

***In vitro* Morphogenesis of *Asparagus racemosus* Willd. and
Rauvolfia serpentina (L.) Benth. ex Kurz.**

**THESIS SUBMITTED TO THE INSTITUTE OF SCIENCE AND
TECHNOLOGY TRIBHUVAN UNIVERSITY, KATHMANDU, NEPAL
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IN BOTANY**

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December, 2011.**

Certificate

This is to certify that the thesis entitled '*In vitro* Morphogenesis of *Asparagus racemosus* Willd. and *Rauvolfia serpentina* Benth. Ex. Kurz.' submitted to Institute of Science and Technology, Tribhuvan University for the degree of Doctor of Philosophy in Botany, is the outcome of original research work carried out by Mr. Krishna Kumar Pant, Lecturer, Department of Environmental Science, Rampur Campus, Chitwan Nepal, under my supervision. Almost all the research work, especially the laboratory experiments on *in vitro* morphogenesis was performed at the Central Department of Botany, Tribhuvan University, Nepal. This work has not been submitted for a degree of any other University.

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DECLARATION

I hereby declare that the work presented in the thesis entitled '*In vitro* Morphogenesis of *Asparagus racemosus* Willd. and *Rauvolfia serpentina* Benth. Ex. Kurz.' has been done by myself, and has not been submitted elsewhere for the award of any degree. All sources of information have been specifically acknowledged by reference to the authors or institutions.

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**DEDICATED TO MY LATE FATHER MR. YAGYA PRASAD
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ABSTRACT

Asparagus racemosus willd. and *Rauvolfia serpentina* Benth. Ex. Kurz. Belonging to the families Lilliaceae and Apocynaceae respectively are among the most important natural resources of Nepal. Both these plants have high medicinal value and are decreasing from the natural forests due to unsustainable collection and destruction of their habitats for various reasons. *R. serpentina* is listed in the “Appendix II” of the CITES and is under the category “E” of IUCN. Similarly, *A. racemosus* is facing towards its extinction due to its increasing demand in the local as well as international markets. Understanding these facts, the GoN has prioritised these species for research in the areas of multiplication and cultivation. Considering these facts, the present research was undertaken to support the national mission of conservation through *ex-situ* way. Plant tissue culture method was selected because it is one of the promising methods of rapid plant propagation in short time and less space without damaging the mother stock. From the present work, most effective explant for the rapid multiplication of these plant species using tissue culture technique has been identified to be the nodes. Calli induced from the nodes are suitable for organogenesis as well as somatic embryogenesis. Induction of buds from the shoots in *A. racemosus* is another potential area where more works are necessary. In the overall experiments, NAA was found to be the most effective hormone among the auxins in all respects and BAP among the cytokinins tested.

From the seed germination experiments of *Asparagus racemosus* on MS basal medium a single seed produced up to 13 shoots. In the tissue culture experiments, the nodes produced more callus when cultured on the medium containing NAA alone in higher concentrations or when combined with BAP. The tough friable calli induced from the nodes on high auxin containing media were capable of inducing somatic embryoids. Generally, high auxin either singally or in combination with higher concentration of cytokinin produced large number of somatic embryoids from the calli. Shoot formation from the calli was supported by lower cytokinin whereas root induction by higher cytokinin levels in the media. In case of multiple shoot induction either a combination of low NAA (0.1 mg/l) and high Kn (1.0 or 2.0 mg/l) concentration or various combinations of IBA and BAP in the MS medium were promising. Similarly, for the root induction, NAA at low concentration (0.1 mg/l) was found to be the best although its other higher concentrations also induced roots at significant levels. Agar

