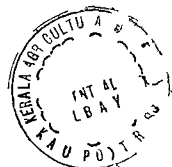
# ASSESSMENT AND INDUCTION OF VARIABILITY IN *PLUMBAGO* SPECIES FOR HIGH PLUMBAGIN CONTENT



By

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**(2005-21-112)**

**THESIS**

Submitted in partial fulfilment of the requirement for the degree of

## Doctor of Philosophy in Agriculture

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Kerala Agricultural University, Thrissur

**Department of Plant Breeding and Genetics**

COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

**2010**

# DECLARATION

I hereby declare that this thesis entitled **Assessment and induction of variability in *Plumbago* species for high plumbagin content** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree diploma associateship fellowship or other similar title of any other University or Society

Vellanikkara

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Certified that this thesis entitled **Assessment and induction of variability in** ***Plumbago*** **species for high plumbagin content** is a record of work done independently by **Mrs Namboodiri Raji Vasudevan** under my guidance and supervision and that it has not previously formed the basis for the award of any degree diploma fellowship or associateship to her

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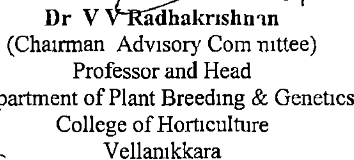

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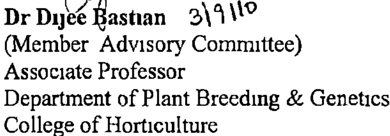

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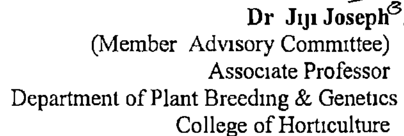

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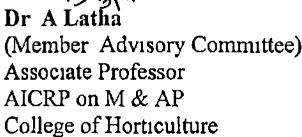

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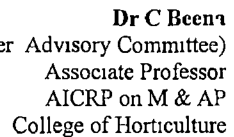

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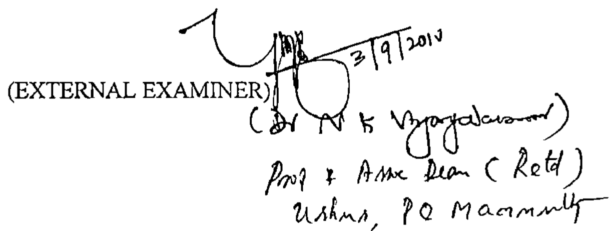

(EXTERNAL EXAMINER)

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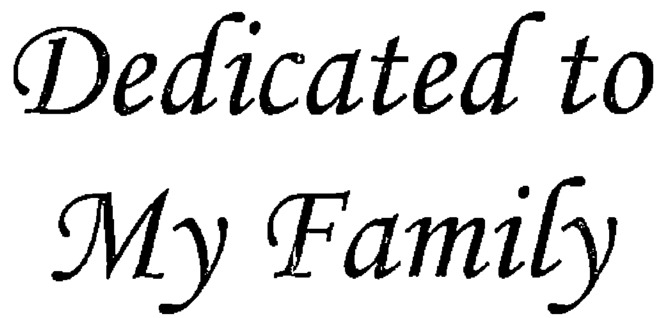
Namboodiri Raji Vasudevan


Dedicated to
My Family

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# *Introduction*

# INTRODUCTION

Medicinal plants have been considered as important therapeutic aid for alleviating ailments of humankind Search for eternal health and longevity as well as remedy to relieve pain and discomfort prompted the early man to explore his immediate natural surrounding In spite of tremendous developments in the field of allopathy medicinal plants and their derivatives still remain one of the major sources of drugs in modern and traditional systems throughout the world playing a major role in medicinal therapy Demand for medicinal plants is increasing due to growing recognition of natural products which are non toxic and having no side effects Furthermore an increasing reliance on medicinal plants in the industrialized societies has been observed *Plu nbago* is one of such plants which is valued for its roots that possess medicinal qualities

*Plumbago* species commonly known as koduveli in Malayalam belongs to Plumbaginaceae which is the only family of the order Plumbaginales The genus *Plumbago* includes 20 species and three species namely *Plumbago rosea* L *Plumbago zeylanica* L and *Plumbago capensis* T are reported from India The red flowered type *Plumbago rosea* a perennial shrub indigenous to Sikkim and Khasi hills is the accepted source of drug in Kerala

Its cultivation is always associated with anthropogenic localit es in both north and south India indicating its use as tribal medicine The white flowered type *Plumbago zeylanica* is considered to be more widespread and common than *P rosea* and is indigenous to South East Asia *Plumbago capensis* producing pale blue flowers is indigenous to South Africa and grown in gardens in India as an ornamental plant

The roots form the officinal part of the plant which enters into the composition of various ayurvedic preparations The active principle in *Plumbago* is plumbagin a naturally occurring naphthoquinone (2 methyl 5 hydroxy 1 4 naphthoquinone) It is extensively used in the treatment of chronic skin diseases like leucoderma It is known to have antifungal antimicrobial anticancerous as well as insecticidal properties It is also reported to cause chromosomal aberrations Among the three species of *Plumbago* *Plumbago rosea* is found to have higher content of plumbagin and is commonly cultivated throughout Kerala There is no seed set in *Plumbago rosea*

However in *Plumbago zeylanica* there is seed set But the seeds fail to germinate without seed treatments like scarification and cutting of micropylar or chalazal end of seed The multiplication of both these species is done through vegetative means and so the variability in them is found to be low Considering the limited natural variability available and perennial nature of *Plumbago* species creation of variability for enhanced root yield as well as increased therapeutic content is essential Genetic improvement through conventional breeding method is difficult in *Plumbago* species especially in *Plumbago rosea* due to lack of sexual reproduction In such a situation mutation and polyploidy breeding are found to be most viable to induce variability in this species for economic traits

Mutation breeding includes both *in vivo* as well as *in vitro* mutagenesis *In vivo* mutagenesis is beset with the problem of chimerism In vegetatively propagated crops it is more difficult to control chimerism and also often the size of the propagule is too big to treat larger population with mutagen Thanks to the technology of regenerating an individual plant from a single cell it is now possible to induce stable and true mutants without chimera Significant progress has been achieved in the last two decades in plant cell and tissue culture Cell and tissue culture techniques are important biotechnological tools now widely utilized to generate variation for plant improvement *In vitro* culture induced variation also known somaclonal variation constitutes an important source of variability for improvement of vegetatively propagated crops

The combination of *in vitro* culture methods with induced mutations can speed up breeding programmes from the generation of variability through selection to multiplication of new genotypes (Maluszynski *et al* 1995) Exploitation of somaclonal variation as a method to create genetic variability is unique When compared to *in vivo* mutagenesis the frequency of recovering useful variants is surprisingly high in case of somaclonal variation Somaclonal variation has been exploited in many crops especially in aromatic grasses for isolation of desirable plant types showing improvement in yield Most of the mutation breeding work in medicinal plants has been centered on *in vivo* mutagenesis Even though several protocols for *in vitro* regeneration of both *Plumbago rosea* and *Plumbago zeylanica* have been reported no systematic breeding programmes have been undertaken in

these species Since the protocols for *in vitro* regeneration are available *in vitro* mutagenesis can be attempted in *Plumbago* species

Polyploidy breeding is another important method used for inducing variability No reports are available on the use of induced polyploidy in *Plumbago* species Hence besides attempting induction of variability through *in vitro* techniques polyploidization using colchicine can be undertaken Induction of variability in the ***Plumbago*** species and the subsequent exploitation of this induced variability for screening genotypes with high plumbagin content will be of great value to the farmers as well as pharmaceutical industry

In light of the above mentioned facts the present study was undertaken with the following objectives

1 To explore and collect ***Plumbago*** species available in different districts of Kerala

2 To evaluate collected accessions of *Plumbago* species for genetic variability in morphological and biometrical traits includmg therapeutically active ingredients

3 To induce variability through *in vitro* mutagenesis and polyploidization in selected accessions

4 To screen the variants obtained from *in vitro* mutagenesis for high plumbagin content and other agronomic traits

# Review of literature

# 2 REVIEW OF LITERATURE

## 2 1 ORIGIN CLASSIFICATION AND GEOGRAPHICAL DISTRIBUTION OF *PLUMBAGO* SPECIES

*Plumbago* species belong to Plumbaginaceae the only family of order Plumbaginales Members of this family are mainly herbs and shrubs (Trease and Evans 1985) It comes under the series Heteromerae (Bentham and Hooker 1884) Hutchinson (1973) and Engler (1973) reported that *Plumbago* comes under the order Plumbaginales It contains 19 genera and 775 species There are seven related genera viz *Plumbago* Plumbagella Ceratostigma Armeria Limonium Goniolimon and Acantholimon reported in the family Plumbaginaceae (Darlington and Wylie 1961) The genus *Plumbago* includes 20 species of herbs under shrubs or shrubs distributed in tropics Three species *Plumbago rosea* L *Plumbago zeylanica* L *Plumbago capensis* T have been recorded from India based on flower colour and the first two species are considered medicinally important The systematic position of genus *Plumbago* is as follows

| | |
|---|---|
| Class | Dicotyledons |
| Sub class | Gamopetalae |
| Series | Heteromerae |
| Order | Plumbagmales |
| Family | Plumbaginaceae |
| Genera | *Plumbago* |
| Species | Rosea Zeylanica and Capensis |

The red flowered *Plumbago rosea* is a shrubby perennial found growing throughout India often as cultivated plant or as a garden escape It is reported to be wild or indigenous to Sikkim and Khasi hills It has been reported only as cultivated or as an escape from cultivation and never been recorded as wild in any areas far from Sikkim or Khasi hills (CSIR 1969) It is highly suited to Kerala conditions and is the accepted source of drug in Kerala (Sivarajan and Balachandran 1994) The white flowered type *Plumbago zeylanica* found as wild in peninsular India and West Bengal

5

is cultivated in gardens throughout India This species is more widespread and common than *P rosea* and is possibly indigenous to South East Asia

The species *Plumbago capaensis* having pale blue flowers is a native of South Africa This species is grown in gardens of India as an ornamental plant (CSIR 1969)

## 2 2 BOTANY

### 2 2 1 Morphological characters

*Plumbago rosea* is shrubby perennial with leaves broadly ovate or elliptic dark green above pale below flower red in terminal and axillary racemes *Plumbago capaensis* native of South Africa is a shrub with oblong or oblong spatulate leaves and pale blue flowers *Plumbago zeylanica* is a perennial sub scandent shrub with ovate leaves glabrous flowers white in elongated spikes capsules oblong pointed contained in viscid glandular persistent calyx It contains 2n–24 chromosomes in contrast to 2n 12 in *P rosea* and other species (CSIR 1969)

Dutta (1970) reported presence of glandular hairs secreting a sticky substance on the leaves branches and fruits of *Plumbago zeylanica*

The morphological anatomical and reproductive characters of the three species of *Plumbago* viz *P rosea P zeylanica* and *P capensis* were evaluated and a key for identification of the three species was proposed by Subha (2000) *P rosea* could be easily distinguished by the presence of smooth stem dark green leaves with reddish tinge on the petiole and midrib and red flowers *P zeylanica* was characterised by the presence of ridged stem with reddish striations along the ridges starting from the node and white flowers *P capensis* was found to be quite different from the other two species due to the presence of spatulate leaves angular stem and compact inflorescence with brilliant blue flowers

### 2 2 2 Reproductive biology

#### 2 2 2 1 Flowering period

Krizek and Semeniuk (1972) studied the effect of day and night temperature on

flowering of *Limonium* an ornamental plant in the family plumbaginaceae They reported that a low temperature of 16 15°C was required for flower initiation

Long days and cool night temperatures increased the percentage of flowering in *Limonium* cultivars (Semeniuk and Krizek 1972) Application of gibberellic acid 500 ppm accelerated flowering of *Limonium* (Wilfret and Raulston 1975) Escher *et al* (1988) reported that *Plumbago indica* requires a day length of nine hours for flowering

Flowering in *P rosea* was observed to be seasonal between October and February while it was continuous in *P zeylanica* (Menon 1999)

**2 2 2 2 Inflorescence**

Inflorescence was observed only in terminal position as a single spike in *P rosea* In *P rosea* inflorescences were single or branched spikes either terminal or axillary Inflorescences were longer in *P rosea* and took longer periods for completion of flowering in a spike as well as produced more number of flowers per inflorescence (Menon 1999)

**2 2 2 3 Floral biology**

Iyer and Komalammal (1960) described the floral biology of *Plumbago zeylanica* and *P rosea* They found that in *P zeylanica* bracts are considerably larger than bracteoles whereas they are nearly equal in size in *P rosea*

Baker (1966) reported the presence of papillate and nonpapillte type of stigmas in several species of Plumbagmaceae

Bahadur (1978) noticed distyly as common feature of several members of family plumbagmaceae

Huang *et al* (1990) studied the organization of embryosac and egg of *Plumbago zeylanica* They reported that egg of *P zeylanica* has combined synergids and gamete functions

Huang and Russell (1993) reported that embryosac of *P zeylanica* consists of 2 to 5 cells including an egg cell a central cell and 0 to 1 antipodal cell depending on the degeneration of lateral and chalazal nuclei during megagametogenesis

Sodmergen *et al* (1995) studied male gametophyte development in *Plumbago zeylanica* and explained cell localization and cell determination in early generative cell of *Plumbago zeylanica*

Sodmergen *et al* (1995) studied male gametophyte development in *Plumbago zeylanica* and explained cell localization and cell determination in early generative cell of *Plumbago zeylanica*

Ca Yajuan *et al* (1997) isolated viable egg of *Plumbago zeylanica* and found that eggs retain viability for 24 hours

Southward *et al* (1997) noticed the existence dimorphic sperms in *Plumbago zeylanica* The plastid rich sperms fused with egg and sperm with fewer plastids fused with the central cell

Subha (2000) compared floral characters of the three species of *Plumbago* and described *P rosea* as having beautiful scarlet red flowers with reddish green bracts and bracteoles fimbricate style and stamens with pink filaments red anthers and creamy white pollen grains *P zeylanica* was having attractive white flowers with cordate bracteoles green calyx tube terete style white filaments carrying purple anthers and white pollen grains *P capensis* produced brilliant blue flowers subtended by lanceolate bracts and bracteoles blue anthers blue filaments and creamy yellow pollen grains

**2 2 2 4 Anthesis anther dehiscence and stigma receptivity**

Subha (2000) observed a peak period of anthesis between 6 a m and 8 a m in *P rosea* whereas in the case of *P zeylanica* it was between 4 a m and 6 a m with flower opening extending over a long period of time She also indicated the presence of protogyny in the species *P rosea* where anther dehiscence was found to occur after flower opening and stigma became receptive before dehiscence of anthers In *P*

**2 2 2 5 Pollen morphology fertility and viability**

Menon (1999) compared pollen fertility in *P zeylanica* and *P rosea* using acetocarmine staining technique and found that *P zeylanica* produced more fertile (89 9%) pollen grains compared to that of *P rosea* (28 13%) This was contrary to the observation made by Subha (2000) who reported very high pollen fertility in the three species of *Plumbago* using the same staining technique

Menon (1999) reported failure of germination of pollen grains of the two species *P rosea* as well as *P zeylanica* under *in vitro* conditions However pollen grains of *P zeylanica* recorded germination under *in vivo* conditions on artificial pollination of the stigma of both the species

**2 2 2 6 Pollination and fruit set**

Frankel and Galun (1977) reported that there exists heteromorphic incompatibility in *Plumbago Ceratostigma* and *Limonium* species of the family Plumbaginaceae Dulberger (1975) pointed out that structural stigma dimorphism may be involved in incompatibility In *P europea* and *P capensis* papillae of the long styled and short styled morphs differ in the way cuticle is attached to the cellulose layer The intermorph pollination is incompatible and inhibition of pollen growth occurs at the stigmatic surface

No fruit set has been reported in *P rosea* (CSIR 1969) However Iyer and Komalammal (1960) and Sivarajan and Balachandran (1994) described the fruits of *Plumbago* species The fruits are enclosed in persistent calyx in one seeded membranous capsule

Menon (1999) reported fruit set in *P zeylanica* alone This species recorded 70 to 80 per cent fruit set on self pollination 85 to 90 per cent fruit set on open pollination and 30 to 40 per cent on natural cross pollination

Arya (1999) reported abundant adhesion pollen grains on papillate stigma in *Plumbago zeylanica* unlike *Plumbago rosea* as one reason for satisfactory seed set in this species

## 2 3 ACTIVE PRINCIPLES AND THEIR PROPERTIES

The roots form the officinal part of the *Plumbago* species The active principle in *Plumbago* is plumbagin a naturally occurring naphthoquinone (2 methyl 5 hydroxy 1 4 naphthoquinone) (CSIR 1969) It is obtained as golden yellow needle shaped crystals (Chopra *et al* 1958) Krishnaswamy and Purushothaman (1980) isolated plumbagin from the roots of *Plumbago zeylanica* and reported that it is a potential anticancer drug and is used in reducing the tumour growth in fibrosarcoma and also against lymphocytic leukaemia Plumbagin causes effective cell growth inhibition induces apoptosis and generates single strand breaks in cancer cells Srinivas *et al* (2004) reported that plumbagin induced cell death in human cervical cancer cell line exhibiting biochemical characteristics of apoptosis Further investigations by Nazeem *et al* (2009) confirmed that plumbagin induces apoptosis in human cancer cells through the redox mechanism involving copper by producing reactive oxygen species (ROS)

The antiviral activity of Liv 52 a powdered mixture of 18 plants including *Plumbago zeylanica* as a component was studied by Singh *et al* (1983) The antifertility activity of plumbagin isolated from the roots of *Plumbago zeylanica* was reported by Bhargava (1984) Gujar (1990) reported the use of plumbagin in the treatment of liver disorders The ethanolic root extract of *Plumbago rosea* showed an increase in life span of mice bearing ascites sarcoma 180 tumor (Solomon *et al* 1993) Devi *et al* (1994) suggested that the ethanolic root extract of *Plumbago rosea* as a good candidate for use with radiation to enhance tumour killing effect Plumbagin obtained from various species of the genus *Plumbago* possesses potent anti fertility activity (Dhar and Rao 1995) The anti inflammatory properties of root extracts of genus *Plumbago* were reported by Rimbau *et al* (1996)

Plumbagin is also reported to exhibit various insecticidal antibacterial and antifungal activities Gujar and Mehrotra (1988) reported that plumbagin possessed high contact toxicity to *Dysdercus koenigii* Joshi *et al* (1988) reported retarded growth delayed metamorphosis deformation and inhibition of moulting on topical treatment of red cotton bug with plumbagin Plumbagin isolated from the roots of *Plumbago indica* was found to have high larvicidal action against mosquito larvae (Chockalingam *et al* 1990) The mortality and survival of the parasitic nematode

*Haemonchus contortus* and the embryonation of *Ascaris sum* were inhibited when plumbagin extracted from the plants of *Plumbago* spp was applied (Fetterer and Fleming 1991) Villavicencio and Perez escandon (1992) reported the antifeedant effect of the extracts of *Plumbago* spp on three species of orthopteran acrid ds Gujar *et al* (1994) studied the bioactivity of the extracts of plants in the genus *Plumbago* and reported that the inhibition of growth by plumbagin in nymphs of *Dysdercus koenigii* was dose dependent and in adults the mating behaviour and reproduction were affected Plumbagin significantly affected both chitin and cuticular protein when applied topically to the larvae of noctuid *Helicoverpa armigera* (Krishnayya and Rao 1995) Saxena *et al* (1996) reported that topical application of plumbagin dissolved in acetone killed the adults of *Musca domestica* a dipteran insect in high concentration and also induced sterility in medium concentrations

Krishnaswamy and Purushothaman (1980) reported that plumbagin extracted from the two species of *Plumbago viz* *rosea* and *zeylanica* showed antibacterial activity It inhibits the growth of both gram positive and gram negative bacteria at 20 mg/ml

The antifungal activity of plumbagin against *Rhizopus nigricans* *Pencillium notatum* *P canadense* *Epidermophyton floccosum* and *Microsporum nanum* at 10 mg/ml was reported by Krishnaswamy and Purushothaman (1980)

The roots are reported to contain other related compounds apart from plumbagin such as sitosterol stigmasterol campeserol and flavanoids Menon (1999) reported the presence of additional compounds in the thin layer chromatogram of cured roots of *P rosea* The compounds belonged to steroid group and flavanoid group

**2 3 1 Therapeutic uses**

Plumbagin is highly caustic and causes blisters on skin Hence the roots of *P rosea* are used only after curing and drying for ayurvedic preparations In ayurvedic preparations *P rosea* is recommended as an excellent remedy as digestive (Deepanam) and stomachic (Pachanam) Plumbagin is pungent astringent diuretic germicidal vesicant and abortifacient It overcomes flattulence oedema piles coughs worms and haemorrhidal anal inflammation It is effective in the treatment of

common warts (Pillai *et al* 1981) and liver disorder (Gujar 1990) The drug also cures enlargement of abdomen anaemia diabetes leucoderma leprosy diaarrhoea dyspepesis and elephantiasis (Sivarajan and Balachandran 1994)

Albeit a lot of research has been done on botany chemical constituents and their properties studies on crop improvement in *Plumbago* species are meager At Kerala Agricultural University studies on cultivat on of *Plumbago rosea* were initiated in 1990 Subha (1990) compared the effect of different levels of spacing and planting materials on yield and quality of *Plumbago rosea* The results indicated that plumbagin content of root in this species varied from 0 16% 0 17% Different spacing levels did not influence the plumbagin content The possibility of successfully cultivating *Plumbago* as an intercrop in young coconut plantation adopting different methods of planting such as ridge and furrow flat bed mound and pit followed mound was observed by Menon (1995) No significant difference was observed in root yield under open and shaded condition But higher shoot weight was observed under shaded condition Even though crude plumbagin was higher under shade condition there was no marked difference in the purified plumbagin content

The available literature on *Plumbago* species is limited Hence the literature citations for the present work were broadened to cover vegetatively propagated crops belonging to a wide range of economic use such as tubers medicinal aromatic and ornamental The literature available is reviewed under the following heads

1 Evaluation of genetic variability in *Plumbago* species for morphological and biometrical traits
2 Induction of variability through *in vitro* mutgenesis in *Plumbago* species
3 Induction of polyploidy in *Plumbago* species

## 2 4 EVALUATION OF GENETIC VARIABILITY IN *PLUMBAGO* SPECIES FOR MORPHOLOGICAL AND BIOMETRICAL TRAITS

### 2 4 1 Genetic variability

#### 2 4 1 1 Morphological and biometric traits

Arya (1999) assessed variability in eight ecotypes of *P rosea* and one ecotype of *P zeylanica* and reported highest values of internode length plant height number of suckers and fresh weight of shoot in *P zeylanica*

