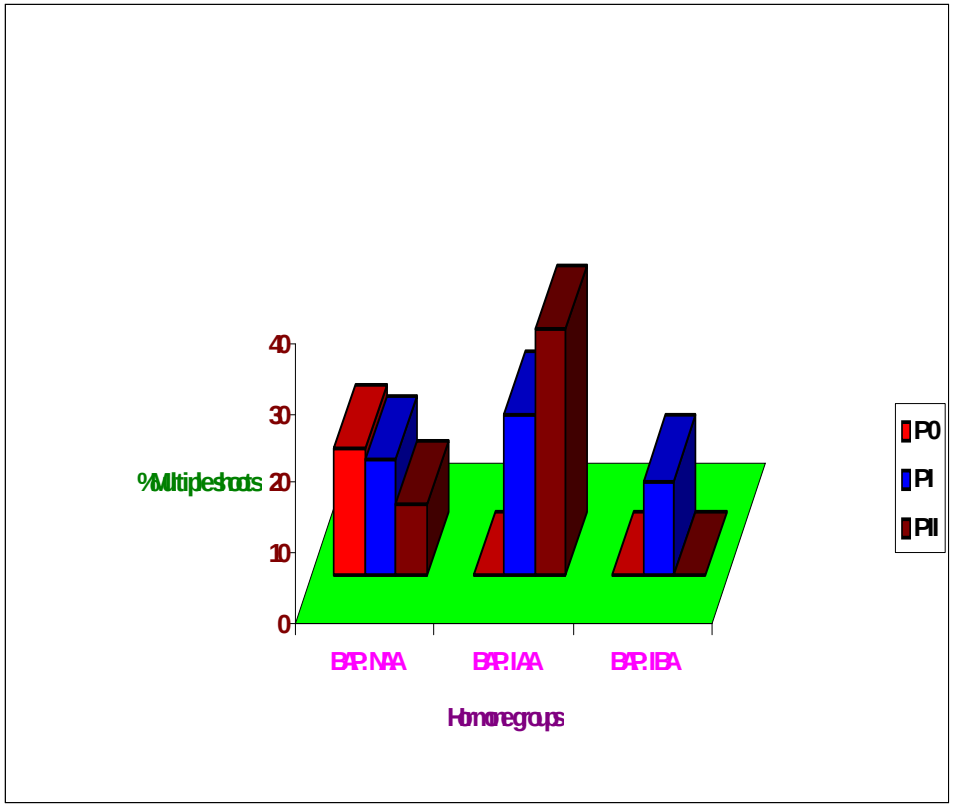
Further multiple shoots development after sub culturing on ½ MS



Elongation of sub cultured shoot on ½ MS

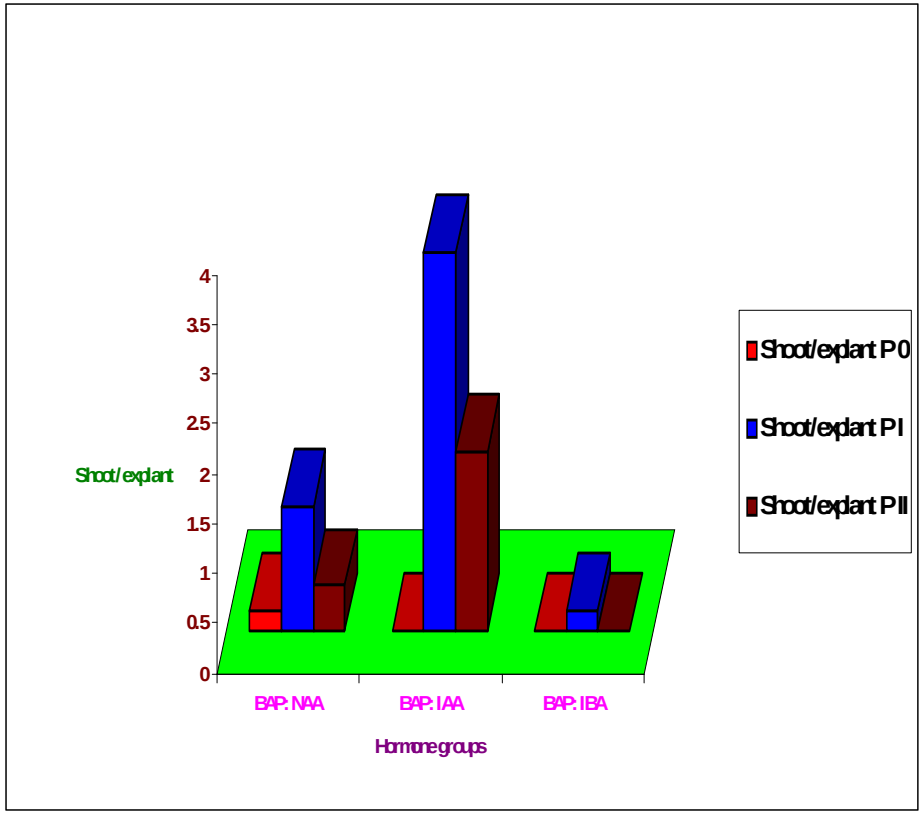
Effect of Hormone on multiple shoot formation (%) on WPM medium