manipulation experiment for the induction of storage roots produced insignificant result. A preliminary study on bud induction from the shoots has shown very interesting results. This can be an alternative method of rapid multiplication of plants. Vitrification of shoots is one of the major problems in tissue culture and generally the vitrified shoots are discarded. We here, have used the vitrified shoots as explants and produced normal shoots using very low amount of cytokinin or auxin in the sub-culture medium. Sand rooting using NAA 100 mg/l pulse treatment, acclimatizing in a shade house on the coco-peat and transferring the acclimatized plantlets on the garden soil in an open environment were the final steps. Another important and threatened medicinal plant *Rauvolfia serpentina* also was taken for the present research. Here, callus induction from the shoot and leaf explants were supported by the incorporation of 2,4-D or NAA above 1.0 mg/l and 0.5 mg/l singly in the MS medium respectively. In combination of auxin and cytokinin, NAA 1.0 mg/l along with all concentrations of BAP yielded highly significant amount of callus especially from the shoots. The calli obtained from the leaf were rarely embryogenic as well as caulogenic. The shoot induced friable green calli were able to go for organogenesis as well as somatic embryogenesis. The multiple shoot induction was generally supported by a lower auxin and higher cytokinin concentrations. The maximum induction was observed on the MS medium incorporated with IBA 0.1 + BAP 2.0 mg/l with 7.83 ± 1.01 shoots per explant. For the induction of roots from the shoots, NAA singly at almost all concentrations were found to be significant, however NAA 0.5 mg/l induced the maximum average roots upto 12.50 per explants after 12 weeks of culture. IAA and IBA did induce some roots when alone but in combination with cytokinins their performances were very poor. Finally, the shoots rooted both *in vitro* and *in vivo* after pulse treatment with 100 mg/l auxin survived in the open environment after acclimatization in the shade house.

CONTENTS

	Page
DEDICATION	i
CERTIFICATE FROM RESEARCH SUPERVISOR	ii
DECLARATION	iii
ACKNOWLEDGEMENTS	iv
ABBREVIATIONS AND ACRONYMS	vi
ABSTRACT	vii
Contents	ix
List of Tables	xii
List of Figures	xiii
1. INTRODUCTION	1-23
1.1 Background	1
1.2 Medicinal plants	2
1.3 Trade and conservation status of the medicinal plants	3
1.4 Species selected for the present investigation	6
1.4.a. <i>Asparagus racemosus</i>	6
1.4.a.i. Uses and Phytochemical contents of <i>Asparagus racemosus</i>	7
1.4.b. <i>R. Serpentine</i>	9
1.4.b.i. Uses and chemical composition of <i>Rauvolfia serpentine</i>	10
1.5 Conservation and awareness for sustainable use	12
1.6 Plant tissue culture (Micropropagation)	13
1.6.1 History of plant tissue culture	13
1.6.2 Importance of plant tissue culture	13
1.6.3 Culture types and their applications	15
1.7 Plant growth regulators (PGRs)	18
1.7.1 Auxins and cytokinins used for this investigation	19
1.8. Justification	20
1.9. Hypothesis	22
1.10 Objectives of the study	23
2. REVIEW OF LITERATURE	24-41
2.1 Importance of the plants selected for the present study	24
2.2 Seed germination	26
2.3 Callus induction and general multiplication	28
2.3.1 <i>Asparagus</i>	28
2.3.2 <i>Rauvolfia</i>	32

2.3.3	Other plants	37
3.	MATERIAL AND METHODS	42-50
3.1	Plant material	42
3.2	Sterilization	42
3.2.1	Surface sterilization of seeds	42
3.2.2	Sterilization of glassware	42
3.2.3	Sterilization of Media and equipments	43
3.3	Preparation of explants	43
3.4	Media	43
3.4.1	Culture media	43
3.4.2	Preparation of stock solutions	45
3.4.3	Preparation of MS medium	45
3.5	Plant growth regulators	46
3.5.1	Preparation of PGRs stock solutions	47
3.6	Inoculation of explants	48
3.7	Culture conditions	48
3.8	Sub-cultures	48
3.9	Agar manipulation in the media (for <i>A. racemosus</i>)	49
3.10	Cytological study of callus	49
3.11	Rooting	49
3.12	Acclimatisation	50
3.13	Data recording and analysis	50
4.	RESULTS	51-103
4.A.	<i>Asparagus racemosus</i>	51
4.A.1	Seed germination	51
4.A.2	Effect of different hormones either alone or in combinations on nodes.	52
4.A.2.1	Effects of auxins on nodal explants	53
4.A.2.2	Effects of cytokinins on nodal explants	55
4.A.2.3.	Effects of NAA and BAP in combinations on nodal explants	56
4.A.2.4.	Effects of NAA and Kinetin in combinations on nodal explants	57
4.A.2.5.	Effects of IBA and BAP in combinations on nodal explants	59
4.A.2.6.	Effects of IBA and Kinetin in combinations on nodal explants	60
4.A.2.7.	Effects of IAA and BAP in combinations on nodal explants	62
4.A.2.8.	Effects of IAA and Kinetin in combinations on nodal explants	63
4.A.2.9.	Response of MS basal medium to different explants	65
4.A.3.	Agar concentration manipulation	65
4.A.4	Somatic embryogenesis and Callus analysis	66
4.A.5	Buds, caulogenesis and vitrification of shoots	67
4.A.6	Rooting and acclimatization	71