Menon (1999) analysed the growth and quality in *P rosea* and *P zeylanica* The analysis of growth characters revealed that *P zeylanica* had significantly higher plant height and total leaf area However *P rosea* recorded higher internodal length Fresh and dry weights of shoot were significantly higher in *P zeylanica* In contrast fresh root and dry root yield root shoot ratio and harvest index were higher in *P rosea*

**2 4 1 2 Plumbagin content**

Plumbagin is present to a maximum of about 0 91 per cent in the roots of all the species of *Plumbago* seen in India (CSIR 1969) The proportion of plumbagin varies with the locality growth age soil conditions and season of the year In general it was found that older the plant and drier the soil the greater is the quantity of active principle found in the roots It has also been reported that the fresh roots yield a much greater proportion of plumbagin than roots which have been stored for longer periods

Menon (1999) recorded a higher content of the active principle plumbagin in *P rosea* ranging from 0 69 to 1 4 per cent (on dry weight basis) than in *P zeylanica* with a plumbagin content ranging from 0 19 to 0 33 per cent (on dry weight basis) In *P rosea* plumbagin level varied significantly at different stages of growth and the maximum value of 1 4 per cent was noticed at 16 MAP

Arya (1999) reported significantly low content of plumbagin in *P zeylanica* when compared to eight ecotypes of *P rosea*

**2 4 1 3 GCV PCV Heritability and Genetic gain**

Heritability in the broad sense refers to the relative proportion of genotypic variance to phenotypic variance Coefficient of variation (CV) is used to compare the relative variation among different metric traits which are measured in different units Lush (1937) and Johnson *et al* (1955a) developed accurate procedures for the calculation of genetic advance under specified intensities of selection which in metric traits largely depends on the heritability phenotypic variability of the trait under selection and the selection differential expressed as phenotypic standard deviation

Kanakamony (1998) reported highest heritability for leaf length followed by leaf breadth and tiller number and lowest for rhizome weight in *Kaempferia galanga*

However high estimates of heritability coupled with genetic gain was noticed only for number of leaves and rhizome number indicating considerable scope for genetic improvement with respect to these traits

Arya (1999) observed high heritability for characters such as plant height internode length number of suckers length of root girth of root number of roots dry weight of root and plumbagin content in roots of *P rosea* However only three characters *viz* plant height root length and dry root yield recorded high values for combined estimates of heritability and genetic gain The character plant height recorded maximum genetic gain (137 90%) and plumbagin content recorded minimum genetic gain (0 39%)

The coefficient of variation heritability and expected genetic advance for 18 important horticultural traits was estimated in 26 accessions of ginger (*Zingiber officinale*) High heritability coupled with high genetic advance as percentage of mean was observed for plant height leaf length suckers per plant number of mother and secondary rhizomes weight of primary rhizome and rhizome yield per plant indicating that desirable improvement in these traits can be brought about through selection (Yadav 1999)

Fifteen accessions of turmeric (*Curcuma longa* L ) were evaluated for variability in thirteen traits namely days to maturity plant height number of suckers/plant length of leaves width of leaves number of rhizomes/plant length of rhizome diameter of rhizome rhizome yield/plant total biomass/plant harvest index rhizome yield/plot and rhizome yield/hectare Higher estimates of both PCV and GCV were observed in order of merit for rhizome yield/plant total biomass/plant rhizome yield/hectare rhizome yield/plot High estimates of heritability combined with genetic advance as per cent over mean was recorded by characters such as number of suckers per plant total biomass per plant and number of rhizomes per plant (Singh *et al* 2008)

**2 4 1 4 Correlation studies**

Correlation studies to determine the inter relationship among various traits are useful in making selection Grafius (1964) noted necessity to estimate the genotypic

and phenotypic correlation coefficient to determine the true association due to genetic causes

In *Kaempferia galanga* a significant positive association of yield with number of leaves tillers leaf length plant spread and rhizome number was noticed (Kanakamony 1998)

Arya (1999) estimated the phenotypic and genotypic correlation coefficients of dry root yield with eleven other characters and their *inter se* associations in *Plumbago rosea* All the genotypic correlations between dry root yield and other characters were positive Eight of the eleven characters namely plant height number of leaves internodal length number of suckers per plant root length root girth number of roots per plant fresh weight of shoot and fresh weight of root exhibited significant genotypic and phenotypic correlation with dry root yield Correlation between pair of characters other than those with yield was also worked out The plumbagin content in roots showed both positive and negative correlations with other characters At genotypic level the plumbagin content had a positive correlation with number of roots plant This character had shown negative correlation with fresh weight of shoot plant height dry weight of shoot and internodal length

Correlation studies in ginger revealed that the genotypic correlation coefficients were higher than the phenotypic ones for almost all the characters The rhizome yield per plant and per plot had significant positive genotypic and phenotypic correlation with number of leaves per shoot (0 615 and 0 413) number of tillers per plant (0 679 and 0 6؟2) leaf length (0 878 and 0 778) leaf breadth (0 970 and 0 801) rhizome length (0 578 and 0 739) and rhizome breadth (0 713 and 0 613)(Tiwari 2003)

In *Chlorophytum borivilianum* correlation analysis for root yield (dependent variable) and seven plant traits (independent variables) revealed that leaf number leaf length and finger number which had contributed highly to divergence had also significant associations with root yield The $D^2$ and correlation results suggested that the variability for the three traits (leaf number leaf length and finger number) could be reliable selection criterion for root yield in *C borivilianum* (Kumar *et al* 2008)

**2 4 1 5 Path coefficient analysis**

Path coefficient analysis is standardized partial regression coefficient analysis and as such measures the direct effect of one variable and indirect effect via other variables on the dependent variable This analysis permits the separation of correlation coefficients into components of direct and indirect effects of independent variables on dependent variable (Dewey and Lu 1959)

Arya (1999) studied the direct and indirect effects of eight quantitative characters on plumbagin content of roots of *P rosea* The characters namely plant height number of suckers root length root girth and fresh root yield exhibited negative d rect effects on plumbagin content However the characters such as internodal length and dry root yield expressed positive direct effect on plumbagin co ntent Fresh root yield was considered as a negat ve factor and dry root yield as a positive factor contributing directly and indirectly to the plumbagin content

**2 4 1 6 Cluster analysis**

Among several statistical methods used to quantify genetic divergence multivariate analysis is potent tool (Rao 1969) Assessment of genetic divergence by use of $D^2$ statistic is useful in choosing parents for any breeding programme (Murty 1965)

Sixty accessions of *Coleus parviflorus* collected from different eco geographical reg ons of Kerala and neighbouring states were grouped into ten clusters based on economic traits The clustering pattern showed no parallelism between genetic d versity and geographical distribution Based on the ranking for important yield attributes the overall best yielder from each cluster was selected and subjected to physical and chemical mutagenesis (Abraham 2002)

Six phenotypic characters and three withanolide markers were assessed in 25 accessions of *Withania somnifera* collected from different states of India for studying genetic variability Based on $D^2$ values and PCA (Principal Component Analysis) of phenotypic traits like plant height number of branches/plant number of seeds/berry root length root diameter and root yield these 25 accessions were grouped in five

Pradesh) AGB006 (J&K) and AGB009 (Punjab) representing clusters 2 and 4 exhibited maximum intra and inter cluster divergence Cluster 5 representing accession AGB053 (Andhra Pradesh) was having mixed traits (Kumar *et al* 2007)

Genetic divergence among 51 genotypes was determined using nine characters of *C borivilianum* of indigenous origin via Mahalanobis $D^2$ statistic The genotypes were grouped into eight clusters Intra cluster distance was largest for cluster VIII (nine genotypes) followed by cluster I (six genotypes) Inter cluster $D^2$ values recorded between cluster II and III and those between cluster III and VI indicated the possibility of raising transgressed hybrids from cross hybridization programs using divergent parents of these four clusters The clustering pattern indicated that geographical diversity was not necessarily related with genetic diversity (Kumar *et al* 2008)

Twenty nine accessions of Brahmi (*Bacopa monnieri*) collected from different geographical regions of Kerala were evaluated for variability Significant genetic variability was expressed by all the characters studied Cluster analysis indicated that there was no parallelism between geographical distribution and clustering pattern of accessions Accession 29 was identified as better plant for higher biomass yield and Bacoside A content followed by Accession 14 (Radhakrishnan *et al* 2008)

## 2 5 INDUCTION OF VARIABILITY THROUGH *IN VITRO* MUTGENESIS IN *PLUMBAGO* SPECIES

### 2 5 1 *In vitro* regeneration in *Plumbago* species

#### i) Establishment of aseptic cultures

Explants collected from field grown plants are usually contaminated by various microorganisms Besides surface contaminants Mathias and Anderson (1987) reported that bacteria fungi or viruses on the surface of the bark glandular hairs at the nodes and internal tissues could cause contamination in the explants Woody plant tissue culture has a serious problem associated with it as it harbours a number of microorganisms within its tissues internally which cause latent contamination Surface sterilisation removes only those microorganisms that are present on the outer surface According to Mallika *et al* (1992) growing stock plants under controlled conditions and regularly spraying the plants with systemic and contact fungicides can reduce or

avoid the problem of contamination to some extent Common sterilising agents like sodium or calcium hypochlorite (5 10%) ethyl alcohol (50–95%) and mercuric chloride (0 01 0 1%) are used to exclude the surface contaminants by washing in the appropriate solution for 10–25 min followed by several rinses in sterile water Rigorous screening of the stock cultures for bacterial contamination is highly essential

**ii) Basic culture media**

Although more than 50 different devised media formulations have been used for the *in vitro* culture of tissues of various plant species (Gamborg *et al* 1976 Huang and Murashige 1977) the formulation described by Murashige and Skoog (1962) (MS medium) is most commonly used often with relatively minor changes Percentage of bud breaks and shoot multiplication was generally higher in MS medium compared to several other media The MS medium is characterised by high concentration of mineral salts Skirvin (1980) and Griffins *et al* (1981) suggested that reducing the strength of MS medium by half was more beneficial for culturing Response of an explant to different media depends on the plant species All the reports available on *in vitro* culture of *Plumbago* species are in MS medium

**iii) Culture environment**

The physical form of the medium whether liquid or semi solid pH other environmental factors like light temperature relative humidity and season of culture play an important role in the *in vitro* growth and differentiation Light requirement for differentiation involves a combination of several components namely intensity quality and duration (Murashige 1974) Broderick *et al* (1974) reported that in onion and tomato callus cultures were formed and maintained in darkness at 23 27° C According to Murashige (1977) the optimum day light period required is 16 hours for a wide range of plants

**2 5 1 1 Direct organogenesis**

*In vitro* culture of medicinal plants has been achieved through rapid proliferation of shoot tips and axillary buds in culture The cells of apical and axillary meristems are uniformly diploid and least susceptible to genotypic changes hence this method

ensures genetic stability of the clones A shoot tip and an axillary bud having preformed meristems usually develop axillary shoots on a high cytokinin concentration Skirvin (1980) pointed out that there is variation among the type of explant within each plant species and the most suitable explant for each plant species should be determined Many efficient protocols for *in vitro* propagation through direct regeneration have been developed in many medicinal plant species including the *Plumbago*

Thomas *et al* (2003) reported direct shoot regeneration from terminal bud and mature nodal explants in *Terminalia arjuna* Roxb a multipurpose tree Nodal explants produced more shoots than terminal apex

Panimalar *et al* (2007) observed that the best explant in *Centella asiatica* was nodal segments *Dioscorea bulbifera* L containing the pharmaceutically important compound diosgenin was regenerated *in vitro* through nodal segments on supplemented Murashige and Skoog medium (Narula *et al* 2007)

***Plumbago* species**

Rout *et al* (1999) developed an efficient protocol for *in vitro* clonal propagation of *Plumbago zeylanica* L through nodal culture Multiple shoots were induced from nodal explants of *P zeylanica* on Murashige and Skoog s (1962) medium supplemented with 0 5 mg l to 1 0 mg mg l 6 benzyladenine and 3% (w/v) sucrose

*In vitro* plant regeneration was achieved by Rout (2002) from leaf explants of *Plumbago rosea* and *Plumbago zeylanica* on MS medium The semi mature leaves produced more shoot buds as compared to the younger leaves Mature leaves did not show any response for shoot bud initiation More than 85% of the semi mature explants produced shoot buds per leaf explant within 4 week of culture

Chaplot *et al* (2006) established protocol for plant propagation through axillary bud proliferation and organogenesis in *Plumbago zeylanica* (L) Multiple shoots emerged from the nodal explants within two weeks of incubation in MS medium

Gopalakrishnan *et al* (2009) established a simple rapid high frequency micropropagation method for *Plumbago rosea* from leaf explants without intervening callus phase

### 2 5 1 2 Effect of growth regulators on shoot proliferation and elongation

Murashige (1974) utilised cytokinins to overcome the apical dominance of shoot to enhance the branching of lateral buds from leaf axils He reported that BAP is the most effective cytokinin for meristem shoot tip and bud culture followed by kinetin Lo *et al* (1980) reported that a high content of cytokinin was deleterious to the initiation and elongation of roots of both monocotyledonous and dicotyledonous plants Cytokinins levels were shown to be the most critical for multiplication of many medicinal plants Upadhyay *et al* (1989) made an in depth study of the influence of cytokinin on the growth characteristics in *Picrorhiza kurroa in vitro* The use of three cytokinins (BA 2ip and kinetin) separately at higher concentration (3 0 5 0 mg $l^{-1}$) used in the medium led to an increase in the abnormalities (vitrification and fasciation) of the shoots However it was observed that cytokinin was required in optimal quantity for shoot proliferation in many genotypes but inclusion of low concentration of auxins along with cytokinin triggered the rate of shoot proliferation Hence for successful organogenesis in some cultures a combination of auxins and cytokinins are used Although exogenous auxins do not promote axillary shoot proliferation culture growth has been improved by its presence (Wang and Hu 1980) Lundergan and Janick (1980) advocated that one of the possible roles of auxin at elongation stage is to nullify the suppressive effect of high cytokinin concentration thereby restoring normal shoot growth Gibberellins are also known to prevent root and shoot formation Gibberellic acid ($GA_3$) is the most frequently used gibberellins in tissue culture But $GA_3$ in presence of cytokinin promoted shoot proliferation as well as elongation Gibberellic acid at 0 1 0 5 mg $l^{-1}$ and adenine sulfate at 50 100 mg $l^{-1}$ had a promising effect on shoot proliferation and elongation The role of the three above mentioned growth regulators *viz* BA adenine sulfate and gibberellic acid have been reviewed in many medicinal plant species including in the *Plumbago* species Apart from these the role of Thidiazuron a synthetic phenylurea derivative with high cytokinin activity has been reviewed in woody plant species

ı) **Benzyl Adenıne (BA)**

Multıple shoots were ınduced from nodal explants of *P zeylanıca* on Murashıge and Skoog s (1962) medıum supplemented wıth 0 5 mg l¹ to 1 0 mg l 6 benzyladenıne and ɔ% (w/v) sucrose Inclusıon of IAA (0 01 mg l¹) ın the culture medıum ımproved the frequency of productıon of multıple shoots (Rout 1999)

Cultures were ınıtıated from nodal segments of mature plants of *Hypeı ıcum perforatum* ınoculated onto MS medıum supplemented wıth 4 5 µM BA kınetın thıdıazuron ındıvıdually or ın combınatıon wıth 0 05 µM NAA Organogenıc explants were observed on medıum wıth eıther BA or kınetın alone or ın combınatıon of these wıth NAA Subculture of organogenıc explants onto the prolıferatıon medıum contaınıng 4 5 µM BA promoted the organogenıc response (Santarem and Astar ta 2003)

Mıcropropagatıon of *Bacopa ı ıonnıeı ı* was achıeved on MS and B5 medıum supplemented wıth BAP and NAA usıng leaf explants and nodal segments Best results were found on MS medıum ın both the explants wıth BAP (2 0 mg l ) showıng hıgher percentage of regeneratıon (Mohapatra and Rath 2005)

In *Tylophoı a ındıca* multıple shoot formatıon was ınduced from nodal explant on MS medıum supplemented wıth dıfferent concentratıons of BAP alone or ın combınatıon wıth NAA However nodal explants cultured on MS medıum supplemented wıth dıfferent concentratıon of BAP alone showed best results The number of shoots formed also varıed wıth the concentratıon of BAP Three to four shoots were formed on lower concentratıon of BAP (1 ppm) Hıghest number of shoots per culture was obtaıned on MS medıum supplemented wıth 2 ppm of BAP where nodal explant produced nearly 45 to 50 shoots But wıth the further ıncrease ın concentratıon of BAP number of shoots formed has waned to 6 to11 shoots on 4 ppm of BAP and 4 shoots per explant on 6 to10 ppm (Nadha 2006)

The nodal explants of *P zeylanıca* when ınoculated ın MS medıum supplemented wıth BA (0 0 8 8 mg l¹) and IAA (0 0 2 88 mg l¹) at dıfferent concentratıons eıther alone or ın combınatıons produced maxımum number of shoots

(12 multiple shoots) in the basal medium with 4 4 mg l BA and 1 4 1 mg $l^{1}$ IAA (Chaplot *et al* 2006)

Leaf explants of *Plumbago rosea* inoculated on MS supplemented with 6 66 µM BAP and 2 69 µM NAA produced numerous (105 ± 0 3) shootlets with an average length of 3 1 ± 0 0 cm Small shootlets were transferred to shoot elongation medium supplemented with 1 11 µM BAP plus1 44 µM $GA_3$ (Gopalakrishnan *et al* 2009)

**ii) Adenine sulphate (Ads)**

It brings about responses similar to cytokinins Usually added at 50 100 ppm concentration In most cultures adenine sulphate acts more as a synergist of cytokin ns The addition of adenine sulfate in the culture medium influenced rapid prolifeiation of shoot multiplication of *Cu cu na longa* cvs Suroma and PTS 28 The frequency of shoot multiplication increased 4 fold at every 4 week interval cult red on MS medium supplemented with 4 0 mg $l^{1}$ BA 1 0 mg $l^{1}$ IAA and 100 mg $l^{1}$ adenine sulfate (Rout *et al* 1995) Similarly the rates of multiplicat on of *Zingibei officinale* cvs Suruchi and Suprabha were higher in a medium containing BA (4 0 6 0 mg $l^{1}$) IAA (1 0 1 5 mg $l^{1}$) and 100 mg/l adenine sulfate (Palai *et al* 1997)

High frequency shoot bud regeneration was achieved was achieved by Rout (2002) from leaf explants of *Plumbago rosea* and *Plumbago zeylanica* in MS medium supplemented with 1 5 mg $l^{1}$ 6 benzylaminopurine 0 25 mg l indole 3 acetic acid 50 mg $l^{1}$ adenine sulfate and 3% (w/v) sucrose

Mult ple shoots were induced from apical meristems of *Curculigo oi chioides* Gaertn grown on Murashige and Skoog (MS) basal medium supplemented with 1 5 mg l 6 benzyladenine (BA) 100 mg $l^{1}$ adenine sulfate and 3% sucrose Inclusion of indole 3 butyric ac d (IBA) or indole 3 acetic acid (IAA) in the culture medium improved the formation of multiple shoots The highest frequency of mult plication was obtained on MS medium supplemented with 1 5 mg $l^{1}$ BA 100 mg l Ads 0 25 mg $l^{1}$ IBA and 3% sucrose (Francis *et al* 2007)

Bud and shoot formation were achieved when nodal segments of *Trichosanthes cucui mina* were cultured in MS medium containing 0 5 mgl $^{1}$ BA Effect of cytokinin

precursor adenine sulphate for enhanced release of axillary buds was also investigated The same medium for culture initiation was found effective for shoot multiplication wherein on an average 6 2 shoots were produced at the end of third subculture The cytokinin precursor adenine sulphate was ineffective in inducing multiple shoots in *Trichosanthes* (Sankar *et al* 2007)

**iii) Thidiazuron (TDZ)**

Thidiazuron (TDZ) is among the most active cytokinin like substances for woody plant tissue culture It facilitates efficient micropropagation of many recalcitrant woody species Low concentrations (<1 µM) can induce greater axillary proliferation than many other cytokinins At concentrations higher than 1 µM TDZ can stimulate the formation of callus adventitious shoots or somatic embryos However TDZ may inhibit shoot elongation In some cases it is necessary to transfer shoots to an elongation medium containing a lower level of TDZ and/or a less active cytokinin Subsequent rooting of microshoots may be unaffected or slightly inhibited by prior exposure to TDZ The main undesirable side effect of TDZ is that cultures of some species occasionally form fasciated shoots The high cytokinin activity and positive response of woody species to TDZ have established it as among the most active cytokinins for *in vitro* manipulation of many woody species (Huetteman and Preece 1993)

Ledbetter and Preece (2004) studied the effects of thidiazuron (TDZ) on adventitious shoot production from oak leaf hydrangea Leaf explants placed *in vitro* on MS medium containing 1 µM IBA and 0 05 0 1 0 5 1 0 or 5 0 µM TDZ formed shoots that were adventitious Lower concentrations of TDZ stimulated the formation of fewer shoots but they elongated The highest concentration resulted in leaf explants that were heavily callused and had many tiny shoots that were slow to elongate

Attempts were made to study the effect of thidiazuron (TDZ) on adventitious shoot induction and plant development in *Paulownia tomentosa* explants derived from mature trees Optimal shoot regeneration was obtained in leaf explants cultured on MS medium containing TDZ (22 7 or 27 3 µM) in combination with 2 9 µM indole 3 acetic acid (IAA) for 2 weeks and subsequent culture in TDZ free shoot development

### 2 5 1 3 Callus mediated regeneration

The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium cell division cell growth and tissue differentiation are induced Several reports are available on the regeneration of various medicinal plants *via* callus culture The callus mediated regeneration in *Plumbago* species as well as other medicinal plant species is reviewed

### 2 5 1 4 Effect of culture environment and growth regulators on callus induction and regeneration

Satheeshkumar and Bhavanandan (1988) could induce callus stem segments from the medicinal plant *Plumbago rosea* L on MS medium containing 2 4 D and kinetin The explants placed horizontally showed MS media supplemented with 2 4 D (2 5 mg l ) and kinetin (1 5 mg $l^{-1}$) production better callus production than those placed vertically Multiple shoot formation was induced on callus subcultured in MS media supplemented with BAP (2 0 mg $l^{-1}$) plus NAA (1 0 mg $l^{-1}$) An 18 h photoperiod or darkness had no different effect on callus induction