Hormones	P 1	P 2	P 3
BAP: NAA	18.1	16.7	10
BAP: IAA	0	22.8	**35**
BAP: IBA	0	13.6	0



Effect of Hormone on multiple shoots/explant on WPM medium

Hormone	P 1	P 2	P 3
BAP: NAA	0.2	**1.25**	0.45
BAP:IAA	0	**3.8**	1.8
BAP: IBA	0	**0.2**	0



Multiple shoots formation (%) during successive passage on different culture media

Treatment	MS		½MS				WPM			
	P 0	P1	P 0	P I	P II	P III	P 0	P I	P II	P III
BAP 0.3: NAA 0.2	0	0	40	10	20	15.4	0	**67**	30	26.7
BAP 1.0: IAA 0.5	0	0	20	0	40	26.7	0	60	**80**	80
BAP 0.5: IAA 1.0	40	0	0	0	10	10	0	0	**40**	20
%Average Multiple shoots	13.3	0	20	3.3	23.3	17.4	0	42.2	**50**	42.2

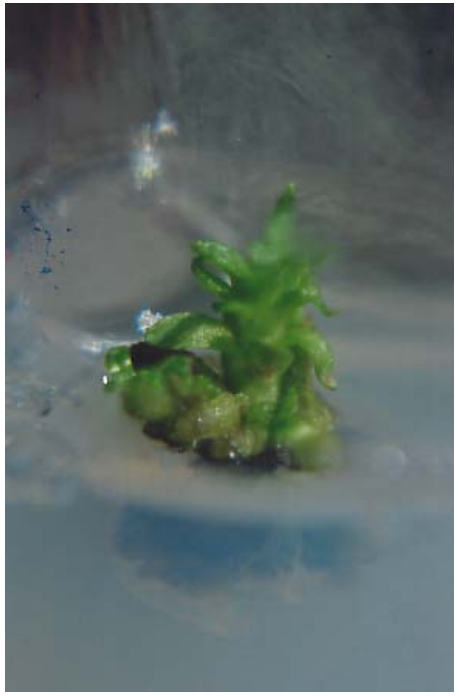
✓**WPM with BAP 1.0 ppm: IAA 0.5 ppm at PII**

Multiple shoots/explant during successive passage on different culture media

Treatment	MS		½MS				WPM			
	P 0	P 1	P 0	P I	P II	P III	P 0	P I	P II	P III
BAP 0.3: NAA 0.2	0	0	0.6	0.3	0.3	0.2	0	**5**	1.7	0.4
BAP 1.0: IAA 0.5	0	0	0.2	0	0.9	0.5	0	**14.6**	6.5	8.7
BAP 0.5: IAA 1.0	0.4	0	0	0	0.1	0.1	0	0	0.4	0.3
Average Shoots/explant	0.13	0	0.27	0.1	0.43	0.27	0	6.5	2.9	3.13

✓**WPM with BAP 1.0 ppm: IAA 0.5 ppm at PI**

Spectrum of multiple shoot development on WPM




PI **PII**

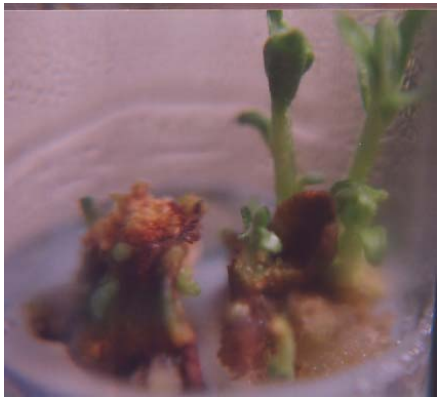
Multiple shoots developed with BAP 0.3 ppm+ NAA 0.2 ppm

Maximum 5 shoots/explant in PI, decreased to 1.7 shoots in PII.