	Photoplates	72-81
4.B.	<i>Rauvolfia serpentina</i>	82
4.B.1	Seed germination experiment	82
4.B.2.	Effects of different PGRs either singly or in combinations on nodes	82
4.B.2.1.	Effects of auxins on nodal explants	82
4.B.2.2.	Effects of cytokinins on nodal explants	84
4.B.2.3.	Effects of NAA and BAP in combinations on nodal explants	85
4.B.2.4.	Effects of NAA and Kn in combinations on nodal explants	86
4.B.2.5.	Effects of IAA and BAP in combinations on nodal explants	88
4.B.2.6.	Effects of IAA and Kn in combinations on nodal explants	89
4.B.2.7.	Effects of IBA and BAP in combinations on nodal explants	91
4.B.2.8.	Effects of IBA and Kn in combinations on nodal explants	92
4.B.2.9.	Effects of hormone free MS medium on shoot explants	94
4.B.3.	Somatic embryogenesis and caulogenesis	94
4.B.2.10.	<i>In Vivo</i> Rooting and Acclimatization	95
	Photoplates	96-103
5.	DISCUSSION	104-126
5.1	<i>Asparagus racemosus</i>	104
5.1.1	Seed germination	104
5.1.2	Callus Induction	105
5.1.3.	Shoot Multiplication and Shoot length	109
5.1.4.	<i>In Vitro</i> Root Induction and Root length	113
5.1.5.	Agar manipulation	114
5.1.6.	Somatic embryogenesis	115
5.1.7.	Buds, Caulogenesis and Vitrification	116
5.1.7.1.	Buds	116
5.1.7.2.	Caulogenesis	117
5.1.7.3.	Vitrification	117
5.1.8.	<i>In Vivo</i> Rooting and Acclematization	118
5.2.	<i>Rauvolfia serpentina</i>	119
5.2.1.	Seed germination	119
5.2.2.	Callus induction	119
5.2.3.	Shoot induction and shoot growth	120
5.2.4.	Root induction and root elongation	123
5.2.5.	Somatic embryogenesis	124
5.2.6.	Caulogenesis	125
5.2.7.	<i>In Vivo</i> Rooting and Acclematization	126
6.	CONCLUSION	127-128
7.	RECOMMENDATIONS	129
8.	SUMMARY	130-132
9.	REFERENCES	133-151

Appendix: Published papers based on present study.

List of Tables

		Page
Table 3.1	Murashige and Skoog (1962) Media Composition	44
Table 3.2	Plant growth regulators (PGRs) used to supplement the basic MS medium.	47
<i>Asparagus racemosus</i> Willd.		52-81
Table 4.1	Variation in seed germination	51
Table 4.2	Effects of various Auxins on nodal explants.	54
Table 4.3	Effects of various Cytokinins on nodal explants.	55
Table 4.4	Effects of various concentration combinations of NAA and BAP on nodes.	57
Table 4.5	Effects of various concentration combinations of NAA and Kinetin on nodes.	58
Table 4.6	Effects of various concentration combinations of IBA and BAP on nodes.	60
Table 4.7	Effects of various concentration combinations of IBA and Kinetin on nodes.	61
Table 4.8	Effects of various concentration combinations of IAA and BAP on nodes.	62
Table 4.9	Effects of various concentration combinations of IAA and Kinetin on nodes.	64
Table: 4.10	Buds, Somatic embryos, Caulogenesis and Vitrification observations.	69
<i>Rauvolfia serpentina</i> Benth. Ex. Kurz.		82-110
Table 4.11	Effects of auxins on shoot explants	83
Table 4.12	Effects of cytokinins on shoot explants	85
Table 4.13	Effects of NAA and BAP in combinations on shoot explants	86
Table 4.14	Effects of NAA and Kn in combinations on shoot explants	87
Table 4.15	Effects of IAA and BAP in combinations on shoot explants	89
Table 4.16	Effects of IAA and Kn in combinations on shoot explants	90
Table 4.17	Effects of IBA and BAP in combinations on shoot explants	92
Table 4.18	Effects of IBA and Kn in combinations on shoot explants	93
Table 4.19	<i>In vivo</i> rooting and acclimatization	94