Tawfik *et al* (1998) developed a protocol to induce organogenic callus in *Rosmarinus officinalis* L High concentrations of TDZ (up to 2 0 mg l ) alone or plus 0 5 mg $l^{-1}$ IAA induced organogenic callus on shoot tip stem and leaf explants cultured on MS medium Compact dark green calli were formed on the abaxial side of the leaf segment These when transferred to a medium supplemented with different concentrations of BA (2 0 4 0 6 0 and 8 0 mg $l^{-1}$) produced multiple shoots

Callus cultures of *Taxus* species were established on $B_5$ medium supplemented with 2 4 D NAA and IBA individually or in combination with kinetin Although callus induction occurred when incubated under a 16 h light/8 h dark day/night regime the amount of callus produced and the overall success rate was higher on explants incubated in total darkness (Wickremsinhe and Arteca 1998)

Callus was initiated from internodal segment leaf root and petiole explants of *Withania somnifera* (L ) Dunal on MS medium supplemented with 2 4 D (0 5 3 0 mg l $^{-1}$) and NAA (0 5 3 0 mg l  ) either alone or along with kinetin (0 5 1 0 mg l  ) Internode segments exhibited highest callusing (up to 80 per cent) response after 30 days of culture on MS medium supplemented with NAA (1 0 mg/l) or 2 4 D (1 0 mg l $^{-1}$) with kinetin (1 0 mg l $^{-1}$) NAA when used at higher concentration (3 0 mg l  ) formed roots along with small amount of callus Multiple shoot regeneration was observed from callus of all the explants except root on MS medium fortified with BAP (0 5 2 5 mg l $^{-1}$) or in combination with IAA (0 5 mg l  ) within two weeks of culture The shoots obtained were dwarf in nature but elongated further upon transfer to 0 1 mg l $^{-1}$ $GA_3$ within a week (Govindaraju *et al* 2003)

Indirect organogenesis was achieved by Satheeshkumar and Seeni (2003) in young stem leaf and root explants collected from 6 9 month old plants of *Plumbago rosea* All the explants responded similarly in a hormonal regime of 2 5 mg l  BA and 1 5 mg l  NAA with the formation of nodular callus in 4 weeks the callus was divided and subcultured at 4 week intervals in the presence of 3 0 mg/l to produce up to 23 5 ± 1 6 shoots in 18 weeks and then at 2 0 mg l $^{-1}$ BA to produce 79 6 ± 1 5 shoots in 23 weeks

Callus initiation was observed from young leaves of *P zeylanica* on MS medium supplemented with BA (0 0  8 8 mg l  ) IAA (0 0  2 88 mg l  ) and Ads (160 mg l $^{-1}$) Leaf callus developed on MS with 6 7 mg l $^{-1}$ BA 1 42 mg l  IAA and 160 mg l $^{-1}$ Ads underwent organogenesis after three weeks of incubation onto various regeneration media containing different concentrations of BA IAA and Ads The highest number of shoots from the leaf callus was observed on MS with 4 4 mg l  BA 1 42 mg l  IAA and 160 mg l  Ads On an average 30 shoots were recorded in callus cultures through organogenesis (Chaplot *et al* 2006)

Paul (2006) observed higher percentage of callusing in pseudostem explants of ginger cultured in MS medium supplemented with 2 4 D 1 0 mg l $^{-1}$ and incubated under dark conditions Callus induced in medium with higher levels of 2 4 D (3 0 4 0 mg l  ) were friable loose and watery with root hairs

Nıkam and Savant (2007) reported callus formatıon ın all the explants (root hypocotyls cotyledons node mternode and leaf) of *Ceropegıa sahyadı ıca* Ans and Kulk on MS med a wıth BAP (18mM) KN(18mM) IAA (7 5 12mM) NAA (5 12 mM) and 2 4 D (0 25 5 mM) eıther alone or ın combmatıon The callus formed ın conjunctıon wıth IAA and NAA occasıonally showed formatıon of fine haıry mass from the surface of callı whıle the callı produced on medıum contaın ng 2 4 D were soft pale yellowısh and non morphogenıc

Inductıon of callus was observed on nodal explants of *Plumbago ı osea* three weeks after ınoculatıon on half strength MS medıum supplemented wıth 2 4 D 1 0 mg l [1] The callı were subcultured on half strength MS medıum supplemented wıth BA (0 5 2 0 3 0 and 4 0 mg l [1]) and IAA (0 0 and 1 0 mg l [1]) Benzyl adenıne 2 0 mg l promoted the prolıferatıon of shoots (23 2) Hıgher concentratıons of BA were found to suppress the growth of shoots ın *Plumbago* (Preetha *et al* 2007)

Callus was ınduced on young buds and pseudostem explants from 4 week old *ın vıtı o* shoot cultures of gınger placed on MS medıum supplemented wıth varyıng concentratıons 2 4 D (0 5 3 0 mg l [1]) and BA 0 5 mg l [1] The cultures were ıncubated and maıntaıned under dark condıtıons Embryogenıc callus was produced ın the medıum s ıpplemented wıth 2 4 D (0 5 mg l ) At hıgher levels of 2 4 D (1 5 3 0 mg l [1]) the callus growth was slow and became rhızogenıc (Suma and Kesavachandran 2007)

Sıvanesan (2007) ınvestıgated morphogenetıc potentıal of leaf callus cultures of *Plı mbago zeylanıca* to develop protocol for shoot regeneratıon and somaclonal varıatıon Callı derıved from leaf explants cultured on MS medıum fortıfied wıth 2 0 mg l BAP when subcultured on MS medıum fortıfied wıth 2 0 mg l BAP 1 5 mg l [1] Kın and 1 0 mg l [1] NAA ınduced somaclonal varıatıon

**2 5 1 5 Rootıng of *ın vıtı o* produced shoots**

*In vıtro* produced shoots can be rooted eıther through *ın vıtro* methods ıtself or through *ex vıtro* methods There are three phases mvolved m rhızogenesıs namely ınductıon ınıtıatıon and elongatıon All cytokınıns ınhıbıt rootıng and auxıns favour ınductıon of rootıng Addıtıon of IAA IBA or NAA to the MS medıum ınduced

rooting of *in vitro* shoots in majority of plant species including *Plumbago* Rooting improved in many woody and herbaceous species when the concentration of macro salts was lowered to half or less and the concentration of sucrose was lowered from 2 or 3% to 0 5% (Webb and Street 1977) Concentration of agar used for rooting varies from zero in liquid medium to 0 9 per cent in solid medium Liquid media facilitates the free diffusion of toxic plant wastes and when used with filter paper bridge sy tem provides excellent aeration for root development (Hu and Wang 1985)

Callus regenerated shoots from stem segments of *P rosea* produced roots on transferring to media containing 1 5 mg l $^{1}$ IBA (Satheeshkumar and Bhavanandan 1988) Rout *et al* (1999) reported induction of rooting in microshoots from nodal cultures of *Plumbago zeyla nca* on half strength MS medium supplemented with 0 25 mg l IBA with 2% sucrose Similar report of root induction in nodal cultures of *P zeyla nca* was made by Selvakumar *et al* (2001) in MS medium containing 4 92 μM IBA

Satheeshkumar and Seeni (2003) reported successful rooting of shoots regenerated from leaf and stem callus cultures of *P rosea* Shoots of 2 0 3 5 cm length were rooted easily in half strength MS agar medium supplemented with 0 1 mg l IBA within four weeks of inoculation

Micro shoots regenerated from nodal segments of *P rosea* via callus culture successfully rooted in med a containing IBA 1 5 mg l $^{1}$ (Preetha *et al* 2007)

Well developed shoots (4 to 5 cm with three nodes) generated through axillary bud proliferation and leaf callus of *P zeylanica* rooted profusely on MS medium with IBA (1 2 mg l ) within 10 days (Chaplot *et al* 2006)

Besides auxins the role of gibberellic acid ($GA_3$) in root morphogenesis is reviewed In *Oxalis* species (*O glaucifolia* and *O rhombeo ovata*) shoots regenerated from internodal callus cultures were successfully rooted using half strength liquid MS med um containing 0 1 mg l $GA_3$ (Ochatt *et al* 1988) Bajaj *et al* (1998) reported high rate of rooting on shootlets developed from shoot tips of *Morus* species on MS medium at half concentration supplemented with 0 5 mg l $^{1}$ IBA and 0 1 mg l $GA_3$

Some workers have reported successful *in vitro* rooting of plant species in the media without any growth regulators Gayathri (2005) reported rooting of multiple shoots of *Woodfordia fruticosa* (L ) Kurz in SH medium devoid of growth hormones

Similar report was made by Gopalakrishnan *et al* (2009) in *P rosea* wherein shootlets developed from leaf explants when transferred to half strength MS basal medium without any plant growth regulator produced 4 3 ± 0 2 rootlets per plants with average root length of 4 0 ± 0 0 cm after 25 days of culture

**2 5 1 6 Hardening and planting out**

Acclimatization is crucial to any micro propagation procedures since shoot and plantlets produced *in vitro* must be readapted to the environmental conditions outside the culture vessels During the period of adaptation changes in both structures and physiology of shoots occur Leaves of *in vitro* cultured plantlets are characterised by the absence or reduced amount of epicuticular wax in comparison to the leaves of the green house or field grown plants (Grout 1975 Sutter and Langhans 1982) This affects the rate of water loss from the leaves During acclimatization as the humidity is gradually lowered the density of wax on leaves increases (Wardle *et al* 1983) Barnes (1979) suggested the method of covering the transplanted plantlet with polythene cover to maintain high humidity

Satheeshkumar and Bhavanandan (1988) reported that when micropropagated plants of *Plumbago rosea* were transferred to pots containing a 1 1 soil and sand mixture under greenhouse conditions about 60 per cent of the plants survived

Rout *et al* (1999) reported that about 95 per cent of micropropagated plantlets of *Plumbago zeylanica* were established in the greenhouse within 2 3 weeks of transfer under 85 per cent relative humidity

Callus regenerated plantlets of *P rosea* established within four weeks when transplanted to pots containing sand and soil mixture and maintained in a shade net house permitting 25 per cent sunlight without hardening (Satheeshkumar and Seeni 2003)

Almost 96 per cent of the plantlets obtained through axillary bud proliferation and organogenesis of *Plumbago zeylanica* Linn survived hardening when transferred to the field (Chaplot *et al* 2006)

Gopalakrishnan *et al* (2009) reported survival of more than 90 per cent of plantlets obtained from direct organogenesis of *P rosea* leaves was observed on hardening in red soil vermiculite and farmyard manure (1 1 1) for one week However the rate of survival decreased to 10 per cent after two to three weeks of acclimatization It was observed that gradual acclimatization of *in vitro* grown plants to external environment is most essential for *P rosea* More than 80 per cent of the plants transferred to pots survived and resumed growth

**2 5 1 7 Field evaluation of somaclones**

Kukreja *et al* (1991) standardized a procedure for selecting *in vitro* raised plants of *Mentha arvensis* based on qualitative and quantitative characters The *in vitro* regenerated plantlets showed a wide range of variation for various agronomic traits such as plant height leaf stem weight ratio herb yield oil yield and four major constituents of essential oil namely menthol menthone isomenthone and methyl acetate Based on initial quantitative and qualitative assessment on individual plant basis 27 somaclones were selected and further evaluated in replicated trial with parent plant CIMAP/Hy 77 as control check Two somaclones SC 93 and SC179 were selected on the basis of better oil content and stability of volatile oil composition

Plants regenerated from cell suspension cultures of palmarosa grass *Cymbopogon martinii* (Roxb ) Wats were analysed by Patnaik *et al* (1999) for somaclonal variation in five clonal generations A wide range of variation in important quantitative traits e g plant yield height tiller number oil content and qualitative changes in essential oil constituents (geraniol geranyl acetate geranyl formate and linalool) were observed among the 120 somaclones screened Eight somaclones were selected on the basis of high herb and oil yield over the donor line and high geraniol content in the oil Based on performance in the field trials three superior lines were selected and maintained for five clonal generations The superior lines exhibited a reasonable degree of stability in the traits selected

Sanchu (2000) studied variability in morphological yield and quality parameters of black pepper cultivar Cheriakaniyakkadan derived through indirect organogenesis She observed variability in leaf area number of lateral branches number of spikes per branch spike length number of berries per spike and recovery of essential oil and piperine She could isolate five calliclones of black pepper tolerant to Phytophthora foot rot disease and a superior somaclone having high yield quality and tolerance to Phytophthora foot rot from the study

Sujatha (2001) studied variability in axillary bud regenerants of black pepper varieties Panniyur 1 2 4 and Subhakara along with parentalclones Out of 61 morphological characters studied 56 were found homogenous within somaclones of each variety Signifiant variation was observed in five traits such as number of branches angle of insertion of branches and area of young leaf and mature leaf of orthotrope and plageotrope

Satheeshkumar and Seeni (2003) evaluated the *in vitro* plants of *Plumbago rosea* obtained through indirect organogenesis against the conventionally propagated plants The micropropagated plants when transferred to experimental plots and cultivated for 10 months produced significantly higher number (18 0 ± 0 5) of larger tuberous roots (137 4 ± 3 4 g fw/plant) compared to conventional rooted cuttings (14 0 ± 1 7 47 9 ± 1 6 g fw/plant) During this period the concentration of the root specific compound plumbagin recorded per g dw (1 5 per cent ) was higher than that of conventionally propagated plants (0 9 1 0 per cent)

Ravindra *et al* (2004) studied somaclonal variation for plant height plant spread leaf shape leaf size leaf form herb yield essential oil content and 10 important constituents of the essential oil in an Indian cultivar Bourbon of rose scented geranium (*Pelargonium graveolens*) Significantly larger variance was observed among *in vitro* regenerated plants of the $SC_1$ generation (first generation following an *in vitro* phase) than among parental plants raised from stem cuttings for herb yield plant height leaf size essential oil content and for the contents of *cis* rose oxide *trans* rose oxide isomenthone and 10 epi γ eudesmol in the essential oil Somaclones selected for plant spread and for the contents of linalool and *trans* rose oxide in their essential oils did not breed true in the $SC_2$ generation However for the

remain ng traits studied 13 100 per cent of the selected clones retained their selected traits in the $SC_2$ generation suggesting that the changes in these somaclones were due to genetic causes

Rathy *et al* (2007) evaluated growth of regenerants of black pepper variety Panniyur 4 derived through axillary bud culture and indirect organogenesis alongwith CP (conventionally propagated) plants Somaclones exhibited superiority over CP plants in morphologial characters such as number of leaves/plant length and breadth of leaves The somaclones produced laterals much earlier than CP plants

**2 5 2 *In vitro* mutagenesis**

Among the various techniques now available in tissue culture the use of tissue culture induced mutagenesis for plant modification is a relatively new and very attractive area of reseaich Bajaj (1983) studied direct and indirect effects of gamma irradiation on the seeds seedlings callus tissue cultures excised shoots ovules and embryos and reported that callus tissue cultures are more radio resistant than the intact seedlings EMS had an inhibitory effect on the shoot regeneration but stimulatory callus growth

According to Vajrabhaya (1977) treatment with chemical or physical mutagens can cause chromosome or gene mutations spontaneously *in vitro* *In vitro* culture techniques allow selection of the desired variants from large population of cells

*In vit o* technology including anther culture ploidy man pulation mutagenesis and a number of other gene manipulation techniques are mainly in their way into scene supplementing classical breeding (Sigbjornsson 1991) *In vitro* mutagenesis has been proved to be effective method of crop improvement in vegetatively propagated crops for inducing variability (Novak 1991) Even though the occurrence of desired mutation is empirical and random the combination of *in vitro* and mutation techniques can speed up crop improvement (Ahloowalia 1995)

Plant improvements based on mutation can change one or few specific traits and thus contribute to crop improvement The relatively high number of research reports compared with lower number of cultivars released suggests that mutagenesis in

combination with tissue culture is either ineffective or has yet to be exploited (Predieri 2001)

**2 5 2 1 Physical Mutagenesis**

Physical mutagens like γ rays are widely used in plant tissue culture for widening the *in vitro* culture induced variability in many crop plants One of the earliest studies on *in vitro* mutagenesis in medicinal plants in India was conducted in *Datura innoxia* by Jain *et al* (1984) Investigations were carried out on growth and differentiation in the anther derived cultures of *Datura innoxia* that had been subjected to varying doses of gamma irradiation ranging from 0 2 to 5 kR Growth of callus cultures was stimulated at 0 2 kR dose of gamma irradiation but it decreased as radiation dose increased Cultures exposed to 5 kR dose turned brown indicat ng a general inhibition of callus growth Shoot regeneration however was stimulated both at 0 2 as well 1 kR radiation doses

Shylaja (1996) reported *in vitro* induction of mutation using gamma irradiation in black pepper cultivars Kalluvally Karimunda Balankotta, Cheriakanyakkadan Panniyur I and Panniyur 2 Calli of the cultivar Kalluvally tolerated higher doze of γ irradiation as compared to other cultivars In all the cultivars callus growth inhibition was found at higher doses of gamma irradiation *viz* 40 and 50 Gy The doze 30 Gy was fixed as maximum dose of gamma irradiation that the calli of different cultivars could withstand The regeneration capacity of irradiated calli was found very low and the regenerated shoots were weak and chlorotic

Gavidia and Bermudez (1999) could isolate variants for cardenolide content from irradiated shoot tips of *Digitalis obscura* Shoot tips excised from the shoot cultures of a high yielding native plant of *D obscura* (genotype T4) exposed to gamma rays (20 100 Gy) Radiosensitivity was assessed and $LD_{50}$ determined was about 60 Gy Shoot tips mutagenized (20 40 Gy) were cultured in the basal MS medium Plantlets developed from irradiated shoot tips presented a high variability in their cardenolide production (878 to 3291 µg/g d w ) including variants with similar or even higher productivities than the native T4 plant

KAU (2001) reported the effect of γ irradiation on growth and multiplication of *in vitro* ginger sprouts The highest doze of γ irradiation that the sprouts could withstand was identified as 20 Gy

Mutations were induced in tissue cultured plants of lotus a sacred ornamental by Arunyanart and Soontronyatara (2002) The plantlets were obtained by culturing rhizomatous buds in half MS medium supplemented with 3 μ M IAA and 15 μ M 2iP The *in vitro* 4 week old plantlets were irradiated with γ irradiation at 0 2 3 4 5 and 6 krad or X ray irradiation at 0 1 2 3 4 and 5 krad The use of γ and X rays to induce mutation resulted in 21 altered characteristics Mutants from 1 and 2 krad of either γ or X rays had long secondary roots and adventitious roots These mutants also exhibited abnormal characteristics including vitrification chlorosis and deformed petioles and in addition had inhibited growth of lateral buds secondary roots and rhizomes

Differential radio sensitivity and post radiation recovery were observed in different Musa clones by Novak *et al* (1990) when they subjected the shoot apices shoots of seven clones of dessert banana plantain and bluggoe cooking banana to γ irradiation dozes of 15 30 45 and 60 Gy at a dose rate of 8 Gy $min^{-1}$ Considerable phenotypic variation was observed among plants regenerated from *in vitro* shoot tips after mutagenic treatment

In banana cult var Basrai (AAA) lower doses of irradiation *viz* 10 and 20 Gy enhanced multiplication rate of *in vitro* multiple shoot cultures (Karmarkar *et al* 2001) Obeidy *et al* (2002) studied the difference in radio sensitivity of banana cultivars Grand Nain Gros Michel and Williams *In vitro* cultures of these cultivars were subjected to γ irradiation (40 and 60 Gy) At 60 Gy reduced survival was observed in all the cultivars The number of shoots was reduced by 65 5 61 8 and 54 3 per cent in Gros Michel Williams and Grand Nain respectively when subjected to γ irradiation doze of 60 Gy There was no significant variation in shoot number at 40 Gy in the three cultivars studied Shoot elongation in Gros Michel decreased with radiation while that of Grand Nam did not significantly vary Longer shoots were recorded in Williams at 40 Gy They also isolated a mutant of Williams which exhibited salt tolerance up to 0 75 per cent NaCl in *in vitro* screening experiments

Irradiation of *in vitro* cultures of banana and subsequent field evaluation trials were done by Hui *et al* (2001 and 2002) They isolated 28 mutants of Grand Nain which exhibited wide variability in morphological characters such as the colour of pseudostem leaf stem ratio and plant shape as compared to control plants The mutants recorded 10 per cent yield increase over control plants

**2 5 2 2 Chemical mutagenesis**

The treatment of plant cells and tissues *in vitro* with chemical mutagens like EMS and NG enhances the spectrum and frequencies of somaclonal variation In any vegetative propagation system including the *in vitro* method of asexual propagation a mutagenic treatment may uncover recessive alleles by mutating or deleting corresponding dominant alleles

Efforts were made to artificially induce variation by subjecting callus cultures of some *Cymbopogon* species to treatment with chemical mutagens like EMS and NG (Patnaik *et al* 2000) As a prerequisite dose response curves for each of the chemical were first established and $I_{50}$ dose (giving about 50% cell growth compared to control) were determined for each of these mutagens In such an experiment with palmarosa the optimal doses of the chemical mutagens EMS and NG determined through established dose response curves were administered to callus cultured in order to obtain possible point mutation Consequently the regenerants from the treated callus exhibited a wide range of variations with respect to herb yield plant height and tiller number But the range of variations in the regenerants was almost in line with those observed among the somaclones obtained from untreated Phenotypically some of the EMS treated callus regenerants had broad leaf and tall plant habit which also had comparatively less number of tillers compared to donor lines Although essential compounds in terms of quantitative differences between different constituents were observed to be significant the major constituents like geraniol geranyl acetate which constitutes about 95% of geraniol oil pool more or less remained same Among the plants obtained from NG treated callus on the other hand the range of variation was not as wide as in the EMS population and there were very few significantly different and desirable variations in population Throughout the growth of regenerants in green

house as well as ın field conditıons for more than one year no albınos or any other chlorophyll mutant could be observed

Swamy *et al* (2005) ınduced streptomycın resıstance ın *Solanum surattense* usıng a sımple procedure ın whıch mutagenesıs (ethyl methane sulphonate and gamma rays) was performed dırectly on cotyledon explants Chloroplast encoded streptomycın resıstant shoots were developed from green (unbleached) sectors of the cotyledons The streptomycın resıstant plants were sımılar to parental plants ın morphology and ploıdy level (2n − 2x 24)

## 2 6 INDUCTION OF POLYPLOIDY IN *PLUMBAGO* SPECIES

Polyploıdy refers to a cond tıon ın whıch there are more than two sets of chromosomes ın the cells of an organısm It has been estımated by Muntzıng (1936) that more than 50 per cent of angıosperms are polyploıds Polyploıdy ıs of two dıfferent kınds ınvolvıng eıther duplıcatıon of the same genome or summatıon of dıfferent genomes referred to as autopolyploıdy and allopolyploıdy respectıvely Polyploıdy has played a very ımportant role ın evolutıon of plants allopolyploıdy beıng the major contrıbutor Autopolyploıdy has contrıbuted to lımıted extent ın evolut on of plant specıes