Further Spectrum of multiple shoot development on WPM

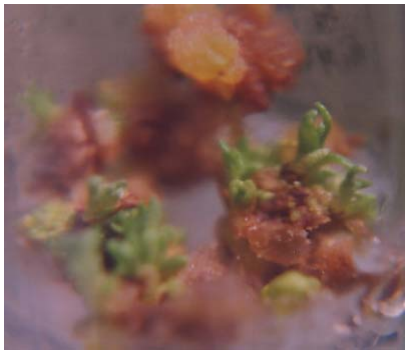
P1

PII

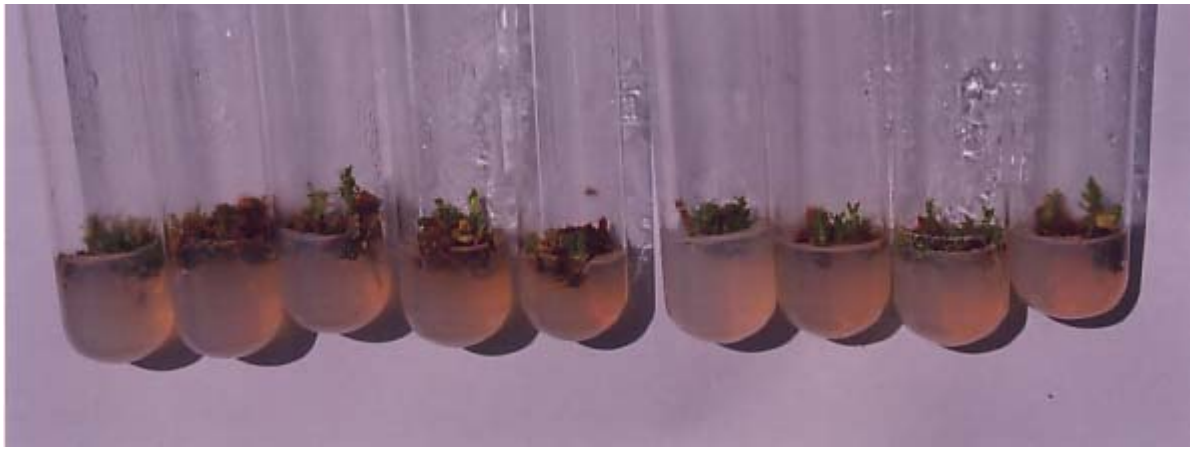


Multiple shoots developed with hormone combination: BAP 1.0 ppm+IAA 0.5 ppm, Maximum shoots of 14.6/explant in PI, decreased to 6.5 in PII **Contd.**