List of Figures

	Page
Fig. 1.1 Active principles of <i>Asparagus racemosus</i>	8
(I)- Chemical structure of Shatavarin.	
(II)- Chemical structure of Sarsasapogenin.	
(III)- Chemical structure of Racemosol.	
(IV)- Chemical structure of Asparagamine.	
Fig. 1.2- Active principles of <i>Rauvolfia serpentina</i>.	12
(I)- Chemical structure of Reserpine.	
(II)- Chemical structure of Ajmaline.	
Fig. 1.3- Plant Growth Regulators used in the Experiment	19
(I)- Chemical structure of IAA.	
(II)- Chemical structure of IBA.	
(III)- Chemical structure of NAA.	
(IV)- Chemical structure of 2,4-D.	
(V)- Chemical structure of Kinetin.	
(VI)- Chemical structure of BAP.	
Fig. 4.A. <i>Asparagus racemosus</i> Willd.	73
Fig. A- Habitat of <i>Asparagus racemosus</i> plant growing in the garden of CDB, TU.	
Fig. A ₁ . Germinating seeds of <i>A. racemosus</i> in the petridish on a moist filter paper.	
Figure 4.A.1: Germination of <i>Asparagus racemosus</i> on MS medium:	73
Fig. 1- Emergence of only root (with no secondary root) and no shoot after 8 weeks of culture.	
Fig. 2- Emergence of only shoot (no root) after 8 weeks of culture.	
Fig. 3- Emergence of one shoot with root after 8 weeks of culture.	
Fig. 4- Equal growth of root and shoot after 8 weeks of culture.	
Fig. 5- Multiple shoot (5) with single root system after 8 weeks of inoculation.	
Fig. 6- Multiple shoot (4) with single root system after 8 weeks of inoculation.	
Fig. 7- Multiple shoots (4) emerging from the single point after 8 weeks of culture.	
Fig. 8 - Multiple shoot induction from the cut bases of shoots after 7 weeks of culture.	
Fig. 9- Multiple shoot and root induction from the cut bases of shoots after 3 weeks of culture.	
Figure 4.A.2: Various effects of Auxins in <i>A. racemosus</i>.	75
Fig. 10 - Callus induction from the node on MS + NAA 0.5 mg/l after 10 weeks of culture.	
Fig. 11- Callus induction from the node on MS + NAA 2.0 mg/l after 10 weeks of culture.	
Fig. 12- Multiple shoot and root from the node on MS + NAA 0.1 mg/l after 9 weeks of culture.	
Fig. 13 - Multiple shoot induction from the node on MS + IBA 0.5 mg/l after 12 weeks of culture.	
Fig. 14- Normal multiple shoot induction from vitrified shoot explant on MS + IAA 0.5 mg/l.	
Fig. 15- Root induction from a single explants on MS + NAA 0.1 mg/l after 8 weeks of culture.	

Fig. 4.A.3: Various effects of Cytokinins on *A. racemosus*.

- Fig. 16- Callus induction from the shoot on MS + Kn 2.0 mg/l after 12 weeks of culture.
Fig. 17- Multiple shoots from the vitrified mass on BAP 0.1 mg/l after 10 weeks of culture.
Fig. 18- Normal multiple shoot formation from the vitrified mass of tissues on MS + BAP 1.0 mg/l.
Fig. 19- Abnormal (vitrification) shoot formation on MS + BAP 2.0 mg/l after 8 weeks of culture.
Fig. 20- Root formation from the marginal cells of the shoot induced callus on MS + Kn 0.5 mg/l.