Polyploıds find several applıcatıons ın crop ımprovement Overcomıng self ıncompatıbılıty makıng dıstant crosses possıble use as a brıdgıng specıes ın gene transfer between two specıes as source of new genetıc varıatıon or use dırectly as crop varıetıes are some to lıst a few

Polyploıdızatıon can be achıeved artıficıally ın two ways mıtotıc or meıotıc The former ınvolves deregulatıon of mıtosıs at any stage ın the vegetatıve cycle resultıng ın doublıng of chromosomes wıth two ıdentıcal gene complements The meıotıc mechanısm ınvolves fusıon of unreduced gametes wıth somatıc chromosome number (2n) Several chemıcals such as acenaphthene chloral hydrate benzene nıtrous oxıde 8 hydroxy quınolıne sulfanılamıde mercurıc chlorıde etc were used ın the earlıer perıods to brıng about mıtotıc arrest for the ınductıon of polyploıdy In 1937 Blakeslee dıscovered the polyploıdızıng property of an alkaloıd colchıcıne extracted from the bulbs and seeds of the plant *Colchıcum autumnale* It was far more

effective than other chemicals and in due course colchicine replaced all the conventional methods of chromosome doubling in plants Blakeslee and Avery (1937) working on *Portulaca Datura* and *Cucurbita* and Nebel and Ruttle (1938) working on *Tradescantia Petunia* Snapdragons and Marigolds noticed that this alkaloid was very effective in doubling the chromosome number These pioneering works paved the way for future use of colchicine as a chromosome doubling agent This can be attributed to its specific advantages namely high effectiveness for making polyploids in many different species and with very little damage to the treated plants High solubility in water non toxicity to plants even at higher doses adds to its merits (Eigsti and Dustin 1955)

**2 6 1 Methods of Colchicine application**

For inducing polyploidy the meristematic region is to be treated with colchicine solution i e actively dividing cells are to be exposed to colchicine Treating germinating seeds treating whole seedlings or apical bud treatment of young seedlings using cotton swab are commonly followed for obtaining polyploids Growing shoot apices are commonly treated with 0 1 to 1 per cent colchicine which is applied by brush or else by placing a small cotton wool piece at the shoot tip which is soaked daily with colchicine solution The treatment is repeated once or twice daily for a few days Kulkarni *et al* (1984) induced autotetraploidy in *Catharanthus roseus* by immersing apical buds of seven days old seedlings in 0 5 per cent colchicine for 19 hours Dhawan and Tyagi (1989) developed two autotetraploids m *Hyosyamus muticus* by soaking the seeds in 0 5 per cent solution of colchicine for 72 hours

Ajithmohan (1995) induced polyploidy in *Kaempferia galanga* by treating the rhizomes with 0 45 per cent colchicine Cochicine was applied in holes drilled close to buds on rhizomes and by cotton swab method i e by covering the bud with a piece of cotton wool soaked in colchicine solution for specified time However only the colchicine application in hole close to rhizome bud yielded polyploid while the cotton swab method was not successful in inducing variability

Polyploidy was successfully induced in the clone PR 107 of para rubber tree (*Hevea brasiliensis*) by Sankariammal and Saraswathyamma (2004) Aqueous

solution of colchicine (0 75%) was applied to young bud sprouts of the clone PR 107 continuously for seven days by cotton swab method

**2 6 2 Morphological and cytological features of polyploids**

Autopolyploidy leads to an increase in general vigour and vegetative growth The features associated with autopolyploidy include increased size of plant parts larger and thicker leaves larger flower and fruits which are usually less in number than diploids Yield will be higher if the economically important part is vegetative Polyploids have larger cell s ze than diploids Guard cells of stomata are larger and the n imber of stomata per unit area is lower in polyploids than diploids Pollen grains of polyploids are generally larger than those of the corresponding diploids However the enhancement in vegetative growth is accompanied by reduced fertility slow rate of growth delayed flowering and germination (Singh 1993)

AutoploypIoidy breeding is attempted with the objective of obtaining enhanced vigour and high vegetative yield Enhancement in some other characters such as alkaloid content, resistence to pests and d seases etc are also expected However there are several reports in which the autopolyploid developed being weak and inferior to original diploid According to Stebbins (1971) the increase of cell size wh ch generally characterizes polyploids does not necessarily lead to increased size of plant as a whole or even of its individual organs because of the reduct on in the number of cell divisions in these plants

Ajithmol an (1995) developed polyploid in *Kaempfe ia galanga* which was characterized by small plant size and reduced vigour Rhizome development was found retarded and per plant yield was less Flowering was not observed in the induced polyploid for two growing seasons

Induced polyploidy has not been reported in the two cultivated species of *Plumbago* namely *P rosea* and *P zeylanica* Polyploidy is worth trying in *Plumbago* since the economically important part is root and the crop is clonally propagated Any improvement in root yield or plumbagin content that may be induced by polyploidy can readily be maintained through clonal propagation

# Materials and Methods

# 3 MATERIALS AND METHODS

## 3 1 Experiment I

### Exploration and collection of different genotypes of *Plumbago* species

### 3 1 1 Materials

Accessions of *Plumbago* species namely *Plumbago rosea* *Plumbago zeylanica* and *Plumbago capensis* collected from different geographical locations in different districts of Kerala The collected accessions were raised as pot culture in the field of AICRP on M& AP College of Horticulture Vellanikkara

### 3 1 2 Methodology

The study was carried out during the period November 2005 to December 2006 The eco geographic diversity of *Plumbago* species was explored in various districts of Kerala during the period November December 2005 The accessions of species *Plumbago rosea* were collected from farmer s fields whereas the accessions of species *P zeylanica* and *P capensis* were collected from private nurseries The planting material was collected in the form of rooted cuttings and a collector s number was given to identify the accession

A preliminary survey of the area to be explored was done before actual collection The location of sampling sites within the collection area was carefully planned in advance and was based on the consideration of change in ecological and agricultural conditions in the area soil pattern and change in agricultural practices The sampling procedure adopted was random in order to collect maximum range of variability present in the *Plumbago* species

The collected accessions were raised as pot culture for a preliminary evaluation and multiplication In the preliminary trial the collected accessions of *Plumbago* were evaluated mainly for descriptors/characters of agronomic importance as well as distinct morphological features The passport data of the collected accessions are presented in Table1 Based on the passport data of information of collected materials IC (Indigenous Collection) numbers were obtained from NBPGR New Delhi The

**Table 1 Passport data of collected accessions of *Plumbago* species**

| Sl No | Collector's No | Genus | Species | Date of Collection | Collection source | Village | District | Latitude | Longitude | Altitude | Main characters |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Acc no 1 | *Plumbago* | *rosea* | 15 11 2005 | Farmer's plot | Vellanikkara | Thrissur | 10°30'N | 76°15E | 15 msl | Low root production bushy nature low biomass and dry matter production |
| 2 | Acc no 2 | *Plumbago* | *rosea* | 12 11 2005 | Farmer's plot | Thamarassery | Wayanad | 11 12'N | 76°15E | 400 msl | Low root production bushy nature low biomass and short internodes |
| 3 | Acc no 3 | *Plumbago* | *rosea* | 12 11 2005 | Farmer's plot | Ambalavayal | Wayanad | 11°12'N | 76 15E | 600 msl | Long internode taller plants with medium production of roots |
| 4 | Acc no 4 | *Plumbago* | *rosea* | 14 11 2005 | Farmer's plot | Thenjipalam | Kozhikode | 11°15'N | 75°49E | 5 msl | Long internode long linear leaves with light green and medium production of roots |
| 5 | Acc no 5 | *Plumbago* | *rosea* | 14 11 2005 | Farmer's plot | Kozhikode | Kozhikode | 11°15'N | 75°49E | 5 msl | Very tall plants with medium internode length higher number of leaves branches and roots |

| Sl No | Collector's No | Genus | Species | Date of Collection | Collection source | Village | District | Latitude | Longitude | Altitude | Main characters |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | Acc no 6 | *Plumbago* | *rosea* | 12 11 2005 | Farmer's plot | Kalpetta | Wayanad | 11 12 N | 76°15 E | 300msl | Tall plants with higher number of leaves and medium internode |
| 7 | Acc no 7 | *Plumbago* | *rosea* | 12 11 2005 | Farmer's plot | Pookode | Wayanad | 11 12 N | 76°15 E | 300msl | Broad leaves with bigger root size |
| 8 | Acc no 8 | *Plumbago* | *rosea* | 14 11 2005 | Farmer's plot | Thuruthissery | Ernakulam | 10 00'N | 76°15 E | 10 msl | Medium sized plant with high root number |
| 9 | Acc no 9 | *Plumbago* | *rosea* | 12 11 2005 | Farmer's plot | Thrissur | Thrissur | 10 30'N | 76 15 E | 15 msl | Medium sized plant with high root number |
| 10 | Acc no 10 | *Plumbago* | *rosea* | 15 11 2005 | Farmer's plot | Vellanikkara | Thrissur | 10°30'N | 76°15 E | 15 msl | Higher root number as well as high plumbagin content |

| Sl No | Collector s No | Genus | Spec es | Date of Collect on | Collection source | Village | D strict | Lat tude | Long tude | Alt tude | Main characters |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | Acc no 11 | *Plu bago* | *rosea* | 08 11 2005 | Farmer s plot | Kottakal | Malappuram | 10°47'N | 75°58E | 10 msl | Tall plants with low branch ng habit and long roots |
| 12 | Acc no 12 | *Plumbago* | *rosea* | 08 11 2005 | Farmer s plot | Kadampuzha | Malappuram | 10°47'N | 75 58E | 20 msl | Plants w th low number of leaves and small leaves as well as roots |
| 13 | Acc no 13 | *Plun bago* | *rosea* | 14 11 2005 | Farmer s plot | Valanchery | Malappuram | 11°12'N | 76°15 E | 20 msl | Dwarf plants with short internodes and low root product on |
| 14 | Acc no 14 | *Plu nbago* | *rosea* | 15 11 2005 | Farmer s plot | Vellan kkara | Thr ssur | 10°30'N | 76 15 E | 15 msl | Med um s zed plant w th low root product on |
| 15 | Acc no 15 | *Plun bago* | *rosea* | 15 11 2005 | Farmer s plot | Vellanikkara | Thr ssur | 10 30'N | 76°15 E | 15 msl | Plants w th long leaves and longer roots |

| Sl No | Collector's No | Genus | Species | Date of Collection | Collection source | Village | District | Latitude | Longitude | Altitude | Main characters |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | Acc no 16 | *Plumbago* | *rosea* | 15 11 2005 | Farmer's plot | Vellanikkara | Thrissur | 10 30'N | 76°15 E | 15 msl | Plants with very high plumbagin content in roots |
| 17 | Acc no 17 | *Plumbago* | *rosea* | 10 11 2005 | Farmer's plot | Ottapalam | Palakkad | 10°46'N | 76°42E | 15 msl | Medium sized plant with medium number of roots |
| 18 | Acc no 18 | *Plumbago* | *rosea* | 10 11 2005 | Farmer's plot | Kollengode | Palakkad | 10°46'N | 76°42E | 15 msl | Medium sized plant with medium number of roots |
| 19 | Acc no 19 | *Plumbago* | *rosea* | 10 11 2005 | Farmer's plot | Nenmara | Palakkad | 10 46'N | 76 42E | 15 msl | Tall plants with long roots |
| 20 | Acc no 20 | *Plumbago* | *rosea* | 10 11 2005 | Farmer's plot | Changaramkulam | Malappuram | 10°47'N | 75 58E | 20 msl | Medium sized plant with medium number of roots |


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| Sl No | Collector s No | Genus | Species | Date of Collection | Collect on source | V llage | D strict | Latitude | Long tude | Altitude | Main characters |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 21 | Acc no 21 | *Plun bago* | *osea* | 10 11 2005 | Farmer s plot | Nell ampathy | Palakkad | 10 46'N | 76 42E | 1000 msl | High plant biomass with thin roots |
| 22 | Acc no 22 | *Plumbago* | *rosea* | 10 11 2005 | Farmer s plot | Santhampara | Idukki | 9 3'N | 77 02E | 500 msl | Med um s zed plant w th med um number of roots |
| 23 | Acc no 23 | *Plu nbago* | *rosea* | 10 11 2005 | Farmer s plot | Pulpara | Idukki | 9 3'N | 77°02E | 500 msl | Very h gh fresh and dry root y eld w th very dark leaves |
| 24 | Acc no 24 | *Plumbago* | *rosea* | 10 11 2005 | Farmer s plot | Munnar | Idukki | 9 3'N | 77 02E | 500 msl | Very h gh fresh and dry root y eld w th very dark leaves |
| 25 | Acc no 25 | *Plumbago* | *rosea* | 09 11 2005 | Farmer s plot | Vellan kkara | Thr ssur | 10 30'N | 76°15 E | 15 msl | Low root weight and root y eld w th very low plumbag n content |


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| Sl No | Collector's No | Genus | Species | Date of Collection | Collection source | Village | District | Latitude | Longitude | Altitude | Main characters |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 26 | Acc no 26 | *Plumbago* | *zeylanica* | 15 11 2005 | Private nursery | Vellanikkara | Thrissur | 10 30'N | 76°15 E | 15 msl | Tall plants producing roots thinner than *Plumbago rosea* and low plumbagin content |
| 27 | Acc no 27 | *Plumbago* | *capensis* | 15 11 2005 | Private nursery | Vellanikkara | Thrissur | 10°30'N | 76°15 E | 15 msl | No tuberous root formation |

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**3 2 Experiment II**

**Assessment of genetic variability of various morphological and biometrical traits in the collected accessions**

**3 2 1 Materials**

The accessions collected in the experiment one were raised in replicated trial in the field of AICRP on M& AP College of Horticulture Vellanikkara for assessing genetic variability of various morphological and biometric traits including plumbagin content The details of the accessions collected such as IC number place of collection and district are given m Table 2

**3 2 2 Methodology**

The study was carried out during the period from January 2007 to February 2008 The experiment was laid out in RBD with three replications Three month old rooted cuttings of the twenty six accessions were raised in cement pots of 35 cm diameter and 45 cm depth filled with potting mixture (soil sand and dried cow dung in equal proportions) The pots were hand weeded as and when necessary The crop was raised as irrigated crop During summer months there was attack of mealy bugs and it was controlled by Ekalux 0 2 per cent spray The accessions were evaluated for different morphological and biometric traits Detailed studies on floral morphology and biology were made Following were the main items of observations made in the field

**3 2 3 Morphological traits**

The observations on the following morphological characters were taken in each accession taken at monthly intervals except for leaf length and leaf breadth which were recorded at quarterly intervals

1 Number of branches per plant

The total number of branches per plant was counted

2 Plant height

The height of plant was measured from the ground level to the tip of the top most leaf and expressed in centimeters This observation was taken in the longest

branch which was tagged

3 Internodal length

The distance between the point of attachment of the second leaf and that of the third leaf from the top of the branch used for measuring height was recorded and expressed in centimeters

**Table 2 Details of collected accessions of *Plumbago* species raised in replicated trial**

| Sl No | Species | Place of Collection | District | Accession No |
|---|---|---|---|---|
| 1 | *Plumbago rosea* | Vellanikkara | Thrissur | IC 566499 |
| 2 | *Plumbago rosea* | Thamarassery | Wayanad | IC 566500 |
| 3 | *Plumbago rosea* | Ambalavayal | Wayanad | IC 566501 |
| 4 | *Plumbago rosea* | Thenjipalam | Malappuram | IC 566502 |
| 5 | *Plumbago rosea* | Kozhikode | Kozhikode | IC 566503 |
| 6 | *Plumbago rosea* | Kalpetta | Wayanad | IC 566504 |
| 7 | *Plumbago rosea* | Pookode | Wayanad | IC 566505 |
| 8 | *Plumbago rosea* | Thuruthissery | Ernakulam | IC 566506 |
| 9 | *Plumbago rosea* | Thrissur | Thrissur | IC 566507 |
| 10 | *Plumbago rosea* | Vellanikkara | Thrissur | IC 566508 |
| 11 | *Plumbago rosea* | Kottakkal | Malappuram | IC 566509 |
| 12 | *Plumbago rosea* | Kadampuzha | Malappuram | IC 566510 |
| 13 | *Plumbago rosea* | Valanchery | Malappuram | IC 566511 |
| 14 | *Plumbago rosea* | Vellanikkara | Thrissur | IC 566512 |
| 15 | *Plumbago rosea* | Vellanikkara | Thrissur | IC 566513 |
| 16 | *Plumbago rosea* | Vellanikkara | Thrissur | IC 566514 |
| 17 | *Plumbago rosea* | Ottappalam | Palakkad | IC 566515 |
| 18 | *Plumbago rosea* | Kollengode | Palakkad | IC 566516 |
| 19 | *Plumbago rosea* | Nenmara | Palakkad | IC 566517 |
| 20 | *Plumbago rosea* | Changaramkulam | Malappuram | IC 566518 |
| 21 | *Plumbago rosea* | Nelhampathy | Palakkad | IC 566519 |
| 22 | *Plumbago rosea* | Santhampara | Idukki | IC 566520 |
| 23 | *Plumbago rosea* | Pulppara | Idukki | IC 566521 |
| 24 | *Plumbago rosea* | Munnar | Idukki | IC 566522 |
| 25 | *Plumbago rosea* | Vellanikkara | Thrissur | IC 566523 |
| 26 | *Plumbago zeylanica* | Vellanikkara | Thrissur | Acc no 26 |

4 Number of leaves per plant

The total number of leaves per plant was counted from planting till harvest

5 Leaf length

Ten fully opened leaves from top middle and bottom of the tagged branch were selected and the length was measured as the distance between the base (point of attachment of the petiole) and the tip of the leaf blade The average length was worked out and expressed in centimeters

6 Leaf breadth

This observation was taken on ten leaves used for taking leaf length The distance between the two widest points of the leaf lamina was measured as leaf breadth and expressed in centimeters

**3 2 4 Biometric traits**

Harvesting was done fifteen months after planting to the pots without damaging the roots The roots were washed in flowing water to remove the adhering soil particles and the data on following parameters were recorded

1 Number of roots

The number of primary roots emerging from the base of the stem was counted

2 Length of root

The length of the longest root was recorded in centimeters

3 Girth of roots

The girth of the thickest root was measured and expressed in centimeters The girth of the thickest root was measured and expressed in centimeters

4 Total biomass

The fresh weight of the entire plant was recorded and expressed in gram

5 Fresh weight of roots

The fresh weight of roots of each plant was recorded and expressed in grams

6 Dry weight of roots

The roots were air dried for one day packed in labeled paper bags and oven dried at 50 $60^{0}$C for three days The dry weight of root was recorded in grams per plant

7 Plumbagin content

a) Quantitative analysis

The dried root of *Plumbago* was ground in a mixer grinder and sieved to obtain fine powder The powdered root sample weighing 500 mg was taken in a conical flask of 100 ml capacity and stoppered To this 20 ml AR grade acetone was added This was kept on a shaker and shaken for twenty minutes at a speed of 150 rpm and later allowed to settle The extract was then decanted and collected into a standard flask (100 ml ) This process was repeated four times The volume of the extract was made up to 100 ml of the standard flask by adding acetone One ml of the extract was taken in a test tube and made up to 10 ml with acetone The absorbance of this extract was read in a spectrophotometer at 410 nm against blank acetone Standard plumbagin (Sigma Aldrich Co Ltd USA) was dissolved in acetone at varying concentiations and the colour development as observed in root samples was noted The optical density (O D ) of the standard plumbagin solutions were taken at 410nm in a spectrophotometer and a calibration curve plotted Based on the standard curve concentration of plumbagin in the dried root samples of *Plumbago* access ons was worked out as

Plumbagin (%)

$$\frac{\text{O D of sample}}{\text{O D of standard}} \times \text{Concentration of standard} \times \frac{1000}{\text{Weight of sample}} \times 10^{4}$$

b) Qualitative analysis of plumbagin by Th n Layer Chromatography (TLC)

The crude acetone extract of dried root samples were subjected to qualitative analysis by TLC using silica gel 60 $F_{254}$ TLC plates The mobile phase consisting of methanol and chloroform in the proportion (1 1 v/v) was used The standard plumbagin was also co chromatographed along with the sample On complet on of the solvent run the TLC plates were dried after marking the solvent front and viewed under UV 354 nm and under visible light Rf value was calculated as

$$\frac{\text{Distance traveled by the sample}}{\text{Distance traveled by the solvent}}$$

**3 2 5 Floral traits**

Flowering in the two species of *Plumbago* commenced in October 2007 and it continued up to first week of February 2008 in *P rosea*

**3 2 5 1 Floral morphology**

The morphological features of inflorescence and flower were closely observed with hand lens and dissection microscope

1 Inflorescence characters

i) Days for opening of first flower in an inflorescence

The first flower bud after visual emergence in both species *Plumbago rosea* and *Plumbago zeylanica* were tagged and days taken for opening of first flower was noted

ii) Number of flowers per inflorescence

Twenty six inflorescences labeled earlier were utilized for recording this character After completion of flowering inflorescences were collected and the number of flowers per inflorescence was recorded by counting the persistent bracts

iii) Days for completion of anthesis per inflorescence

The inflorescence labeled for studying the number of days required for opening of the first flower were observed till completion of anthesis The day of completion of anthesis was noted

2 Flower characters

Five fully opened flowers from twenty six accessions were collected and observation on length of flower length of calyx length of corolla tube and length of style were recorded and expressed in centimeters The mean was worked out for each genotype

**3 2 5 2 Floral biology**

For the detailed study of reproductive biology in *Plumbago rosea* and *Plumbago zeylanica* ten plants from each of the species were planted in plastic pots during May 2007 The observations were taken from November 2007 to February

2008 In order to find out the causes for the failure of fruit set in *Plumbago rosea* detailed observations on the following aspects were done

1 Time of flower opening

Five inflorescences each from ten plants of *Plumbago rosea* and *Plumbago zeylanica* were tagged The number of flowers opened in each inflorescence was recorded at hourly intervals from 4 a m to 4 p m Percentage of flowers opened at hourly intervals were estimated for determining the peak time of flower opening and peak period of anthesis in an inflorescence

2 Time of anther dehiscence and stigma receptivity

The colour and appearance of the anthers were examined under stereo microscope at hourly intervals in fully matured flower buds of each species to find out the time of anther dehiscence in a flower The stigmatic surfaces were also observed for any change in colour or appearance in the same buds at same intervals of time to find out stigma receptivity