Further spectrum of multiple shoots developed on WPM



PIII

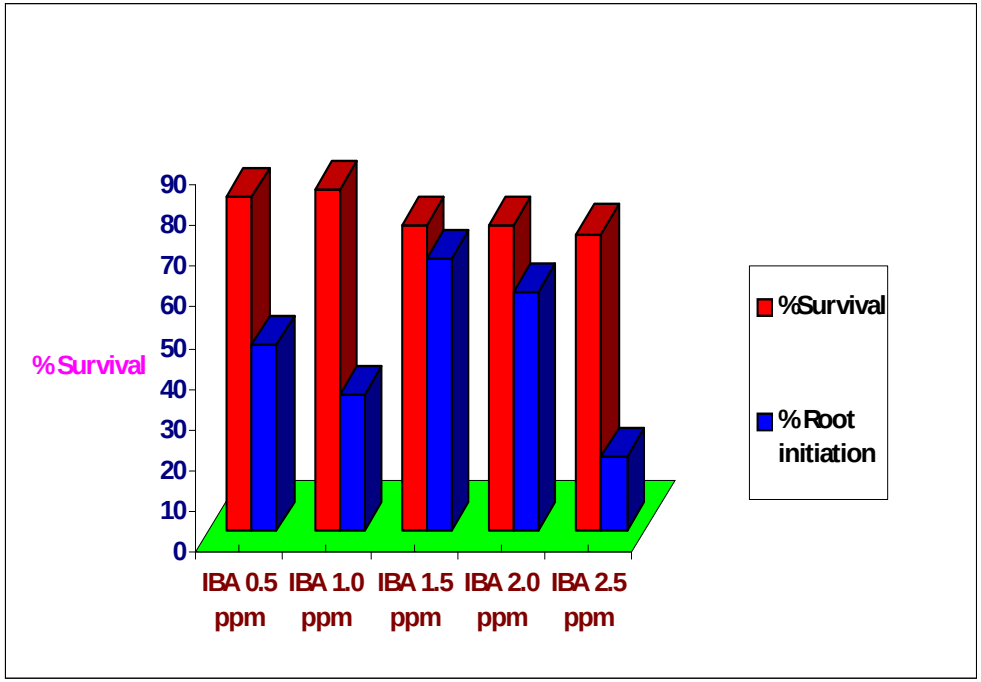


✓**Multiple shoots/explant achieved = 8.7, on BAP 1.0 ppm+**

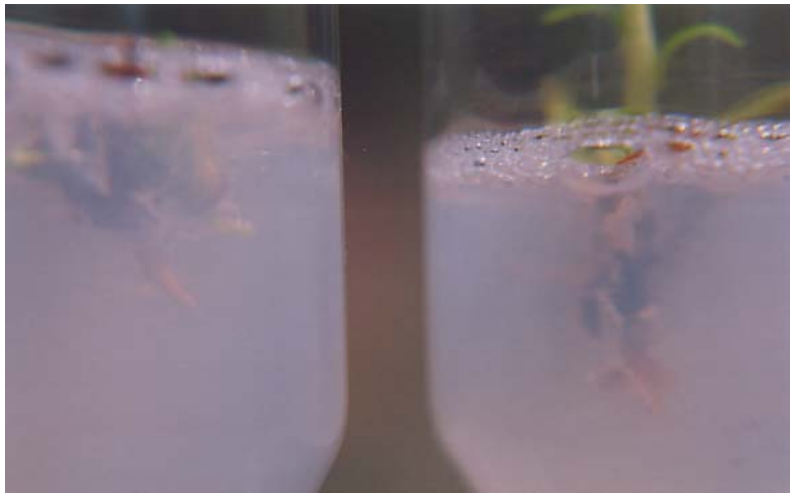
IAA 0.5 ppm in PIII

Effect of IBA on shoot survival and rooting rate on WPM medium

Treatment	%Survival	% Root formation
IBA 0.5 ppm	81.8	45.5
IBA 1.0 ppm	**83.3**	33.3
IBA 1.5 ppm	75	**66.7**
IBA 2.0 ppm	75	58.3
IBA 2.5 ppm	72.7	18.2
Average	77.6%	44.4%