Fig. 4.A.4: Various effects of NAA and BAP in combination.

75

- Fig. 21- Hard callus and somatic embryoids formation on MS + NAA 0.1 + BAP 2.0 mg/l.
Fig. 22- Normal shoots from a vitrified shoot after sub culture on NAA 0.1 + BAP 2.0 mg/l.
Fig. 23- Root and shoot with callus at the base from a node on MS + NAA 1.0 + BAP 0.1 mg/l.
Fig. 24- Vitrified shoots from shoot induced callus on MS + NAA 1.0 + BAP 2.0 mg/l.
Fig. 25- Root induction from a shoot explants on MS + NAA 1.0 + BAP 0.1 mg/l after 10 weeks.

Fig. 4.A.5: Various effects of NAA and Kinetin in combination:

75

- Fig. 26- Friable callus induction from a node on MS + NAA 0.5 + K 0.5 mg/l.
Fig. 27- Multiple shoots from a node on MS + NAA 0.1 + K 2.0.
Fig. 28- Multiple shoots from a node on MS + NAA 0.1 + K 1.0 mg/l.
Fig. 29- Healthy and branched multiple shoots from nodes on MS + NAA 1.0 + K 0.5 mg/l.
Fig. 30- Multiple shoots from the node with callus at the base on MS + NAA 1.0 + Kn 2.0 mg/l.
Fig. 31- Multiple root induction from the shoot induced callus on MS + NAA 0.5 + K 0.5 mg/l.

Fig. 4.A.6: Various effects of IBA and BAP in combination.

77

- Fig. 32- Primary and secondary callus from a node on MS + IBA 1.0 + BAP 2.0 mg/l.
Fig. 33- Multiple shoot induction from a node on MS + IBA 1.0 + BAP 1.0 mg/l.
Fig. 34- Long multiple shoots induced from a node on MS + IBA 0.1 + BAP 0.1 mg/l.
Fig. 35- Multiple shoots with heavy branching from a node on MS + IBA 0.5 + BAP 0.5 mg/l.
Fig. 36- Root induction from a node on MS + IBA 1.0 + BAP 1.0 mg/l.

Fig. 4.A.7: Various effects of IBA and BAP in combinations.

77

- Fig. 37- Green callus formation on the upper surface from a node on MS + IBA 1.0 + K 2.0 mg/l.
Fig. 38- Branched healthy multiple shoots induced from nodes on MS + IBA 0.5 + K 2.0 mg/l.
Fig. 39- Vitrified shoots initially induced from the node on MS + IBA 0.1 + K 2.0 mg/l.
Fig. 40- Root induction from the shoot explant with callus on MS + IBA 1.0 + K 2.0 mg/l.

Fig. 4.A.8: Various effects of IAA and BAP in combinations.

77

- Fig. 41- Callus induction from the surface of a shoot explant on IAA 1.0 + BAP 1.0 mg/l.
Fig. 42- Long and healthy multiple shoot formation on IAA 0.1 + BAP 0.1 mg/l.
Fig. 43- Vitrified shoots induced from the normal node explant on IAA 0.5 + BAP 1.0 mg/l.
Fig. 44- Normal as well as vitrified shoots from a node explant on IAA 0.1 + BAP 0.1 mg/l.
Fig. 45- Weak multiple roots induced from the shoot explants on IAA 1.0 + BAP 1.0 mg/l..

Fig. 4.A.9: Various effects of IAA and Kn in combinations.

77

- Fig. 46- Friable callus formed from the shoot (node) explant by IAA 1.0 + K 1.0 mg/l..
Fig. 47- Long multiple shoot induction from the node on IAA 0.1 + K 2.0 mg/l.
Fig. 48- Normal healthy shoots induced from the node explants on IAA 0.5 + K 2.0 mg/l.

Fig. 49- Multiple roots induced from the node induced callus on IAA 1.0 + K 2.0 mg/l.

Fig. 50- Root differentiation from the shoot induced callus on IAA 0.1 + K 0.1 mg/l.