3 Pollen fertility

Pollen fertility was assessed on the basis of stainability of pollen grains m aceto carmine glycerine mixture Pollen grains were collected from the matured buds and stained with a drop of aceto carmine glycerine mixture on a clean slide and kept aside for ten minutes All the pollen grains that were well filled and stained were counted as fertile and others as sterile Three fields of five different slides prepared from each species were observed under microscope and the values expressed as percentage

4 Pollen viability

i) *In vitro* studies

Pollen grains were collected from freshly dehisced anthers from both the species and kept for germination in the following media

20 % sucrose

40 % sucrose

60 % sucrose

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Pollen grains were placed in a drop of the culture solution on a slide and incubated in the following manner A pair of petridishes was lined with moist filter paper Two match sticks were placed parallel in the bottom half on which two slides containing a drop of germination medium with the pollen grains were placed and was covered by the top half This was left undisturbed at room temperature for twenty four hours The percentage of pollen grains germinated was recorded after staining in one percent acetocarmine The germinated grains were counted from ten different fields per treatment per replication and the percentage of germination was calculated as

$$\text{Percentage of germination} = \frac{\text{No of pollen grains germinated}}{\text{Total no of pollen grains}} \times 100$$

ii) *In vivo* studies

To study pollen germ nation and tube growth *in vivo* artificial pollination was undertaken in the two species of *Plumbago* The study of anthesis in *Plumbago rosea* had revealed that the anther dehiscence never coincided with stigma receptivity Besides the anther dehiscence was delayed by three hours So the following method was adopted when *P rosea* was used as pollinator parent The same plants for observing anthesis were used for pollination The plants were placed in an enclosed area In order to hasten the anthesis and dehiscence a sixty watt electric bulb was placed at an appropriate height above the inflorescence of *P rosea* plant in the pot The warm light from the bulb resulted in early opening of flower as well as the dehiscence of the anther Such freshly dehisced anthers were used for pollinating both the species of *Plumbago* The mature flower buds of the two species of *Plumbago* about to open the next day were emasculated and bagged one day prior to anthesis The freshly dehisced anthers by the method mentioned earlier were rubbed on to the stigma of the emasculated flower buds and again covered with a butter paper cover The retention of pollinated flower buds was observed at the intervals of 24 48 72 and 96 hours after pollination The stigma of a few such pollinated buds from both *P rosea* and *P zeylanica* were excised after 24 and 48 hours and placed in a petridish lined with moist filter paper The stigma was then transferred to a clean slide with a few drops of acetocarmine A cover glass was placed on the material gently pressed and observed under microscope

To determine the mode of pollination and the percentage fruit set under natural conditions selfing and open pollination were carried out Twenty inflorescences of

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uniform age were tagged before the commencement of anthesis Out of these ten were covered with butter paper to encourage self pollination and the remaining ten left uncovered so as to favour open pollination The extent of fruitset in each case were determined and expressed as percent

**3 2 6 Statistical analysis**

The data collected for the various morphological and biometric traits were subjected to statistical analysis Analysis of variance and covariance was performed using the software SPAR I (Statistical Package for Agriculture Research work IASRI NewDelhi) The components of variation such as coefficients of variation heritability genetic advance correlation and path coefficient were estimated as per Nadarajan and Gunasekaran (2005)

**3 2 6 1 Components of variation**

i) Variability

Phenotypic variance ($\sigma^2p$)

$\sigma^2p - \sigma^2g + \sigma^2e$

$\sigma^2e$ – Environmental variance

$\sigma^2g$ Genotypic variance

$$\sigma^2g\ (G) \quad \frac{MSt - MSe}{r}$$

Where MSt – Mean sum of squares due to treatments
MSe Mean sum of squares due to error
r Number of replications

The phenotypic and genotypic coefficients of variation were calculated as per the formula suggested by Burton and Devane (1953) for each character by making use of the estimates of $\sigma^2g$ and $\sigma^2p$ mentioned above

$$\text{Phenotypic coefficient of variation (PCV \%)} - \frac{\sigma p}{\text{Mean}} \times 100$$

$$\text{Genotypic coefficient of variation (GCV \%)} \quad \frac{\sigma g}{\text{Mean}} \times 100$$

Where $\sigma p$ and $\sigma g$ are phenotypic and genotypic standard deviations respectively The PCV and GCV were classified as follows

Low < 10 per cent

Moderate 10 20 per cent

High > 20 per cent

ii) Heritability

Heritability in broad sense was estimated using the following formula suggested by Burton and Devane (1953)

$$\text{Heritability } (h^2) - \frac{\sigma^2 g}{\sigma^2 p} \times 10$$

The heritability values were categorized as follows

Low < 30 per cent

Moderate 30 60 per cent

High > 60 per cent

iii) Genetic advance

The expected genetic advance under selection was estimated by the following formula suggested by Johnson *et al* (1955a)

GA k x h2x σp

Where k standardized selection differential at particular level of selection intensity which is equal to 2 06 in the case of five per cent selection in large samples (Falconer 1967)

$h^2$ – heritability of the character under consideration

σp – phenotypic standard deviation of the original population

iv) Genetic gain

It is the genetic advance expressed as percentage of mean

$$\text{GA (\%)} \quad \frac{\text{Genetic Advance}}{\text{Mean}} \times 100$$

The range of GA as per cent of mean was classified as follows

Low < 10 per cent

Moderate 10 20 per cent

High > 20 per cent

v) Phenotypic and genotypic correlations

Using var ances and covariances the genotypic and phenotypic correlation coefficients between the therapeutic content plumbagin and vario is morphological as well as biometric characters were calculated as per Johnson *et al* (1955b) The significance of correlation coefficients were ascertained from the table r value at (n 2) degrees of freedom where n is the number of pairs of observations used

vi) Path coefficient analysis

The correlation between plumbagin content and other characters were split nto direct and indirect effects using path analysis technique suggested by Dewey and Lu (1959) The direct and indirect effects were classified on the scale given by Lenka and Misra (1973)

| | |
|---|---|
| 0 00 0 09 | Negligible |
| 0 10 0 19 | Low |
| 0 20 0 29 | Moderate |
| 0 30 1 00 | High |
| >1 00 | Very high |

vii) Cluster analysis

Genetic diversity plays an important role in crop improvement The concept of $D^2$ statistics (Mahalonobis 1928) is one of the potent techniques of measuring genetic divergence in plant breeding The $D^2$ values were estimated by the method suggested by Tocl er (Rao 1952) and the genotypes were grouped into clusters The cluster analysis was performed with the help GENSTAT (software developed at College of Horticulture KAU) Twenty six accessions of *Plumbago were* grouped in clusters based on eleven characters The average inter and intra cluster distances of the clusters were calculated Cluster means were calculated for individual characters on the basis of performances of the genotypes included in that cluster The accessions in each cluster were ranked on the basis of two yield characters *viz* dry root weight and plumbagm content The selection of accessions for inducing variability was made on the basis of their ranking in a cluster

**3 3 Experiment III**

**Induction of variability through *in vitro* regeneration and mutagenesis of *Plumbago* species**

**3 3 1 Materials**

Three accessions of *P rosea* selected on the basis of cluster analysis in the experiment II and one accession of *P zeylanica* were used for this experiment

**3 3 2 Methodology**

The study was carried out during March 2008 to July 2009 The experiment was conducted in two phases The first phase consisted of standardization of *in vitro* regeneration and the second phase *in vitro* mutagenesis

**3 3 2 1 Standardisation of *in vitro* regeneration in *Plumbago* species**

The *in vitro* regeneration was carried out in two ways *viz* direct regeneration and callus mediated organogenesis The basic procedures employed for both the methods of *in vitro* regeneration were as follows

**3 3 2 1 1 Preparation of stock plants for explant collection**

For carrying out the standardisation work three month old rooted cuttings of three accessions of *P rosea* and one accession *P zeylanica* were planted in pots during October 2007 and maintained in a green house These were sprayed with Ekalux 0 2 per cent as well as drenched with 2 0 per cent Bavistin at regular intervals prior to explant collection

**3 3 2 1 2 Selection of explant**

The explants were collected from six to nine months old stock plants of *P rosea* and three to six months old stock plants of *P zeylanica* Stem segments nodal segments and leaf discs were taken as explants for the study For preparing leaf discs semi mature leaves from second and third node were taken

**3 3 2 1 3 Culture medium**

The response of the explants was studied in the Murashige and Skoog s (MS)

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medium (Murashige and Skoog 1962) Composition of this medium is given in Table 3 The basal media was supplemented with different levels of growth regulators

**Table 3 Composition of basal medium (MS) used for *in vitro* culture**

| Stock | Ingredients (mg l$^{1}$) | Mg litre$^{1}$ | Stock concentration | Stock |
|---|---|---|---|---|
| | Inorganic constituents | | | |
| I | $(NH_4)NO_3$ | 1650 | 50X | 82 5 g l$^{1}$ |
| | $KNO_3$ | 1900 | | 95 0 g l$^{1}$ |
| | $KH_2PO_4$ | 170 | | 8 5 g l$^{1}$ |
| | $MgSO_4$ $7H_2O$ | 370 | | 18 5 g l$^{1}$ |
| II | $CaCl_2$ $2H_2O$ | 440 | 50X | 22 0 g l$^{1}$ |
| III | $FeSO_4$ $7H_2O$ | 27 8 | 100X | 2 8 g l$^{1}$ |
| | $Na_2EDTA$ | 37 3 | | 3 7 g l$^{1}$ |
| IV | $MnSO_4$ $4H_2O$ | 22 3 | 100X | 2 2 g l$^{1}$ |
| | $ZnSO_4$ $7H_2O$ | 8 6 | | 860 0 mg l$^{1}$ |
| | $H_3BO_3$ | 6 2 | | 620 0 mg l$^{1}$ |
| | KI | 0 83 | | 83 0 mg l$^{1}$ |
| | $Na_2MoO_4$ $2H_2O$ | 0 25 | | 25 0 mg l$^{1}$ |
| | $CuSO_4$ $5H_2O$ | 0 025 | | 2 5 mg l$^{1}$ |
| | $CoCl_2$ $6H_2O$ | 0 025 | | 2 5 mg l$^{1}$ |
| | Organic constituents | | | |
| V | Vitamins | | | |
| | Nicotinic Acid | 0 5 | 100X | 50 0 mg l$^{1}$ |
| | Pyridoxine HCl | 0 5 | | 50 0 mg l$^{1}$ |
| | Thiamine HCl | 0 1 | | 10 0 mg l$^{1}$ |
| | Glycine | 2 | | 200 0 mg l |
| | Myo inositol | 100 | | 0 01 g l$^{1}$ |
| | Sucrose | 30000 | | 30 0 g l$^{1}$ |

**i) Preparation of stock solutions for basal medium**

Standard procedures (Gamborg and Shyluk 1981) were followed for the preparation of the medium Stock solutions of the major and minor nutrients were prepared separately by dissolving the required quantity of chemicals in double distilled water and stored under refrigerated conditions in amber coloured bottles Stock solutions for the major and minor nutrients were prepared afresh every three months The vitamin stock solutions were prepared fresh every six to eight weeks

**ii) Preparation of stock solutions for growth regulators**

Auxins *viz* 2 4 D NAA IBA and IAA were dissolved in few drops of 1N NaOH and gradually diluted to 100ml with double distilled water Cytokinins *viz* BA Kinetin and Thidiazuron were dissolved in few drops of 1N HCl and gradually diluted to 100ml with double distilled water

**iii) Preparation of the culture medium**

All chemicals of AR grade were used m the preparation of culture medium Specific quantities of the stock solution were pipetted out into a beaker The required quantities of growth regulators were also added Sucrose and inositol were added fresh dissolved well and volume made up to the required level using double distilled water The pH of the solution was adjusted in between 5 5 and 5 8 using 1 0 N NaOH or 1 0 N HCl For obtaining a semisolid medium agar was added at 0 75 per cent level and the medium was boiled till a clear solution was obtained About 15 ml of this molten medium was dispensed into the culture tubes (15x2 5cm or 20x2 5cm sizes) In case of liquid medium strips of No 1 Whatman filter paper were used to support the explants The filter paper strips made in the form of M shaped bridge were inserted into the tubes

**iv) Sterilisation of the culture medium**

The tubes were plugged with non absorbent cotton and autoclaved at 121°C and 15 psi (1 06 kg/cm$^2$) for 20 minutes The medium was allowed to cool to room temperature and stored in a cool dry place

**v) Preparation of the explants**

For nodal explants stem cuttings consisting of four to five nodes were excised carefully from the plants Stem segments of 1 5 cm size cut into bits with one node each The internodal portion of 0 5 to1 0 cm size was taken as stem segment For leaf explants semi mature leaves from second and third node were collected The leaf d scs of 1 0 to 1 5 cm$^2$ size consisting of midrib were excised from basal and middle portion of the leaf lamina The explants were immersed in 1 0 per cent Teepol solution for ten minutes and thoroughly washed in running tap water to remove all

traces of the chemical The explants were also given fungicidal treatment by immersing in 0 2 per cent Bavistin solution for thirty minutes

**vi) Transfer area and aseptic manipulations**

All the aseptic manipulations such as surface sterilisation of the explants inoculation of the explants and subsequent subculturing were carried out in a clean laminar airflow chamber The working table of the laminar airflow chamber was initially surface sterilized with absolute alcohol and then by switching on the ultraviolet light for 30 minutes The petri dishes forceps knives and other inoculation aids were initially autoclaved and then flame sterilized before each inoculation The hands were washed thoroughly with soap under running tap water After drying they were wiped with absolute alcohol before inoculation

**vii) Surface Sterilisation**

Surface sterilisation was carried out under perfect aseptic conditions in the laminar airflow chamber The explants were put into the sterilant and kept immersed for the required period They were continuously agitated manually to ensure thorough contact of the explants with the chemical The different sterilisation treatments tried for the explants are listed in Table 4 The explants after surface sterilisat on were rinsed five times thoroughly with sterilized distilled water to remove traces of the sterilant from the surface of the explants

**viii) Inoculation of explant**

The surface sterilised explants were inoculated under perfect aseptic conditions into the different basal media supplemented with varying quantities of growth regulators and cultured

**ix) Culture conditions**

The cultures were incubated at 25+ 2°C in an air conditioned culture room with 16 hours photoperiod (1000 lux) supplied by cool white fluorescent light For inducing callus complete darkness was provided by covering with dark cloth Relative humidity in the culture room varied between 60 and 80 per cent according to the climate prevailing

**Table 4 Different surface sterilisation treatments carried out for the explants of *Plumbago* species before inoculation**

| Sterilant | Concentration (%) | Duration (minutes) |
|---|---|---|
| $HgCl_2$ | 0 1 | 2 |
| | 0 1 | 4 |
| | 0 1 | 6 |
| | 0 1 | 8 |
| | 0 1 | 10 |
| $HgCl_2$ | 0 2 | 1 |
| | 0 2 | 2 |
| | 0 2 | 3 |
| | 0 2 | 4 |
| | 0 2 | 5 |
| Ethyl Alcohol | 100 | 2 |
| | 90 | 2 |
| | 70 | 2 |
| Ethyl Alcohol and $HgCl_2$ | 70 0 1 | 2 4 |
| | 70 0 1 | 2 6 |
| | 70 0 1 | 2 8 |
| | 70 0 2 | 2 1 |
| | 70 0 2 | 2 2 |
| | 70 0 2 | 2 3 |

**x) Standardisation of explants with medium supplements for culture initiation**

The MS medium was supplemented with growth hormones to initiate culture in *Plumbago* species (Table 5) The response of each explant in the basal medium was recorded internodal segments nodal segments and leaf discs were used for establishing cultures The best media supplement and explant were identified based on the cultures showing maximum response as well as the type of response and carried over to two methods of *in vitro* regeneration *viz* direct regeneration and callus mediated organogenesis The following observations were recorded

a) Percentage of cultures showing response

This was calculated using the number of explants showing culture initiation to the number of explant inoculated

b) Number of days for culture initiation per explant inoculated

The number of days required for initiation of culture from the date of inoculation of explant

**Table 5 Different combinations of growth regulators for culture initiation in *Plumbago* species**

| Treatment | Medium |
|---|---|
| T1 | MS + BA 1 0 mg l$^{1}$ + NAA 0 5 mg l$^{1}$ |
| T2 | MS + BA 2 0 mg l$^{1}$+ NAA 1 0 mg l$^{1}$ |
| T3 | MS + BA 2 5 mg l$^{1}$+ NAA 1 5 mg l$^{1}$ |
| T4 | MS + 2 4 D 1 0 mg l$^{1}$ + Kin 0 5 mg l$^{1}$ |
| T5 | MS + 2 4 D 2 0 mg l$^{1}$+ Kin 1 0 mg l$^{1}$ |
| T6 | MS + 2 4 D 2 5 mg l$^{1}$+ Kin 1 5 mg l$^{1}$ |
| T7 | MS + BA mg l$^{1}$+ Ads 25 mg l$^{1}$ |
| T8 | MS + BA 1 0 mg l$^{1}$ + Ads 50 mg l$^{1}$ |
| T9 | MS + TDZ 0 01 mg l$^{1}$ |
| T10 | MS + TDZ 0 03 mg l$^{1}$ |
| T11 | MS + TDZ 0 05 mg l$^{1}$ |
| T12 | MS + TDZ 0 07 mg l$^{1}$ |
| T13 | MS + TDZ 0 1 mg l$^{1}$ |
| T14 | MS + TDZ 0 5 mg l$^{1}$ |
| T15 | MS + TDZ 1 0 mg l$^{1}$ |

### 3 3 2 2 Direct regeneration

Among the three explants tried namely stem segment nodal segments and leaf discs the explant showing maximum response was carried over for further organogenesis Different concentrations of growth regulators were tried for multiple shoot induction and proliferation

#### 3 3 2 2 1 Effect of medium supplements on shoot multiplication

Studies were conducted to determine the effect of various growth regulators on the induction of multiple shoots from explants Details of the treatments conducted are presented in Table 6 Surviving cultures from the culture initiation medium were subcultured at an interval of two to three weeks to the multiplication medium The response of the cultures in each subculture was observed and the following observations were recorded

#### 3 3 2 2 2 Observations

i) Percentage of cultures showing shoot proliferation

The percentage was calculated from the number of cultures showing multiple shoots to the total number of cultures inoculated to multiplication medium

ii) Number of days taken for shoot proliferation

The number of days required for shoot multiplication from the date of subculture was recorded

iii) Effect of different levels of growth regulators on shoot proliferation

The data on number of shoots produced per explant the length of shoot and the average number of leaves produced were recorded and subjected to statistical analysis

**3 3 2 2 3 Effect of media supplements on shoot elongation**

Multiple shoots in the proliferation medium were subcultured to same media combinations for shoot elongation The data on average increase in the shoot length and the increase in number of well developed leaves were subjected to statis cal analysis The treatments that were tried for multiple shoot elongation are presented in Table 6

**Table 6 Treatment combination of growth regulators for shoot multiplication and elongation under direct organogenesis**

| Treatment | Medium |
|---|---|
| T16 | ½ MS + BA 1 0 mg $l^{-1}$ |
| T17 | ½ MS + BA 2 0 mg $l^{-1}$ |
| T18 | MS + BA 3 0 mg $l^{-1}$ |
| T19 | MS + BA 4 0 mg $l^{-1}$ |
| T20 | MS + BA 1 0 mg $l^{-1}$ + Ads 50 mg $l^{-1}$ |
| T21 | MS + BA 1 0 mg $l^{-1}$ + Ads 25 mg $l^{-1}$ |

**3 3 2 2 4 Effect of media supplements on root induction**

Shoots of length more than 2 5 cm were excised from the elongated shoot cultures and were subjected to *in vitro* rooting The shoots were cultured in different concentrations of the auxin IBA Treatments tried for root induction is given in Table 7 Root induction was also attempted in media devoid of growth hormones The cultures were observed at weekly intervals for rooting

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**Table 7 Treatments with varying concentration of IBA for *in vitro* rooting under direct organogenesis**

| Treatment | Medium |
|---|---|
| T22 | ½ MS + IBA 0 25 mg l$^{-1}$ + sucrose 2% |
| T23 | ½ MS + IBA 0 50 mg l$^{-1}$ + sucrose 2% |
| T24 | ½ MS + IBA 1 0 mg l$^{-1}$+ sucrose 3% |
| T25 | ½ MS+ IBA1 5 mg l$^{-1}$+ sucrose 3% |
| T26 | MS + No growth regulators + sucrose 3% |

**3 3 2 2 5 Observations**

i) Percentage of cultures showing root induction

The percentage was calculated using the number of cultures showing root initiation to the cultures inoculated in rooting medium

ii) Number of days required for root initiation

The number of days required for initiation of rooting from the date of inoculation

iii) Number of days for completion of rooting

The number of days from the date of inoculation to the date of transfer of the plantlet from culture tube to sand

iv) Effect of different concentrations of rooting hormone on rooting

Rooted plants were removed from the culture vessels after sufficient number of roots was formed The plants that were taken out of the culture tubes were washed in running tap water to remove all the remnants of agar The observations on root length of plantlets from the tubes were recorded The data was analyzed statistically as per standard procedure

**3 3 2 2 6 Hardening and acclimatisation**

After completion of rooting the culture tubes were removed from culture room and placed under high light intensity and less humid conditions of the laboratory for one week to harden Rooted plants were removed from the culture vessels The plants that were taken out of the culture tubes were washed m running tap water to remove all the remnants of agar The plants were transferred to disposable cups containing

autoclaved fine river sand and watered regularly Individual cup with the plant was covered with transparent polythene cover to ensure sufficient relative humidity within the system After one week polythene cover was removed and the plantlets maintained in cups for four weeks in the laboratory In order to compare the survival rate of *in vitro* regenerated plants with conventionally propagated plants cuttings of the accession IC 566508 were planted in bags at the same time and maintained in net house and their survival recorded after three months of planting The *in vitro* plants established in cups were transferred to polythene bags containing fresh potting mixture and reared in a shade net house under irrigation The percentage survival was recorded after eight weeks

**3 3 2 2 7 Observations**

i) Percentage survival in cups (Primary hardening) The number of plants surviving in cups after hardening to the number of plants transferred

ii) Percentage survival in bags (Secondary hardening) Number of plants surviving in bags after hardening to the number of plants transferred

**3 3 2 2 8 Establishment in field**

The *in vitro* regenerated plants obtained from direct organogenesis were evaluated in the field Their performance was compared with the conventional propagated plants from rooted stem cuttings of the accession IC 566508 The *in vitro* as well as the conventional rooted cuttings of same age (three month old) were transplanted to pots and maintained as pot cultures laid out in CRD in the field in September 2008 The observations on various biometrical and morphological characters of the both plants were recorded