Root development in explants



Root development on WPM rooting medium with different concentrations of IBA

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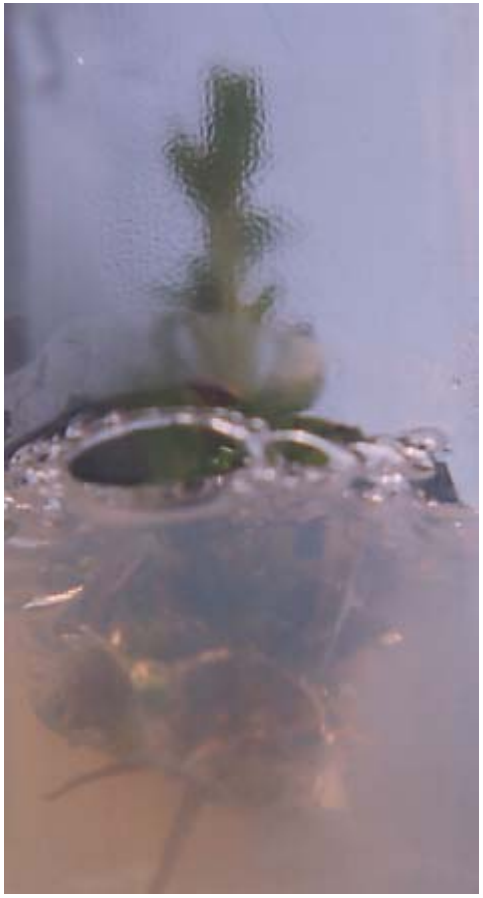
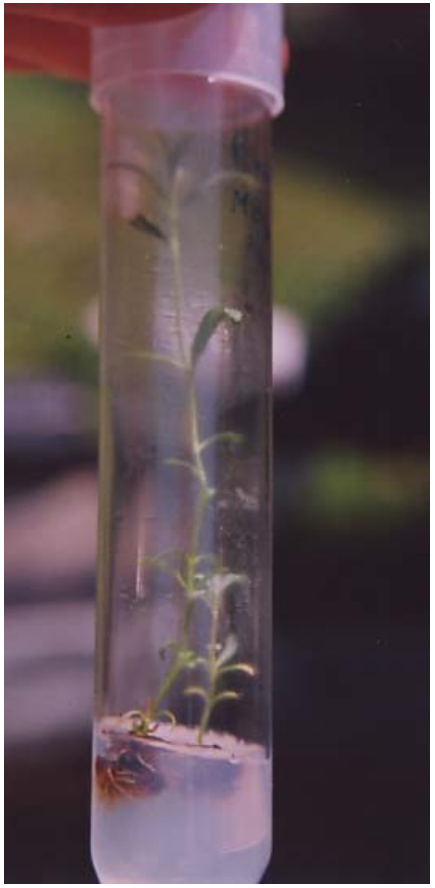
Further spectrum of root development





Roots developed on WPM rooting medium with hormone IBA 1.5 ppm

- **Maximum root induction (66.7%) was observed with IBA (1.5 ppm) on WPM rooting medium**

Comparison with other studies

Present study Singh & Gupta (2007)	Y. Yao (1994)	P. Lummerding (2001)
Shoots/explants : 14.6	2.5	5.5
Hormones: BAP 1.0 ppm: IAA 0.5 ppm on WPM	BAP: 0.4-1.0 ppm on WPM	BAP 1 ppm: IBA 0.01 (on Modified MS)
Rooting rate: 66.7%	33%	12%
Hormone: IBA 1.5 ppm on WPM	Spontaneously on WPM	BAP 0.5 ppm: NAA 0.003 ppm
Source:	Source	Source
Project on Biochem. Charact. & Micro propagation --(DBT), N. Delhi,India	Ph.D. Thesis, Univ. of Helsinki, Finland	Agri-Food Innovation Fund Project # 19980162, Canada

Conclusions

- **Surface sterilization with Tee- Pol (0.1%), 250 mg Tetracycline, Ethenol (70%), $HgCl_2$ (0.1%) was effective to initiate aseptic cultures from active and dormant buds.**
- **Explant treatment with citric acid + ascorbic acid solution, followed by cold treatment, reduced phenolics accumulation.**
- **Media pH of 5.7-5.8 was found to be optimal for reducing explant phenolics accumulation.**
- **Preculturing of explant on plain agar supplemented with Sucrose & Inositol, pH 5.8, before transferring explant on to plant tissue culture media helped in controlling phenolics accumulation and dormancy breaking of dormant buds.**
- **WPM medium was found to be best in controlling explant vitrification.**

Contd.

Conclusions

- **Explant survival was found to be highest (80.6%) on WPM medium**
- **Multiple shoot, shoot/explant was found to be highest on WPM medium.**
- **Hormone combination of BAP 1.0: IAA 0.5 had maximum shoots (80% -P-II), followed by BAP 0.3: NAA 0.2 (67%-PI)**
- **Hormone combinations BAP 1.0: IAA 0.5, led to development of maximum number of 14.6 shoots/explant on WPM medium.**
- **Highest root induction (66.7%) was observed with IBA 1.5 ppm on WPM medium.**

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Thank you