Fig. 4.A.10:- Various effects of MS hormone free medium on different explants. 79

Fig. 51- Slight growth of callus from the shoot induced calli explants on hormone free MS medium.

Fig. 52- Normal shoot induction and branching from the node explant on hormone free MS medium.

Fig. 53- Normal multiple shoot formation from a node on hormone free MS medium.

Fig. 54- Acclematization of *A. recemosus* shoot on the sand soil mixture 50% each.

and 55. Acclematization of *A. recemosus* shoot on 100% coco-peat after *in vivo* rooting with NAA.

Fig. 56- A well established *A. recemosus* plant growing on the garden soil after acclematization.

Fig. 57- Effect of agar and NAA concentration on callus induction.

Fig. 58- Effect of agar and NAA concentration on root induction.

Fig. 59- Effect of agar and NAA concentration on root length.

Fig. 4.A.11: Callus and root induction by NAA and agar concentrations. 79

Fig. 60- Root formation from the shoot induced callus on MS + NAA 1.0 mg/l with 0.6% agar.

Fig. 61- Heavy callus formation from the shoot explant on MS + NAA 2.0 mg/l + 0.6% agar.

Fig. 62- Heavy rooting from the node explant on MS + NAA 0.1 mg/l with 1.0% agar.

Fig. 63- Callus induction from node and root formation from the callus on NAA 0.5 mg/l + 1.0% agar.

Fig. 64- Heavy induction of friable callus from a node on NAA 1.0 mg/l + 1.0% agar.

Fig. 65- Callus induced from shoot and root formed from same callu on NAA 2.0 mg/l + 1.0% agar.

Fig. 4.A.12: Somatic embryoid induction and germination. 79

Fig. 66- Induction of somatic embryoids from friable callus on MS + NAA 0.1 + BAP 1.0 mg/l.

Fig. 67- Formation of somatic embryoids from tough callus on MS + NAA 0.1 + BAP 2.0 mg/l.

Fig. 68- Germination of embryoids MS + IBA 1.0 + BAP 1.0 mg/l.

and 69- A germinated embryoid with distinct shoot and root on NAA 0.1 mg/l.

Fig. 4.A.13. Photomicrographs: 81

Fig. 70- Embryogenic cells of callus spread induced on MS + BAP 1.0 mg/l (10×4).

Fig. 71 and 72- Actively dividing embryogenic cells of the callus (10×40).

Fig. 73 and 74- Longitudinal cell divisions forming 4 celled pro embryoids (10×40).

Fig. 75-78- Transverse and vertical divisions to give octant pro-embryoids (10×40).

Fig. 79- A pro embryoid showing a big houstorium cell (10×40).

Fig. 80-81- Globular embryoids (10×40).

Fig. 82-83- Torpedo stages of somatic embryoids (10×40).

Fig. 84. A cotyledonary stage somatic embryoid and

Fig. 85- A mature somatic embryo exposed (10×4).

Fig. 4.A.14- Bud induction from the nodes. 81

Fig. 86- Different stages of buds induced from node induced shoots on MS IBA 0.5 + BAP 0.5 mg/l.

Fig. 87- Multiple and different shapes of buds at the nodes from MS + BAP 1.0 mg/l.

Fig. 88- A cone shaped bud from the MS + BAP 3.0 mg/l after 8 weeks of culture.

Fig. 89- Multiple buds of different lengths induced from a node on MS + NAA 0.1 + BAP 2.0 mg/l.

Fig. 90- Emergence of shoot from the bud (inside the bud).