**3 3 2 2 9 Observations in field**

The observations on the morphological characters namely number of branches per plant plant height internodal length number of leaves leaf length and leaf breadth were recorded at quarterly intervals The plants were harvested in August 2009 twelve months after planting in the field The observations on the following biometric traits were recorded after harvest namely fresh plant weight number of roots per plant root length root girth fresh root weight dry root weight and

plumbagın content The qualıty of plumbagın ın the *ın vıtıo* plants and the convent onal plants was assessed by TLC All the observatıons were recorded as per the methodology mentıoned ın experıment II The data were subjected to statıstıcal analysıs as per standard procedure

**3 3 2 3 Regeneratıon through callus medıated organogenesıs**

As leaves alone showed callusıng response ıt was carrıed forward for callus medıated organogenesıs Leaf dıscs of *P rosea* and *P zeylanıca* were cultured ın MS medıa for callusıng Sınce no callusıng was observed wıth auxın cytokının combınatıon ın the standardızatıon treatments of explant basal medıum wıth varıous levels of auxın and cytokının ındıvıdually were trıed

**3 3 2 3 1 Effect of gı owth regulators on cıllus ınductıon**

MS medıum was supplemented wıth dıfferent levels of auxıns and cytokınıns respectıvely to ınduce callusıng ın the cultures Auxıns namely NAA and 2 4 D ındıvıdually or ın combınatıon wıth phloroglucınol an auxın synergıst were used to ınduce the callus and theır effect was studıed Detaıls are gıven ın Table 8 Cultures were ıncubated ın complete darkness at 25+2° C at a relatıve humıdıty of about 60 to 80 per cent dependıng on the external clımatıc condıtıons The relatıve performance of cultures foı callus ınductıon and prolıferatıon ın varıous treatments was observed at weekly ıntervals

**3 3 2 3 2 Observatıons**

Observatıons on the followıng parameters were recorded on callus ınductıon

ı) Number of days taken for callus ınıtıatıon The number of days from ınoculatıon of explant to the ınıtıatıon of callus

ıı) Percentage of cultures showıng callusıng The percentage was worked out usıng the number of cultures ınoculated to the number of cultures showıng callus ınductıon

ııı) Growth rate of callus Growth rate was measured on the basıs of a score Scorıng was done based on the spread of callus and a maxımum score of four was gıven to

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those calli that have occupied the whole surface of the media six weeks after incubation

Score 1 – callus occupies ¼ of the media surface

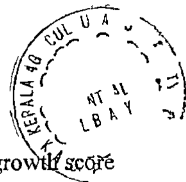
Score 2 – callus occupies ½ of the media surface

Score 3 callus occupies ¾ of the media surface

Score 4 – callus occupies complete media surface

**Table 8 Treatment combinations of growth regulators for callus induction in leaves of *Plumbago* species**

| Treatment | Medium |
|---|---|
| T27 | MS + 2 4 D 1 0 mg l$^{1}$ |
| T28 | MS + 2 4 D 2 0 mg l$^{1}$ |
| T29 | MS + 2 4 D 3 0 mg l$^{1}$ |
| T30 | MS + NAA 1 0 mg l$^{1}$ |
| T31 | MS + NAA 2 0 mg l |
| T32 | MS + NAA 3 0 mg l$^{1}$ |
| T33 | MS + NAA 4 0 mg l$^{1}$ |
| T34 | ½ MS + NAA 1 0 mg/l |
| T35 | ½ MS + NAA 2 0 mg l$^{1}$ |
| T36 | MS + NAA 0 5 mg l$^{1}$ + Phloroglucinol 50 mg l$^{1}$ |
| T37 | MS + NAA 0 5 mg l$^{1}$ + Phloroglucinol 75 mg l$^{1}$ |
| T38 | MS + NAA 0 5 mg l$^{1}$ + Phloroglucinol 100 mg l$^{1}$ |
| T39 | MS + NAA 0 5 mg l$^{1}$ + Phloroglucinol 125 mg l$^{1}$ |
| T40 | MS + NAA 0 5 mg l$^{1}$ + Phloroglucinol 150 mg l$^{1}$ |
| T41 | MS + BA 0 5 mg l$^{1}$ |
| T42 | MS + BA 1 0 mg l$^{1}$ |
| T43 | MS + BA 1 5 mg l$^{1}$ |
| T44 | MS + BA 2 0 mg l$^{1}$ |
| T45 | MS + BA 2 5 mg l$^{1}$ |
| T46 | MS + TDZ 1 0 mg l$^{1}$ |
| T47 | MS + TDZ 1 5 mg l$^{1}$ |
| T48 | MS + TDZ 2 0 mg l$^{1}$ |
| T49 | MS + TDZ 2 5 mg l$^{1}$ |



iv) Callus Index (CI) It was worked out as below

CI – P x G  Where P – percentage of callus initiation and G growth score

v) Callus morphology The morphology of callus was recorded on the basis of physical appearance colour and texture

vi) Nature of callus The nature of callus was noted based on its mode of regeneration i e whether embryogenic or non embryogenic

**3 3 2 3 3 Effect of media supplements on organogenesis from the callus**

Callusing was observed only in the leaves of species *Plumbago rosea* Hence further studies on callus regeneration were carried out only in this species The calli so obtained from the above mentioned treatments were subcultured in MS media containing different levels of growth regulators for shoot induction The callus was divided into smaller pieces and inoculated into regeneration medium The various treatments tried to induce multiple shoots from the calli are presented in Table 9 and the response of the calli was recorded at weekly intervals

**Table 9 Treatment combinations of growth regulators for callus regeneration in *Plumbago rosea***

| Treatment | Medium |
|---|---|
| T50 | MS+ BA 1 0 mg $l^{-1}$+ Ads 50 mg $l^{-1}$ |
| T51 | ½ MS + BA 2 0 mg $l^{-1}$ ( semi solid) |
| T52 | ½ MS + BA 2 0 mg $l^{-1}$ (liquid) |
| T53 | MS + BA 3 0 mg $l^{-1}$ |
| T54 | MS + BA 4 0 mg $l^{-1}$ |
| T55 | MS + BA 0 2 mg $l^{-1}$ + IAA 0 1 mg $l^{-1}$ |

**3 3 2 3 4 Observations**

i) Percentage callus cultures showing proliferation and multiple shoot initiation Percentage was worked out from the number of calli showing proliferation and shoot initiation to number of pieces of calli inoculated

ıı) Number of days for multıple shoots ınıtıatıon and prolıferatıon The number of days from first subculture to the second subculture to shoot elongatıon medıum

ııı) Effect of dıfferent growth regulators on multıple shoot generatıon from callus The effect of dıfferent growth regulators were noted on the average number of shoots produced average length of shoots and the average number of leaves produced per callus subcultured

**3 3 2 3 5 Shoot elongatıon**

Multıple shoots ın the prolıferatıon medıum were subcultured to dıfferent combınatıons of medıa for shoot elongatıon Varıed concentratıons of cytokının were trıed for shoot elongatıon Besıdes cytokının gıbberllıc acıd was also used for elongatıon The treatments that were trıed for multıple shoot elongatıon are presented ın Table 10 The cultures were observed for shoot elongatıon at weekly ıntervals

**Table 10 Treatment combınatıons of growth regulators for elongatıon of multıple shoots from callı of *Plumbago rosea***

| Treatment | Medıum |
|---|---|
| T56 | MS + BA 5 0 mg l 1 |
| T57 | MS + BA 6 0 mg l |
| T58 | MS + BA 7 0 mg l |
| T59 | MS + BA 8 0 mg l 1 |
| T60 | MS + BA 9 0 mg l |
| T61 | MS + BA 10 0 mg l |
| T62 | MS + $GA_3$ 0 1 mg l 1 |

**3 3 2 3 6 Root ınductıon**

The regenerated callı when placed ın the medıum contaınıng $GA_3$ not only showed elongatıon but also rootıng The elongated shoots were cultured ın dıfferent concentratıons of $GA_3$ as well as ın IBA Treatments trıed for root ınductıon are gıven ın Table 11 The excısed shoots were transferred to semı solıd medıum and lıquıd medıum of $GA_3$ ın test tubes as well as to semı solıd medıum of $GA_3$ ın con cal flasks/jam bottles Observatıons on root ınductıon and shoot elongatıon were recorded after completıon of rootıng just before plantıng out

**Table 11 Treatments combmatıon of growth regulators for root ınductıon ın shoots regenerated from callı of *Plumbago rosea***

| Treatment | Medıum |
|---|---|
| T62 | MS + $GA_3$ 0 1 mg l (semı solıd) |
| T63 | MS + $GA_3$ 0 1 mg $l^{1}$ (lıquıd) |
| T64 | MS + $GA_3$ 0 5 mg $l^{1}$ (semı solıd) |
| T65 | MS + $GA_3$ 0 7 mg $l^{1}$ (semı solıd) |
| T66 | MS + $GA_3$ 0 9 mg $l^{1}$ (semı solıd) |
| T67 | MS + IBA 1 5 mg $l^{1}$ (semı solıd) |

**3 3 2 3 7 Observatıons**

ı) Number of days for root ınductıon The number days from the date of ınoculatıon ın the rootıng medıum to the date of transfer to the sand were counted

ıı) Length of shoots The length of shoots per culture tube/flask was recorded and the mean worked out

ııı) Number of leaves per shoot The number of leaves ın each shoot per culture was recorded and the mean worked out

ıv) Length of roots The length of root was recorded from the shoots per culture and average worked out

**3 3 2 3 8 Hardenıng and acclımatızatıon**

Rooted plants were removed from the culture vessels after formatıon of suffıcıent number of roots and transferred to dısposable cups contaınıng autoclaved fine rıver sand and watered regularly The procedure of hardenıng mentıoned for nodal regeneratıon was followed for plantlets obtaıned through callus regeneratıon Due to tıme constraınt the plants obtaıned from callus regeneratıon were not evaluated ın field

**3 3 2 3 9 Observatıons**

The calculatıons on the followıng ıtems of observatıons were done as mentıoned earlıer ın the dırect regeneratıon

ii) Percentage survival in bags ( Secondary hardening)

After the first phase for standardization of *in vitro* regeneration in *Plumbago* species the investigation proceeded to the second phase of this experiment i e *in vitro* mutagenesis

**3 3 2 4 *In vitro* mutagenesis**

In the second phase *in vitro* mutagenesis was carried out to induce variability in *Plumbago* species Since the protocol for *in vitro* regeneration could be standardized only in *Plumbago rosea* accessions of *P rosea* alone were subjected to *in vitro* mutagenesis Both nodal as well as callus cultures were subjected to mutagenesis using physical and chemical mutagens

**3 3 2 4 1 Physical mutagenesis**

**a) Source**

Ionizing radiation gamma rays (wave length 0 1$^0$A) from $^{60}$Co of the Gamma chamber 900 of BARC Mumbai installed at Radio Tracer laboratory of the College of Horticulture Vellanikkara was used as the source of physical mutagen Its mode of action is ionization

**b) Dose**

The nodal segments of *P rosea* accessions selected from cluster analysis were cultured in the best medium identified in the standardization procedure Ten tubes of two week old nodal cultures of *P rosea* were subjected to different doses of gamma radiation (Table 12) Leaf discs from semi mature leaves of *P rosea* were inoculated in the best callus induction medium earlier identified from the standardization procedure Ten callus pieces from six weeks old calli cultures of *P rosea* were placed in the calli multiple shoot regeneration medium and subjected to different doses of gamma radiation

**c) Protocol**

**Nodal culture**

- The irradiated sprouted nodes were immediately transferred to fresh proliferation medium where they were allowed to proliferate This was to

avoid formation of toxic compounds and enhance radiation efficiency

- After proliferation the treated culture along with untreated culture were transferred to elongation medium
- After four weeks the elongated shoots were then cultured in rooting medium
- Regeneration percentage was used to determine the $LD_{50}$ value based on which doses lower than $LD_{50}$ were fixed as optimum doses
- The above procedure was repeated with the optimum doses

**Table 12 Doses of gamma rays used for *in vitro* treatment**

| Treatment | Gamma rays(Gy) | Duration of treatment |
|---|---|---|
| $D_0$ (Control) | 00 | 00 minutes 00 seconds |
| $D_1$ | 10 | 05 minutes 32 seconds |
| $D_2$ | 20 | 11 minutes 4 seconds |
| $D_3$ | 30 | 16 minutes 36 seconds |
| $D_4$ | 40 | 32minutes 12 seconds |
| $D_5$ | 50 | 55 minutes 24 seconds |

**Callus culture**

- The irradiated callus was transferred to fresh regeneration medium After six weeks of proliferation the treated and the untreated proliferated calli were placed in elongation cum rooting medium

- Regeneration percentage was used to determine the $LD_{50}$ value based on which doses lower than $LD_{50}$ were fixed as optimum The above procedure was repeated with the optimum doses

**3 3 2 4 2 Chemical mutagenesis**

**a) Source**

EMS (Specific gravity 1 204 g/cc Molecular weight 124 16g) was used for *in vitro* chemical mutagenesis The mode of action is alkylation

**b) Dose**

The method of explant agitation (Rajendran 2005) was tried Here calli pieces from six week old cultures and two week old sprouted nodal cultures were subjected to treatment by soaking in various concentrations of EMS solutions for a particular duration The EMS solution was prepared freshly in sterile distilled water in the laminar flow and the treatment given under aseptic conditions The details of concentrations tried are given in Table 13 Ten tubes of nodal as well as calli cultures of *P rosea* accessions selected from cluster analysis were subjected to each of the concentration mentioned in the table

**Table 13 Concentration of EMS used for *in vitro* treatment**

| Treatment | Concentration (%) | Duration of treatment(Minutes) |
|---|---|---|
| $DE_0$(Control) | 0 00 | 60 |
| $DE_1$ | 0 05 | 60 |
| $DE_2$ | 0 10 | 60 |
| $DE_3$ | 0 25 | 60 |
| $DE_4$ | 0 50 | 60 |
| $DE_5$ | 0 75 | 60 |

**c) Protocol**

**Nodal culture**

- After soaking for one hour the sprouted nodal segments (two week old cultures) were washed with sterile water to remove residue of EMS which was followed by drying and later transferred to fresh multiplication medium for proliferation
- After four weeks of multiplication the shoots were transferred to elongation medium
- After four weeks the elongated shoots were then cultured in rooting medium
- The regeneration percentage was used to determine the $LD_{50}$ value based on which doses lower than $LD_{50}$ were fixed as optimum The above procedure was repeated with the optimum doses

**Callus culture**

- After soaking for one hour the callus pieces were washed with sterile water to remove residue of EMS which was followed by drying and later transferred to multiplication medium for proliferation
- After four weeks of multiplication the shoots were transferred to rooting cum elongation medium
- The regeneration percentage was used to determine the $LD_{50}$ value based on which doses lower than $LD_{50}$ were fixed as optimum The above procedure was repeated with the optimum doses

**3 3 2 4 3 Observations**

i) Regeneration percentage for estimation of $LD_{50}$ value

For fixing the $LD_{50}$ value of mutagenesis the regeneration based on the multiple shoot initiation and root formation in the nodal as well as calli cultures was recorded The percentage was calculated based on the number of cultures showing multiple shoot initiation and the number of cultures showing root induction

ii) Regeneration percentage for optimum dose

Percentage was worked out from the number of cultures showing multiple shoot initiation elongation and rooting to number of calli/ explant inoculated after treatment with optimum doses of physical and chemical mutagens

iii) Plant regeneration percentage after *in vitro* mutagenesis

Percentage was calculated from the number of plants produced to the number of cultures subjected to mutagenesis

iv) Length of shoot per plantlet

The length of shoot was recorded in each plant and the mean worked out

v) Number of leaves per plantlet

The number of leaves in each plantlet was recorded and the mean worked out

vi) Length of roots per plantlet

The length of root was recorded from each plantlet and the average worked out

### 3 3 2 4 4 Hardening and establishment

The plantlets were hardened and the survival percentage from each dose of radiation was recorded after three months of rearing in net house The observations on morphological characters such as plant height internode length number of leaves leaf length and leaf breadth were recorded The data statistically analysed in CRD with unequal replications for each accession Based on the analysis of variance superior plants from each accession for various levels of mutagenesis were selected for field evaluation Before field establishment the selected somaclones were compared with the rooted cuttings of *P rosea* accessions

### 3 3 2 4 5 Comparison of plants obtained from *in vitro* mutagenesis with conventionally rooted cuttings of *P rosea* in bags

Three superior plants of each accession from *in vitro* mutation were selected for field establishment and their corresponding conventional rooted cuttings of same age were used for recording observations The data obtained on the characters *viz* plant height internodal length number of leaves leaf length and leaf breadth were subjected to statistical analysis

### 3 3 2 5 Statistical Analysis

All the statistical analyses for standardization of *in vitro* regeneration as well as *in vitro* mutagenesis were carried out in completely randomized design (CRD) The Duncan s multiple range test (DMRT) was performed using software package SPSS version 13 0

## 3 4 Experiment IV

**Induction of polyploidy in the *Plumbago* species**

### 3 4 1 Materials

One accession of *P rosea* and one accession of *P zeylanica* selected on the basis of cluster analysis in experiment II were used

**3 4 2 Methodology**

The study was carried out from January 2008 to March 2009 The most effective and widely used chemical colchicine supplied by SRL Ltd Mumbai was used to induce polyploidy in the present investigation

**3 4 2 1 Standardization of colchicine application technique**

**3 4 2 1 1 Preparation of vegetative material for colchicine application**

The sprouted cuttings of *Plumbago rosea* and *Plumbago zeylanica* were used for the standardization Two noded cuttings excised from the semi hardwood portion of both *Plumbago* species were planted with one node above soil surface in polybags (12 x 18 cm with 150 gauge thickness) filled with potting mixture (soil sand and dried cow dung in equal proportions) In each bag three cuttings were planted The planted bags were kept in the net house and watered regularly The cuttings sprouted after three months from the date of planting were used for colchicine treatment The bud emerging from the axil of the leaf on the node just below the apical leaf of the sprouted branch of the cuttings was selected for colchicine application Before colchicine application the sprouted branch was decapitated by excising the apical leaf bud In five days the axillary bud emerged in both *Plumbago* species

**3 4 2 1 2 Method of colchicine application**

Cotton swab method was tried for applying colchicine The emerging axillary bud was completely covered by a small piece of cotton wool fixed temporarily with a cello tape wound to the stem The cotton swab was soaked intermittently with colchicine solution by the help of dropper

**3 4 2 1 3 Fixing doses of colchicine**

To find out the optimum dose of colchicine a preliminary trial was conducted by treating the just emerging axillary buds of three month old sprouted cuttings of *P rosea* and *P zeylanica* The axillary buds were covered with cotton swab soaked in aqueous solution of colchicine of concentrations ranging from 0 0 ($C_0$) to 2 0 per cent ($C_6$) for three hours ($D_1$) and six hours ($D_2$) duration on two consecutive days respectively The details of the duration and concentration of colchicine application

are given in Table 14 In case of control 2ml of glycerol was dissolved in distilled water and volume made up to 20 ml The same procedure was repeated on the second day by preparing fresh stock solution of colchicine The cotton swab was removed after colchicine application for the required duration and the treated axillary bud was observed for sprouting for a period of two weeks The number of days for sprout ng and the percentage sprouting in the two species of *Plumbago* were recorded Based on the sprouting percentage the optimum dose of colchicine was fixed For each concentration of colchicine three bags with three cuttings each of the respective *Plumbago* species were used

**Table 14 Concentration and duration of colchicine treatment in the preliminary trial**

| Treatment No | Concentration (%) | Weight of colchicine (g) | Volume of glycerol (ml) | Volume of distilled water (ml) | Duration | No of days |
|---|---|---|---|---|---|---|
| $C_0$ | 0 00 | 0 00 | 2 | 18 | $D_1$ 3 hours<br>$D_2$ 6 hours | 2 consecutive days |
| $C_1$ | 0 25 | 0 05 | 2 | 18 | | |
| $C_2$ | 0 50 | 0 10 | 2 | 18 | | |
| $C_3$ | 0 75 | 0 15 | 2 | 18 | | |
| $C_4$ | 1 00 | 0 20 | 2 | 18 | | |
| $C_5$ | 1 50 | 0 30 | 2 | 18 | | |
| $C_6$ | 2 00 | 0 40 | 2 | 18 | | |

**3 4 2 1 4 Optimum dose of colchicine application in the *Plumbago* species**

Based on the preliminary trial optimum dose of colchicine to be used for application was decided A weighed quantity of commercially available colchicine powder was mixed with a small quantity of glycerol (10 % of the final volume) and dissolved in distilled water and then the volume was made up to achieve the required concentration Just emerging axillary buds of the two *Plumbago* species were treated with this aqueous solution of colchicine for seven days using the cotton swab method The treatment was done for the following two periods of duration per day

D 3 hours from 7 a m to 10 a m for seven consecutive days

The cotton swab was soaked with colchicine solution intermittently at an interval of one hour Three bags with three cuttings each of the two respective *Plumbago* species were used for the treatment with optimum doses of colchicine In the experiment absolute control i e cuttings without any treatment were used for comparison After seven days of treatment the treated cuttings along with the control were kept in laboratory till the sprout emerged from the bud The plants were then removed from the bags and planted in cement pots The plants were maintained in the net house with regular mist irrigation The plants were observed regularly for the growth of treated axillary bud sprouts Only the branch emerging from the treated bud was allowed to grow whereas all the other branches were removed to avoid their smothering effect This procedure was followed for the control plant as well The observations on growth morphological characters and floral characters were recorded

**3 4 2 1 5 Observations**

The observations on the following morphological characters were recorded at quarterly intervals during the period from April 2008 to March 2009

**a) Morphological characters**

Length/height of the branch

Internode length

Leaf length

Leaf breadth

**b) Study of leaf epidermal cells**

The size of epidermal cells is an indication of the ploidy level of plants Any change in the size of epidermal cells results from change in the number of chromosomes Hence to measure the size of epidermal cells this study was undertaken six months after planting in the pots (second quarter) Free hand sections of leaf from the third node of the branch from treated as well as control plants were taken The sections were stained with a drop of acetocarmine on a clean slide and covered with a cover slip The slides were examined under microscope for the

epidermal cells The length and breadth of ten cells from leaf sections of each species were recorded with the help of ocular micrometer after calibration

**c) Stomatal study**

Stomata size is found to bear a direct correlation with the ploidy level of plants An increase in the size of stomata is an indication of increased cell size which results from increase in the number of chromosomes For observing stomata peeling of the lower epidermis of the leaf from the second node of the branch of treated as well as control plants of both the species was taken The length and the width of the stomatal opening were measured with the help of ocular micrometer after calibration The measurements of stomata from ten different microscopic fields were recorded for leaf from each treatment In order to ascertain the stomatal number peelings of the lower epidermis were taken from the base middle and tip of the leaf lamina and the number of stomatal openings per microscopic field for ten microscopic fields in each treatment in both the species of *Plumbago*

**d) Floral characters**

All the treated as well as control plants were observed for flowering in both the species Flowers in both the species were observed for pollen fertility as well as pollen size

i) Pollen fertility Pollen fertility was assessed by aceto carmine staining technique as mentioned in experiment II The values obtained for each treatment were expressed as percentage

ii) Pollen size Pollen diameter was measured using ocular micrometer after calibration The diameter of pollen (10 nos ) from three mature buds from each colchicine treated as well as control plants of both the species of *Plumbago* was recorded Mean size of the pollen from treated as well as control plants was computed

**3 4 2 2 Statistical analysis**

The data obtained from all the above observations were subjected to statistical analysis as per standard procedure

### 3 4 2 3 Identification of variants/polyploids

The axillary branch of the colchicine treated plants was observed for any marked phenotypic difference from the control plants in both the species of *Plumbago* Besides visual observation the treatments showing significant differences in the statistical analysis were identified

## 3 5 Experiment V

**Evaluation of samples of *Plumbago* species screened from *in vitro* mutagenesis and polyploidy**

### 3 5 1 Materials

The samples of the *Plumbago* species obtained from *in vitro* mutagenesis and polyploidy were evaluated in the field From experiment III soma clones (three months old) obtained from each dose of mutation were planted in pots laid out in CRD along with conventionally rooted cuttings of same age in the field in August 2009

### 3 5 2 Methodology

The field evaluation study was carried out during the period August 2009 to May 2010 The observations on the morphological characters *viz* plant height internodal length number of branches number of leaves leaf length and leaf breadth were recorded at four months after planting and eight months after planting (MAP) The somaclones and the conventional rooted cuttings were also observed for flowering The morphological traits were recorded as per the methodology mentioned in experiment II The data were subjected to statistical analysis in CRD with three replications

# Results

# 4 RESULTS

The results of the various experiments carried out for the Assessment and induction of variability in *Plumbago* species for high plumbagin content are presented under the following heads

1 Exploration and collection of different genotypes of *Plumbago* species
2 Assessment of genetic variability of various morphological and biometrical traits in the collected accessions
3 Induction of variability through *in vitro* regeneration and mutagenesis of *Plumbago* species
4 Induction of polyploidy in *Plumbago* species
5 Evaluation of samples of *Plumbago* species screened from *in vitro* mutagenesis and polyploidy

## 4 1 Exploration and collection of different genotypes of *Plumbago* species

On the basis of eco geographic survey conducted in various districts of Kerala 27 accessions of *Plumbago* were collected and established in the field as pot culture for preliminary evaluation Twenty five accessions of *Plumbago rosea* were collected from different districts of Kerala one accession of *Plumbago zeylanica* and one accession from of *Plumbago capensis* from Thrissur Passport data of the collected accessions was prepared and the same is presented in Table 1 The table gives detailed information on the place of collection date of collection the altitude latitude and longitude of the place of collection The table also reveals important agronomic characters as well as distinct morphological features of the accessions from the three species of *Plumbago* Among the three species evaluated *P rosea* produced tuberous roots with higher plumbagin content than the other two species The accession no 27 (*Plumbago capensis*) was very distinct and produced very thin and non tuberous roots with negligible plumbagin content This species is mainly cultivated for ornamental purposes for its beautiful blue flowers and therefore not included in further investigation Twenty six accessions of two species were carried over to experiment II for detailed evaluation of morphological biometric and floral traits in replicated trial The district wise distribution of *Plumbago* genotypes collected from the exploration is presented in Table 15

**Table 15 Distirct wise distribution of *Plumbago* accessions**

| Sl No | Species | District | Place of Collection | Accession No |
|---|---|---|---|---|
| 1 | *Plumbago rosea* | Ernakulam | Thuruthissery | IC 566506 |
| 2 | *Plumbago rosea* | Idukki | Santhampara | IC 566520 |
| 3 | *Plumbago rosea* | | Pulppara | IC 566521 |
| 4 | *Plumbago rosea* | | Munnar | IC 566522 |
| 5 | *Plumbago rosea* | Kozhikode | Kozhikode | IC 566503 |
| 6 | *Plumbago rosea* | Malappuram | Thenjipalam | IC 566502 |
| 7 | *Plumbago rosea* | | Kottakkal | IC 566509 |
| 8 | *Plumbago rosea* | | Kadampuzha | IC 566510 |
| 9 | *Plumbago rosea* | | Valanchery | IC 566511 |
| 10 | *Plumbago rosea* | | Changaramkulam | IC 566518 |
| 11 | *Plumbago rosea* | Palakkad | Ottappalam | IC 566515 |
| 12 | *Plumbago rosea* | | Kollengode | IC 566516 |
| 13 | *Plumbago rosea* | | Nenmara | IC 566517 |
| 14 | *Plumbago rosea* | | Nelliampathy | IC 566519 |
| 15 | *Plumbago rosea* | Thrissur | Vellanikkara | IC 566499 |
| 16 | *Plumbago rosea* | | Thrissur | IC 566507 |
| 17 | *Plumbago rosea* | | Vellanikkara | IC 566508 |
| 18 | *Plumbago rosea* | | Vellanikkara | IC 566512 |
| 19 | *Plumbago rosea* | | Vellanikkara | IC 566513 |
| 20 | *Plumbago rosea* | | Vellanikkara | IC 566514 |
| 21 | *Plumbago rosea* | | Vellanikkara | IC 566523 |
| 22 | *Plumbago zeylanica* | | Vellanikkara | Acc No 26 |
| 23 | *Plumbago rosea* | Wayanad | Thamarassery | IC 566500 |
| 24 | *Plumbago rosea* | | Ambalavayal | IC 566501 |
| 25 | *Plumbago rosea* | | Kalpetta | IC 566504 |
| 26 | *Plumbago rosea* | | Pookode | IC 566505 |

As seen in the table maximum number of seven accessions of *P rosea* and one accession of *P zeylanica* were collected from Thrissur district followed by five accessions from Malappuram district The distribution of *P rosea* was seen mainly in

places of medium and high altitude especially in the midlands of Kerala Hence accessions of *P rosea* were collected from district located in these areas

**4 2 Assessment of genetic variability of various morphological and biometrical traits in the collected accessions**

Twenty six accessions of *Plumbago* were evaluated in replicated trial for various morphological biometric and floral traits The data obtained for these traits were subjected to statistical analysis and the results are presented below

**4 2 1 Genetic variability**

The extent of genetic variability with respect to thirteen characters including therapeutic content plumbagin m twenty six diverse accessions of *Plumbago* was estimated and the results are presented in Table 16 The analysis of variance revealed significant differences for all the characters except root length and root girth

**4 2 2 Mean performance of twenty six *Plumbago* accessions**

The mean performance of twenty six *Plumbago* accessions for thirteen characters is given in Table 17 The accession of *P zeylanica* had the maximum number of branches (76 67) Among the *P rosea* accessions IC 566504 had the maximum number of branches (29 67) followed by IC 566513 IC 566521 and IC 566522 (29 0) The lowest value of 7 0 for this trait were recorded by two accessions *viz* IC 566509 and IC 566511 Among the twenty five *P rosea* accessions IC 566504 had the maximum height (131 67 cm) followed by IC 566511 (112 3cm) The accession IC 566511 had recorded lowest value of 39 0cm for this trait The *P zeylanica* recorded maximum height of 166 3 cm among all the accessions studied

The result revealed significant variation for the character internodal length among the different *Plumbago* accessions The maximum mean value was recorded by two accessions IC 566501 and IC 566504 (9 33 cm) whereas the accession IC 566511 recorded minimum value (4 33cm) Among all the accessions IC 566504 had highest number of leaves (455 3) followed by the *P zeylanica* accession (453 3) The accession IC 566511 recorded lowest number of leaves The accession IC 566513 had the longest leaf of 10 62 cm followed by IC 566506(10 55 cm) The accession IC

Table 16 ANOVA for morpl ological and biometric traits studied in *Pl umbago* accessions

| Source of var at on | Degrees of freedom | Mean sum of squares | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No of branches plant | Plant he ght (cm) | Internodal length (cm) | No of leaves | Leaf length (cm) | Leaf breadth (cm) | No of roots plant | Root length (cm) | Root g rth (cm) | To al b omass(g) | Fresh root we gh (g) | Dry root we ght (g) | Plumbag n content (%) |
| Repl cat on | 2 | 949 47 | 5170 25 | 24 27 | 127459 9 | 5 49 | 0 97 | 288 15 | 1234 9 | 2 18 | 291285 75 | 25362 81 | 3928 23 | 4 72 |
| Treatn ent | 25 | 519 36** | 1620 27** | 4 63* | 32975** | 5 27** | 1 48** | 65 77** | 209 78 | 0 292 | 29166 61** | 4686 62** | 470 82** | 1 69** |
| Error | 50 | 85 53 | 384 17 | 2 36 | 8784 16 | 1 14 | 0 28 | 38 86 | 319 98 | 0 185 | 11927 24 | 2524 15 | ?58 64 | 0 63 |

* S gn f cance at 5% level

** S gn f cance at 1% level

Table 17 **Mean Performance of twenty s1x access1ons of *Plumbago***

| Access on No | No of branches plant | He ght (cm) | Internodal length (cm) | No of leaves | Leaf length (cm) | Leaf breadth (cm) | No of roots plant | Root Length | Root g rth | Total b omass (g) | Fresh root we ght (g) | Dry Root wt (g) | Plumbag n content (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IC 566499 | 15 00 | 79 67 | 6 67 | 219 00 | 8 47 | 4 78 | 20 00 | 69 00 | 2 20 | 260 00 | 133 33 | 42 00 | 2 65 |
| IC 566500 | 7 67 | 80 33 | 6 67 | 112 00 | 5 69 | 3 31 | 8 33 | 59 67 | 2 10 | 100 00 | 50 00 | 17 33 | 1 99 |
| IC 566501 | 14 00 | 90 33 | 9 33 | 161 33 | 8 10 | 4 17 | 14 67 | 63 00 | 2 27 | 240 00 | 110 00 | 37 33 | 2 14 |
| IC 566502 | 13 33 | 73 33 | 6 33 | 126 67 | 6 23 | 3 91 | 19 33 | 64 00 | 2 00 | 266 67 | 153 33 | 46 00 | 2 62 |
| IC 566503 | 17 00 | 102 67 | 6 67 | 168 00 | 7 63 | 4 80 | 24 33 | 80 33 | 2 93 | 366 67 | 196 67 | 60 67 | 2 79 |
| IC 566504 | 29 67 | 131 67 | 9 33 | 455 33 | 9 10 | 5 47 | 20 33 | 74 67 | 2 63 | 490 00 | 126 67 | 43 67 | 2 75 |
| IC 566505 | 23 33 | 105 67 | 6 33 | 270 67 | 8 18 | 4 45 | 20 67 | 66 00 | 2 33 | 370 00 | 143 33 | 45 00 | 2 70 |
| IC 566506 | 17 67 | 97 33 | 6 33 | 203 33 | 10 55 | 5 90 | 17 67 | 68 67 | 3 03 | 333 33 | 123 33 | 37 67 | 2 88 |
| IC 566507 | 16 00 | 89 00 | 7 33 | 282 33 | 6 93 | 3 75 | 26 33 | 74 50 | 2 50 | 316 67 | 150 00 | 51 00 | 2 75 |
| IC 566508 | 17 00 | 92 00 | 6 17 | 237 33 | 7 20 | 4 20 | 26 33 | 69 00 | 2 37 | 373 33 | 193 33 | 59 00 | 4 62 |
| IC 566509 | 7 00 | 95 67 | 5 67 | 115 33 | 7 46 | 4 10 | 10 67 | 70 33 | 2 03 | 150 00 | 73 33 | 22 00 | 2 41 |
| IC 566510 | 11 33 | 51 67 | 7 00 | 114 67 | 7 10 | 4 36 | 17 67 | 61 67 | 2 90 | 280 00 | 156 67 | 45 67 | 3 46 |
| IC 566511 | 7 00 | 39 00 | 4 33 | 64 33 | 5 01 | 3 00 | 13 00 | 56 33 | 2 33 | 220 00 | 90 00 | 23 33 | 3 79 |
| IC 566512 | 20 00 | 01 67 | 7 00 | 197 33 | 6 12 | 3 85 | 11 33 | 84 00 | 2 40 | 223 33 | 83 33 | 25 33 | 3 27 |
| IC 566513 | 29 00 | 112 33 | 8 33 | 305 33 | 10 62 | 4 88 | 17 00 | 68 67 | 2 60 | 386 67 | 150 00 | 45 00 | 3 37 |
| IC 566514 | 20 33 | 99 33 | 7 33 | 240 33 | 7 13 | 3 87 | 21 00 | 65 67 | 2 07 | 326 67 | 130 00 | 46 00 | 4 11 |
| IC 566515 | 25 67 | 101 33 | 5 67 | 330 00 | 8 32 | 4 91 | 20 67 | 76 00 | 2 63 | 406 67 | 143 33 | 46 33 | 2 81 |
| IC 566516 | 21 67 | 87 33 | 7 00 | 206 67 | 6 82 | 4 08 | 20 00 | 72 33 | 2 60 | 316 67 | 146 67 | 48 33 | 3 87 |
| IC 566517 | 15 00 | 107 67 | 4 73 | 236 00 | 8 41 | 5 27 | 19 33 | 64 33 | 2 47 | 376 67 | 193 33 | 55 00 | 2 39 |
| IC 566518 | 19 67 | 81 00 | 5 37 | 263 00 | 8 77 | 5 34 | 17 33 | 94 67 | 2 63 | 303 33 | 136 67 | 43 67 | 3 68 |
| IC 566519 | 24 33 | 96 00 | 7 67 | 374 00 | 8 33 | 4 81 | 24 67 | 63 00 | 2 23 | 433 33 | 173 33 | 57 67 | 3 43 |
| IC 566520 | 20 67 | 96 67 | 6 33 | 345 33 | 6 67 | 3 87 | 20 67 | 62 33 | 1 87 | 283 33 | 110 00 | 35 33 | 3 50 |
| IC 566521 | 29 00 | 93 33 | 6 00 | 351 33 | 8 63 | 5 31 | 25 67 | 75 33 | 2 43 | 493 33 | 186 67 | 57 33 | 2 97 |
| IC 566522 | 29 00 | 94 33 | 7 33 | 367 00 | 8 01 | 4 61 | 20 00 | 76 50 | 2 40 | 476 67 | 200 00 | 68 67 | 3 02 |
| IC 566523 | 18 00 | 86 00 | 8 50 | 311 33 | 7 49 | 4 36 | 19 33 | 63 67 | 2 30 | 280 00 | 110 00 | 35 33 | 2 89 |
| Acc no 26 | 76 67 | 166 33 | 8 17 | 453 33 | 6 77 | 3 90 | 15 67 | 65 67 | 1 80 | 423 33 | 70 00 | 38 00 | 0 87 |
| Mean | 20 91 | 94 29 | 6 83 | 250 44 | 7 68 | 4 43 | 18 92 | 69 59 | 2 39 | 326 80 | 135 9 | 43 56 | 2 98 |
| SE | ±5 33 | ±11 32 | ±0 88 | ±54 11 | ±0 62 | ±0 31 | ±3 59 | ±10 33 | ±0 24 | ± 63 05 | ±29 00 | ±9 28 | ±0 45 |
| CD 0 05 | 14 77 | 31 36 | 2 5 | 149 98 | 1 69 | 0 85 | 9 95 | NS | NS | 174 73 | 8 37 | 25 72 | 1 24 |
| CV(%) | 44 21 | 20 78 | 22 52 | 37 42 | 13 79 | 12 04 | 32 94 | 25 71 | 18 06 | 33 42 | 36 65 | 36 92 | 26 52 |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566511 had the shortest leaf of 5 01 cm The accessions IC 566506 had the maximum leaf breadth of 5 90 cm followed by the accession IC 566504 (5 47 cm) The accession IC 566511 recorded the minimum value of 3 00 cm for this trait

After harvest it was observed that the accessions IC 566507 and IC 566508 produced maximum numbers of roots per plant (26 33) while the accession IC 566500 produced the least number of roots (8 33) The accession IC 566518 had longest root of 94 67 cm followed by accession IC 566512 (84 00 cm) The accession IC 566511 produced shortest root of 56 33 cm The accession IC 566506 had the thickest root of 3 03cm followed by accession IC 566510 (2 93 cm) The *P zeylanica* had thinnest root (1 80cm) and among the *P rosea* accessions IC 566520 had the thinnest root (1 87 cm)

The accession IC 566521 had recorded maximum value of 493 33 g for total biomasss followed by the accession IC 566504 (490 00 g) while the accession IC 566500 had the minimum value (100 00 g) for this trait The accession IC 566522 recorded the maximum fresh root weight and dry root weight (200 00 g 68 67g) followed by the accession IC 566503 (196 60 g 60 67g) The accession IC 566500 had recorded lowest fresh root weight as well as dry root weight (50 00 g 17 33 g)

The accession IC 566508 had maximum plumbagin content (4 62 %) in the crude acetone extract of dried root followed by the accession IC 566514 (4 11 %) The *P zeylanica* accession had the minimum plumbagin content of 0 87 % Among the *P rosea* genotypes the accession IC 566500 had the lowest plumbagin content (1 99 %)

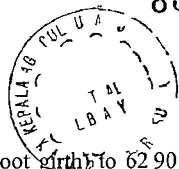
### 4 2 3 Phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV)

The estimates of PCV GCV heritability genetic advance and genetic gain for twelve characters are presented in Table 18

**Table 18 Components of variability for morphological and biometric characters**

| Characters | GCV | PCV | Heritability (%) | Genetic advance | Genetic gain (%) |
|---|---|---|---|---|---|
| No of branches/plant | 57 50 | 72 54 | 62 90 | 19 64 | 93 70 |
| Height (cm) | 21 52 | 29 93 | 51 80 | 30 09 | 31 91 |
| Internodal length(cm) | 12 73 | 25 86 | 24 20 | 0 88 | 12 88 |
| No of leaves | 35 06 | 51 83 | 47 90 | 127 97 | 51 09 |
| Leaf length (cm) | 15 40 | 20 82 | 54 70 | 1 79 | 23 30 |
| Leaf breadth (cm) | 14 00 | 18 47 | 57 50 | 0 97 | 21 89 |
| No of roots/plant | 15 83 | 36 55 | 18 80 | 2 67 | 14 11 |
| Root girth(cm) | 7 89 | 19 71 | 16 00 | 0 16 | 6 69 |
| Total biomass (g) | 23 20 | 40 68 | 32 50 | 89 04 | 27 24 |
| Fresh root weight(g) | 21 75 | 42 63 | 26 04 | 31 77 | 23 37 |
| Dry Root weight (g) | 19 30 | 41 66 | 21 50 | 8 03 | 18 43 |
| Plumbagin content (%) | 19 99 | 33 21 | 36 20 | 0 74 | 24 83 |

The PCV values were higher than the GCV values for all the characters studied Maximum PCV and GCV were recorded for number of branches (72 54 57 50) followed by number of leaves (51 83 35 06) High phenotypic coefficient of variation was recorded by the morphological characters such as plant height (29 93) internodal length (25 86) leaf length (20 82) and the biometric characters such as fresh root weight (42 63) dry root weight (41 66) total biomass (40 68) and number of roots (36 55) Moderate PCV values were exhibited by leaf breadth (18 47) and root girth (19 71) High GCV values were exhibited by the morphological traits *viz* number of leaves (35 06) and plant height (21 52) Moderate GCV values were recorded by leaf length (15 40) leaf breadth (14 0) and internodal length (12 73) Among the biometric traits those exhibiting high genotypic coefficient of variation were total biomass (23 20) and fresh root weight (21 75) Moderate GCV values of 19 30 and 15 83 were exhibited by dry root weight and root number respectively The root girth recorded lowest GCV value of 7 89 Plumbagin content recorded a high PCV of 33 21 and a moderate GCV of 19 99



### 4 2 4 Heritability

The broad sense heritability estimates varied from 16 00 (root girth) to 62 90 (number of branches) as seen in the Table 18 High broad sense heritability was recorded in number of branches per plant (62 90) The characters such as plant height (51 80) number of leaves per plant (47 90) leaf length (54 70) leaf breadth (57 50) total biomass (32 50) and plumbagin content (36 20) exhibited moderate broad sense heritability values while characters such as internodal length (24 20) number of roots per plant (18 80) fresh root weight (26 04) dry root weight (21 50) and root girth (16 00) exhibited low heritability values

### 4 2 5 Genetic advance and Genetic gain

Genetic advance expressed as percentage of mean was maximum for number of branches per plant (93 70) and minimum for root girth (6 69) The number of branches per plant exhibited high heritabihty coupled with high genetic gain Moderate estimates of heritability (30 0 to 60 0 per cent) coupled with high genetic gain (more than 20 0 per cent ) was observed for plant height number of leaves leaf length leaf breadth total biomass fresh root weight and plumbagin content (Table 18)

### 4 2 6 Correlation

Phenotypic and genotypic correlations of plumbagin content with eleven characters *viz* number of branches per plant plant height internodal length number of leaves per plant leaf length leaf breadth total biomass number of roots per plants dry root weight root girth and their inter association were worked out The estimate of correlations both at phenotypic and genotypic levels are given in Table 19

Plumbagin content exhibited significant negative genotypic and phenotypic correlation with the morphological traits such as number of branches ( 0 550 0 293) and plant height ( 0 639 0 355) but a significant positive correlation at both levels with the biometric traits such as number of roots per plant (0 407 0 322) and dry root weight (0 349 0 201) It had significant positive genotypic correlation with root girth (0 517) It had negligible correlation at both genotypic and phenotypic level with total biomass

Among the morphological traits plant height exhibited high positive genotypic (0 930) and phenotypic correlation (0 650) with number of branches per plant The internode length had significant positive genotypic and phenotypic correlation with number of branches per plant (0 529 0 267) and plant height (0 698 0 264)

Number of leaves per plant showed significant genotypic and phenotypic correlation with number of branches per plant (0 806 0 685) plant height (0 780 0 654) and internode length (0 716 0 252)

Leaf length exhibited significant genotypic correlation and phenotypic correlation with plant height (0 414 0 205) and number of leaves per plant (0 458 0 270) It had significant positive genotypic correlation with internode length (0 569) Leaf breadth showed significant negative genotypic as well as phenotypic correlation with number leaves ( 0 536 0 289) and leaf length ( 0 915 0 775) It had significant positive genotypic correlation with plant height (0 430)