Fig. 4.A.15- Caulogenesis (Shoot and root formation from the callus).	81
Fig. 91- Multiple shoots from the callus after 12 weeks of culture on MS + NAA 0.1 mg/l.	
Fig. 92- Massive root formation from the callus on the MS + NAA 2.0 mg/l.	
Fig. 93- Emergence of shoot (meristem) from callus cultured on MS + NAA 0.1 + BAP 2.0 mg/l.	
4.B. <i>Rauvolfia serpentine</i> Benth. Ex. Kurz.	
Fig. 4B. 4B₁: Habit and Habitat of <i>Rauvolfia serpentina</i>.	97
Fig. B- Natural habitat of <i>Rauvolfia serpentina</i> growing in the forest of Nawalparasi.	
Fig. B ₁ - A flowering and fruiting branch on the natural habitat.	
Fig. 4.B.1: Various effects of auxins at different concentrations.	97
Fig. 101- Heavy friable callus formation from the leaf explant on MS + 2,4-D 2.0 mg/l.	
Fig. 102- Primary and secondary callus formation from shoot explant on MS + 2,4-D 2.0 mg/l.	
Fig. 103- Healthy multiple shoot as well as roots induced from a node on MS + NAA 0.1 mg/l.	
Fig. 104- Heavy root induction from an <i>in vitro</i> multiplied shoot on MS + NAA 0.5 mg/l.	
Fig. 105- Roots induced from an <i>in vitro</i> multiplied shoot on MS + IBA 1.0 mg/l.	
Fig. 106- Root induction from an <i>in vitro</i> multiplied shoot on MS + IAA 1.0 mg/l.	
Fig. 4.B.2: Various effects of Cytokinins at different concentrations.	97
Fig. 107- Heavy and hard callus formation at the shoot base on MS + Kn 2.0 mg/l.	
Fig. 108- Multiple shoot induction from the node on MS + BAP 1.0 mg/l.	
Fig. 109- Long and healthy multiple shoots from pieces of calli on MS + BAP 2.0 mg/l.	
Fig. 110- Healthy multiple shoots induced from noded on MS + BAP 1.5 mg/l.	
Fig. 111- Fine long roots from the <i>in vitro</i> multiplied shoot on MS + K 1.0 mg/l.	
Fig. 4.B.3: Various effects of NAA and BAP in combinations.	99
Fig. 112- Callus from node and root from callus on MS + NAA 1.0 + BAP 0.5 mg/l.	
Fig. 113- Multiple shoots induced from the node on MS + NAA 0.1 + BAP 1.0 mg/l.	
Fig. 114- Long healthy shoots with callus at the base from a node on MS + NAA 0.5 + BAP 1.0 mg/l.	
Fig. 115- Root induction from an <i>in vitro</i> multiplied shoot base on MS + NAA 0.5 + BAP 0.5 mg/l.	
Fig. 4.B.4: Various effects of NAA and Kinetin in combinations.	99
Fig. 116- Callus formation from the shoot explant on MS + NAA 1.0 + K 2.0 mg/l.	
Fig. 117- Multiple shoots induced from a node on MS + NAA 0.1 + K 2.0 mg/l.	
Fig. 118- Healthy multiple shoots induced from a node on MS + NAA 0.1 + K 2.0 mg/l.	
Fig. 119- Roots induced from the shoot base along with callus on MS + NAA 1.0 + K 2.0 mg/l.	
Fig. 120- Long roots induced from the shoots on MS + NAA 0.5 + K 1.0 mg/l.	
Fig. 4.B.5: Various effects of IAA and BAP in combinations.	99
Fig. 121- Callus formation from a shoot on MS + IAA 1.0 + BAP 2.0 mg/l.	
Fig. 122- Multiple shoots induced from a node on MS + IAA 1.0 + BAP 2.0 mg/l.	
Fig. 123- Long healthy shoots induced from nodes on MS + IAA 1.0 + BAP 0.1 mg/l.	
Fig. 4.B.6: Various effects of IAA and Kinetin in combinations.	99
Fig. 124- Callus induction from a shoot on MS + IAA 1.0 + K 2.0 mg/l.	
Fig. 125- Multiple shoot induced from a node on MS + IAA 0.5 + K 2.0 mg/l.	

Fig. 126- Long and healthy shoots induced from nodes on MS + IAA 0.5 + K 0.5 mg/l.

Fig. 127- Root induction from the *in vitro* grown shoots on MS + IAA 1.0 + K 0.1 mg/l.

Fig. 4.B.7: Various effects of IBA and BAP in combinations:

101

Fig. 128- Callus formation from the shoot explant on MS + IBA 0.5 + BAP 2.0 mg/l.

Fig. 129- Multiple shoot induction from the node explant on MS + IBA 0.1 + BAP 2.0 mg/l.

Fig. 130- Long multiple shoots induced from a node on MS + IBA 0.1 + BAP 1.0 mg/l.

Fig. 131- Multiple long and short shoots induced from a node on MS + IBA 1.0 + BAP 2.0 mg/l.

Fig. 132- Root induction from the *in vitro* multiplied shoot on MS + IBA 0.1 + BAP 1.0 mg/l.

Fig. 4.B.8: Various effects of IBA and Kinetin combinations:

101

Fig. 133- Callus formation from the shoot explant on MS + IBA 1.0 + K1.0 mg/l.

Fig. 134- Multiple shoots induced (tips wilting) from a node on MS + IBA 1.0 + K 2.0 mg/l.

Fig. 135- Root induction from a shoot on MS + IBA 0.1 + K 1.0 mg/l.

Fig. 136- Fine long roots induced from a shoot on MS + IBA 0.1 + K0.1 mg/l.

Fig. 137- Multiple shoots with a single long root from a node on MS + IBA 1.0 + K 0.1 mg/l.

Fig. 4.B.9: Various effects of hormone free MS medium:

101

Fig. 138- Multiple shoot formation along with slight amount of callus on MS.

Fig. 139- Initiation of multiple shoot from a node on MS.

Fig. 140- Formation of root from the *in vitro* multiplied shoot.

Fig. 141- Formation of roots from the multiple shoots.

Fig. 4.B.10: Caulogenesis.

101

Fig. 142- Massive root induction from the shoot induced callus on MS + NAA 0.5 mg/l.

Fig. 143- Hairy roots coming directly from leaves on media containing MS + NAA 1.0 + BAP 0.1 mg/l.

Fig. 144- Roots from the shoot induced callus after subculture on MS + NAA 1.0 + BAP 1.0 mg/l.

Fig. 145- Profuse rooting with hairs from the shoot induced callus on MS + NAA 1.0 + BAP 1.0 mg/l.

Fig. 146- Roots and shoots formation from the callus on MS + NAA 1.0 + BAP 0.1 mg/l.

Fig. 4.B.11: Photomicrographs:

103

Fig. 147- Embryogenic callus from NAA 0.5 + BAP 1.0 mg/l.

Fig. 148- Actively dividing embryogenic cell (10×40).

Fig. 149- A Globular stage of somatic embryoid (10×40).

Fig. 150 and 151- Elongation of pro-embryois (10×40).

Fig. 152-. Embryoids at different stages (Globular, heart and torpedo at 10×10).

Fig. 153- T. S. of a mature embryo (10×10).

Fig. 154- A typical mature germinating somatic embryo with two distinct cotyledons (10×10).

Fig. 4.B.12: Rooting and acclimatization.

103

Fig. 155- Numerous thick roots formed from the *in vitro* multiplied shoot on MS + NAA 1.0 mg/l.

Fig. 156- Roots from the shoot on NAA 0.5 + BAP 0.5 mg/l.

Fig. 157- Sand rooting of shoots with 2 or more nodes after pulse treatment with different auxins.

Fig. 158- Sand rooted shoot with NAA 100 mg/l pulse treatment after 10 weeks.

Fig. 159- Acclimatization in the glass house on different substrates (soil: sand, coco-peat etc).

Fig. 160- A well acclimatized plant after 12 weeks in the shade house on coco-peat.

ABBREVIATIONS AND ACRONYMS

°C	Degree Celsius
µl	micro litre
µm	micro meter
2,4-D	2,4-Dichlorophenoxyacetic Acid
ANOVA	Analysis of Variance
BA	Benzyl Adenine
cm	centimetre
CTAB	Cetyl TrimethylAmmonium Bromide
cZ	cis Zeatin
DMRT	Duncan's Multiple range test
DNA	DeoxyriboNucleic Acid
Fig	Figure
FAO	Food and Agriculture Organisation
GA	Gibberellic Acid
M	molar
mM	milli molar
mm	milli meter
mRNA	messenger RiboNucleic Acid
MS	Murashige and Skoog
NAA	α-Naphthalene Acetic Acid
NPK	Nitrogen, Phosphorus, Kalium (Potassium)
PCR	Polymerase Chain Reaction
RNA	RiboNucleic Acid
tZ	trans Zeatin
UV	Ultraviolet