The biometric traits also exhibited significant correlation with the morphological traits Number of roots per plant exhibited significant genotypic correlation with plant height (0 367) It had significant correlation at genotypic and phenotypic level with number of leaves (0 766 0 291) leaf length (0 380 0 195) and leaf breadth (0 574 0 259)

Root girth exhibited negative genotypic correlation with following morphological traits *viz* number of branches per plant ( 0 465) plant height ( 0 312) number of leaves per plant ( 0 283) and leaf breadth ( 0 007) However it had significant positive correlation at both genotypic and phenotypic level with leaf length (0 867 0 234) Root girth exhibited significant positive correlation at phenotypic level with leaf breadth (0 323) and number of roots (0 284)

Total biomass had highly significant and positive correlation both at genotypic and phenotypic levels with most of the characters *viz* number of branches per plant (0 637 0 569) plant height (0 730 0 344) internode length (0 278 0 188) number of leaves (0 989 0 620) leaf length (0 717 0 286) and root girth(0 330 248) However t had very insignificant negative genotypic correlation and a significant phenotypic correlation with leaf breadth ( 0 008 0 376) Total biomass had an insignificant

Table 19 Genotypic and phenotypic correlation between plumbagin content and other traits in *Plumbago* accessions

| | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | X9 | X10 | X11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| X1(G)<br>(P) | 1 000 | | | | | | | | | | |
| X2(G)<br>(P) | 0 930**<br>0 650** | 1 000 | | | | | | | | | |
| X3(G)<br>(P) | 0 524**<br>0 267** | 0 698**<br>0 264** | 1 000 | | | | | | | | |
| X4(G)<br>(P) | 0 806**<br>0 683** | 0 780**<br>0 654** | 0 716**<br>0 252* | 1 000 | | | | | | | |
| X5(G)<br>(P) | 0 163<br>0 112 | 0 414**<br>0 205* | 0 569**<br>0 074 | 0 458**<br>0 270** | 1 000 | | | | | | |
| X6(G)<br>(P) | 0 142<br>0 140 | 0 430**<br>0 162 | 0 160<br>0 037 | 0 536**<br>0 289** | 0 915**<br>0 775** | 1 000 | | | | | |
| X7(G)<br>(P) | 0 157<br>0 123 | 0 367<br>0 051 | 0 075<br>0 045 | 0 766**<br>0 291** | 0 380**<br>0 195* | 0 574**<br>0 259** | 1 000 | | | | |
| X8(G)<br>(P) | 0 465**<br>0 096 | 0 312**<br>0 111 | 0 095<br>0 037 | 0 283**<br>0 020 | 0 867**<br>0 234* | 0 007<br>0 323** | 0 075<br>0 284** | 1 000 | | | |
| X9(G)<br>(P) | 0 637**<br>0 569** | 0 730**<br>0 344** | 0 278**<br>0 188* | 0 995**<br>0 620** | 0 717**<br>0 286** | 0 008<br>0 376** | 0 025<br>0 630** | 0 330**<br>0 248* | 1 000 | | |
| X10(G)<br>(P) | 0 176<br>0 263** | 0 360**<br>0 019 | 0 085<br>0 130 | 0 674**<br>0 302** | 0 700**<br>0 163 | 0 003<br>0 309** | 1 086**<br>0 742** | 0 314**<br>0 316** | 0 839**<br>0 780** | 1 000 | |
| X11(G)<br>(P) | 0 550**<br>0 293** | 0 639**<br>0 355** | 0 255*<br>0 233* | 0 235*<br>0 085 | 0 011<br>0 024 | 0 053<br>0 067 | 0 407**<br>0 322** | 0 517**<br>0 170 | 0 004<br>0 096 | 0 340**<br>0 201* | 1 000 |

X1 No of branches X 2 Plant height X3 Internodal length X 4 No of leaves X5 Leaf length X6 leaf breadth X 7 No of roots /plant X8 Root girth X9 Total biomass X10 Dry root weight X11 Plumbagin content G Genotypic correlation P Phenotypic correlation ** Significant at 1%level *Significant at 5% level

genotypic correlation but highly significant phenotypic correlation with number of roots (0 025 0 630)

Dry root weight exhibited maximum significant positive correlation both at genotypic and phenotypic levels with number of roots (1 086 0 742) followed by total biomass (0 839 0 780) number of leaves (0 674 0 302) and root girth (0 314 0 316) plant height (0 360) It had significant positive phenotypic correlation with number of branches per plant (0 263) and leaf breadth (0 309) The dry root weight exhibited significant positive genotypic correlation with plant height (0 359) and leaf length (0 700) but insignificant negative genotypic correlation with leaf breadth ( 0 003)

**4 2 7 Path coefficient analysis**

Genotypic correlations of all the characters with plumbagin content were further partitioned into direct and indirect effects utilising path coefficient analysis The result of path analysis of various characters is presented in the Table 20 The values present on the diagonal are the direct effects and the residual effect is 0 5430

An examination of the path coefficient analysis at genotypic level showed that the highest positive direct effect on plumbagin content was exhibited by number of leaves per plant (1 040) followed by leaf breadth (0 808) and leaf length (0 491) The highest negative direct effect on plumbagin content was exhibited by plant height ( 0 785) followed by number of branches per plant ( 0 738) and internode length ( 0 417) The negative direct contributions of these characters are also indicated by their significant negative genotypic correlation with plumbagin content Among the biometric traits dry root weight exerted high positive direct effect (0 352) on plumbagin content It also exhibited significant positive genotypic correlation The highest negative direct effect on plumbagin content was exhibited by number of roots ( 0 555) followed by root girth ( 0 457) Total biomass exhibited negligible direct effect on plumbagin

The indirect effects of all the ten characters on plumbagin content through other component traits were also examined Number of branches per plant had high negative indirect effect on plumbagin content through plant height ( 0 730) and moderate negative indirect effect through internode length ( 0 219) However its

indirect effect through number of leaves (0 819) was high and positive The number of branches had negligible indirect effect on plumbagin content though all other traits

Plant height exhibited negative indirect influence on plumbagin through number of branches ( 0 686) internode length ( 0 291) and number of roots ( 0 208) It had very high positive indirect effect and a moderate positive indirect effect through number of leaves (0 811) and leaf length (0 208) respectively Similar positive indirect contribution was made by internode length through number of leaves (0 745) and leaf length (0 280) Internode length also exerted negative indirect effect on plumbagin content through number of branches ( 0 387) and plant height ( 0 548) Both plant height and internode length exhibited low to neglige positive indirect effects on plumbagin content through root girth and total biomass respectively Plant height had positive indirect influence through dry weight whereas internode length had negative and very negligible indirect influence through the same on plumbagin content

The genotypic correlation of number leaves with plumbagin content was negative contrary to its very high positive direct effect The high negative indirect effects through number of branches ( 0 595) plant height ( 0 612) and number of roots ( 0 425) had resulted in its negative correlation with plumbagin content However number leaves had moderate but positive indirect influence through leaf length (0 225) and dry root weight (0 237)

The leaf characters such as leaf length and breadth exhibited considerable posit ve direct influence on plumbagm content but insignificant positive genotypic correlation with plumbagin content Leaf length had exerted negative indirect influence through most of the character such as number of branches ( 0 120) plant height ( 0 325) internode length ( 0 238) leaf breadth ( 0 003) number of roots ( 0 211) and root girth ( 0 362) It had exhibited high positive indirect effect through number of leaves alone (0 476) whereas its positive indirect effect through dry root weight (0 246) was moderate Leaf breadth had exhibited negative indirect influence on plumbagin content through plant height ( 0 368) number of leaves ( 0 550) and dry root weight ( 0 269) as opposed to leaf length Even though it had high positive

**Table 20 Direct and indirect effects of various morphological and biometric traits on plumbagin content**

| | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | X9 | X10 | r(G) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| X1 | **0 738** | 0 730 | 0 219 | 0 839 | 0 080 | 0 001 | 0 087 | 0 194 | 0 050 | 0 062 | 0 550** |
| X2 | 0 686 | **0 785** | 0 291 | 0 811 | 0 208 | 0 001 | 0 208 | 0 130 | 0 057 | 0 126 | 0 639** |
| X3 | 0 387 | 0 548 | **0 417** | 0 745 | 0 280 | 0 001 | 0 042 | 0 122 | 0 022 | 0 030 | 0 255* |
| X4 | 0 595 | 0 612 | 0 299 | **1 040** | 0 225 | 0 002 | 0 425 | 0 110 | 0 078 | 0 237 | 0 235* |
| X5 | 0 120 | 0 325 | 0 238 | 0 476 | **0 491** | 0 003 | 0 211 | 0 362 | 0 056 | 0 246 | 0 011 |
| X6 | 0 105 | 0 368 | 0 070 | 0 550 | 0 449 | **0 808** | 0 318 | 0 457 | 0 069 | 0 269 | 0 053 |
| X7 | 0 116 | 0 288 | 0 031 | 0 797 | 0 187 | 0 002 | **0 555** | 0 031 | 0 064 | 0 382 | 0 407** |
| X8 | 0 343 | 0 245 | 0 122 | 0 295 | 0 426 | 0 003 | 0 042 | **0 417** | 0 026 | 0 111 | 0 517** |
| X9 | 0 470 | 0 573 | 0 116 | 1 035 | 0 352 | 0 003 | 0 457 | 0 137 | **0 078** | 0 295 | 0 004 |
| X10 | 0 130 | 0 280 | 0 035 | 0 701 | 0 344 | 0 002 | 0 603 | 0 131 | 0 065 | **0 352** | 0 350** |

Bold figures on the diagonal indicate direct effects residual =0 5430 r(G) Genotypic correlation with plumbagin content X1 No of branches X 2 Plant height X3 Internodal length X 4 No of leaves X5 Leaf length X6 Leaf breadth X 7 No of roots X8 Root girth X9 Total biomass X10 Dry root weight

indirect effect on plumbagin content through number of roots (0 318) and root girth (0 457) these were cancelled by the high negative indirect effects through leaf length ( 0 449) and internode length ( 0 328)

The biometric characters such as number of roots and root girth exhibited significant positive genotypic correlation with plumbagin content in contrast to very high negative direct effect on plumbagin content The very high and positive indirect effects of number of roots through number of leaves (0 797) and dry root weight (0 382) on plumbagin content had resulted in its positive significant genotypic correlation with plumbagin content In contrast to number of roots root girth had exhibited high positive indirect effects through number of branches (0 343) and leaf length (0 426) and a moderate indirect effect through internode length (0 245) which contributed to its significant genotypic correlation with plumbagin content The indirect contribution of root girth through number of leaves was negative ( 0 295)

Among all the traits total biomass had exhibited the maximum indirect effect on plumbagin content through number of leaves (1 035) and leaf length (0 352) However these positive indirect effects were negated by the high negative indirect effects through number of branches ( 0 470) plant height ( 0 573) and leaf breadth ( 0 003) resulting in an insignificant genotypic correlation with plumbagin content The dry root weight also had exerted a very high and positive indirect influence through number of leaves (0 744) and leaf length (0 344) Even though the indirect effects through number of branches ( 0 130) plant height ( 0 280) number of roots ( 0 603) and root girth ( 0 131) were negative the high and positive direct influence of dry root weight on plumbagin content resulted in their significant positive genotypic correlation

**4 2 8 Cluster analysis**

Twelve characters were included for this analysis Based on the relative magnitude of $D^2$ values twenty six *Plumbago* accessions were grouped into seven clusters The $D^2$ values computed for twenty six *Plumbago* accessions are presented in Table 21 The $D^2$ value computed varied from 12 38 to 354 98 showing high divergence among accessions The clustering pattern revealed that cluster VI was the

largest consisting of five accessions which were indigenous to varied ecogeographical regions The cluster V was the smallest with two accessions

**Table 21 Inter and Intra cluster distances of 26 accessions of *Plumbago* species**

| | Cluster I | Cluster II | Cluster III | Cluster IV | Cluster V | Cluster VI | Cluster VII |
|---|---|---|---|---|---|---|---|
| Cluster I | 18 11 | | | | | | |
| Cluster II | 158 71 | 14 75 | | | | | |
| Cluster III | 157 58 | 261 36 | 28 97 | | | | |
| Cluster IV | 56 36 | 100 89 | 118 32 | 12 38 | | | |
| Cluster V | 211 95 | 63 24 | **354 98** | 153 49 | 17 28 | | |
| Cluster VI | 136 39 | 208 45 | 47 95 | 74 08 | 257 31 | 27 86 | |
| ClusterVII | 146 84 | 75 36 | 151 36 | **52 10** | 93 9 | 90 03 | **36 73** |

Average intra d–26 27 No of clusters 7 No of iterations 2

The remaining clusters *viz* cluster I II III and IV had four accessions each and cluster VII had three accessions The inter cluster distance was found to be more than intra cluster distance The values on the diagonal represent intra cluster distances The maximum inter cluster distance was between cluster V and cluster III ($D^2$ 354 98) and the minimum inter cluster was between cluster VII and cluster IV ($D^2$ 52 10) The maximum intra cluster distance was observed in cluster VII indicating h gh variability existing among the accessions of this cluster

The clustering pattern of the twenty six accessions of *Plumbago* species is given in Table 22 As seen in the table instead of forming a separate cluster the species *Plumbago zeylanica* got grouped in cluster II along with the accessions of *Plumbago rosea* It was noted that genotype habituating the same location were grouped into different clusters

Of the seven accessions of *Plumbago rosea* belonging to Thrissur district three accessions *viz* IC 566499 IC 566508 and IC 566523 were grouped into cluster VI two accessions IC 566507 and IC 566513 got grouped into cluster IV and the remaining two accessions IC 566512 and IC 566514 were grouped into different clusters such as cluster VII and cluster III respectively In general accessions

belongıng to dıfferent regıons were grouped ın the same cluster and accessıons belongıng to the same regıon were grouped ın dıfferent clusters

**Table 22 Clusterıng pattern of 26 *Plumbago* accessıons**

| Cluster | Number of genotypes ıncluded ın each cluster | Accessıon numbers along wıth theır dıstrıct of collectıon |
|---|---|---|
| I | 4 | IC 566501(Wayanad) |
| | | IC 566504(Wayanad) |
| | | IC 566519(Palakkad) |
| | | IC 566522(Idukkı) |
| II | 4 | IC 566500(Wayanad) |
| | | IC 566515(Palakkad) |
| | | IC 566518(Malappuram) |
| | | P 26(Thrıssur) |
| III | 4 | IC 566502(Malappuram) |
| | | IC 566505(Wayanad) |
| | | IC 566514(Thrıssur) |
| | | IC 566520(Idukkı) |
| IV | 4 | IC 566507(Thrıssur) |
| | | IC 566510(Malappuram) |
| | | IC 566513(Thrıssur) |
| | | IC 566516(Palakkad) |
| V | 2 | IC 566503(Kozhıkode) |
| | | IC 566521(Idukkı) |
| VI | 5 | IC 566499(Thrıssur) |
| | | IC 566508(Thrıssur) |
| | | IC 566511(Malappuram) |
| | | IC 566517(Palakkad) |
| | | IC 566523(Thrıssur) |
| VII | 3 | IC 566506(Wayanad) |
| | | IC 566509(Malappuram) |
| | | IC 566512(Thrıssur) |

#### 4 2 8 1 Cluster means

The cluster mean values for the twelve characters are presented ın Table 23 From the table ıt ıs seen that cluster II recorded hıghest mean values of 31 83 and 106 11 for number of branches and plant heıght and the cluster VI recorded lowest mean values of 14 27 and 79 82 for these characters For ınternodal length cluster I recorded hıghest mean value (8 42) followed by cluster IV (7 42)

**Table 23 Cluster mean values of eleven characters of 26 *Plumbago* accessions**

| Cluster | No of branches | Height (cm) | Internodal length(cm) | No of leaves | Leaf length (cm) | Leaf breadth (cm) | No of roots | Root girth (cm) | Total biomass (g) | Fresh root weight (g) | Dry Root wt (g) | Plumbagin content (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cluster I | 24 25 | 103 06 | 8 42 | 339 42 | 8 39 | 4 76 | 19 92 | 2 38 | 410 00 | 152 50 | 51 83 | 2 83 |
| Cluster II | 31 83 | 106 11 | 6 47 | 289 58 | 6 95 | 4 36 | 15 50 | 2 29 | 308 33 | 100 00 | 36 33 | 2 34 |
| Cluster III | 19 42 | 93 70 | 6 58 | 264 50 | 7 05 | 4 02 | 20 42 | 2 07 | 311 67 | 134 17 | 43 08 | 3 25 |
| Cluster IV | 19 33 | 85 05 | 7 42 | 227 25 | 7 89 | 4 27 | 20 25 | 2 65 | 325 00 | 150 84 | 47 50 | 3 36 |
| Cluster V | 23 00 | 97 95 | 6 34 | 259 67 | 8 14 | 5 05 | 25 00 | 2 68 | 430 00 | 191 67 | 59 00 | 2 88 |
| Cluster VI | 14 27 | 79 82 | 6 08 | 213 60 | 7 32 | 4 32 | 19 60 | 2 33 | 302 00 | 144 00 | 42 93 | 3 27 |
| ClusterVII | 14 89 | 98 17 | 6 33 | 172 00 | 8 04 | 4 62 | 13 22 | 2 49 | 235 56 | 93 33 | 28 33 | 2 85 |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The cluster VI again recorded lowest mean value (6 08) for this character also Cluster I recorded highest mean value of 339 42 for number of leaves per plant and leaf length (8 59) Cluster V recorded the highest value of 5 05 and cluster III recorded lowest value (4 02) for leaf breadth

Among the six biometric traits studied cluster V recorded highest mean values for five characters It recorded highest mean value for number of roots (25 00) root girth (2 68) total biomass (430 00) fresh root weight (191 67) and dry root weight (59 00) Cluster VII recorded the lowest value mean values for four biometric traits viz number of roots (13 22) total biomass (302 00) and dry root weight (28 33) Cluster III recorded lowest value for root girth (2 07) For plumbagin content the cluster IV recorded the maximum value (3 36) and cluster II recorded minimum value (2 34)

**4 2 8 2 Ranking of genotypes in clusters**

The accessions in each cluster were evaluated on the basis of two yield characters *viz* dry root weight and plumbagin content (Table 24) These were sorted in the descending order for each of these characters and assigned ranks in that order The mean rank obtained by each accession was calculated which helped to identify the overall best yielder in each cluster In cluster I the accessions IC 566519 and IC 566522 ranked first In cluster II the accessions IC 566515 and IC 566518 ranked first The accession IC 566514 ranked first in the cluster III In cluster IV the accession IC 566516 ranked first In cluster V both the accessions IC 566503 and IC 566521 had the same rank In cluster VI the accession IC 566508 ranked first based on the overall mean for two characters In cluster VII the accession IC 566506 ranked first based on the overall mean for the two yield attributes Three accessions *viz* IC 566503 IC 566508 and IC 566514 from the clusters V VI and III respectively were selected based on their superior ranking These three clusters also had maximum inter cluster distance showing maximum divergence The three selected accessions were carried over to experiment three for inducing variability Besides the lone accession of *P zeylanica* belonging to cluster II was also selected for induction of variability

**Table 24 Ranking of *Plumbago* accessions in each cluster based on yield attributes**

| Cluster I | Dry Root wt (g) | Plumbagin content (%) | Dry root wt rank | Plumbagin content rank | Overall mean rank |
|---|---|---|---|---|---|
| IC 566501 | 37 33 | 2 14 | 4 | 4 | 4 |
| IC 566504 | 43 67 | 2 75 | 3 | 3 | 3 |
| IC 566519 | 57 67 | 3 43 | 2 | 1 | 1 5 |
| IC 566522 | 68 67 | 3 02 | 1 | 2 | 1 5 |
| Cluster II | | | | | |
| IC 566500 | 17 33 | 1 99 | 4 | 3 | 3 5 |
| IC 566515 | 46 33 | 2 81 | 1 | 2 | 1 5 |
| IC 566518 | 43 67 | 3 68 | 2 | 1 | 1 5 |
| P 26 *P zeylanica* | 38 00 | 0 87 | 3 | 4 | 3 5 |
| Cluster III | | | | | |
| IC 566502 | 46 00 | 2 62 | 2 | 4 | 3 0 |
| IC 566505 | 45 00 | 2 70 | 3 | 3 | 3 0 |
| IC 566514 | 46 00 | 4 11 | 1 | 1 | 1 0 |
| IC 566520 | 35 33 | 3 50 | 4 | 2 | 3 0 |
| Cluster IV | | | | | |
| IC 566507 | 51 00 | 2 75 | 1 | 4 | 2 5 |
| IC 566510 | 45 67 | 3 46 | 2 | 2 | 2 0 |
| IC 566513 | 45 00 | 3 37 | 3 | 3 | 3 0 |
| IC 566516 | 48 33 | 3 87 | 4 | 1 | 2 5 |
| Cluster V | | | | | |
| IC 566503 | 60 67 | 2 79 | 1 | 2 | 1 5 |
| IC 566521 | 57 33 | 2 97 | 2 | 1 | 1 5 |
| Cluster VI | | | | | |
| IC 566499 | 42 00 | 2 65 | 3 | 4 | 3 5 |
| IC 566508 | 59 00 | 4 62 | 1 | 1 | 1 0 |
| IC 566511 | 23 33 | 3 79 | 5 | 2 | 3 5 |
| IC 566517 | 55 00 | 2 39 | 2 | 5 | 3 5 |
| IC 566523 | 35 33 | 2 89 | 4 | 3 | 3 5 |
| Cluster VII | | | | | |
| IC 566506 | 37 67 | 2 88 | 1 | 3 | 2 0 |
| IC 566509 | 22 00 | 2 41 | 3 | 2 | 2 5 |
| IC 566512 | 25 33 | 3 27 | 2 | 1 | 1 5 |

### 4 2 9 Floral morphology

#### 4 2 9 1 Inflorescence characters

Inflorescence is a spike in both species of *Plumbago* studied The inflorescence characteristics in the two species (Table 25) clearly showed that these differed with respect to flowering time length of inflorescence and number of flowers per inflorescence In *Plumbago rosea* flowering is seasonal from October to February with peak period being December January The flowering is continuous in *Plumbago zeylanica* usually commencing from October with no clear cut peak

The mean values of inflorescence characters are presented in Table 26 The *P zeylanica* genotype had the maximum number of floral branches (44 7) Among the *P rosea* genotypes IC 566522 recorded maximum number of floral branches (29) and IC 566511 the lowest (2) The length of inflorescence in *P zeylanica* was 8 7 cm It ranged between 8 5 and 37 9 cm among *P rosea* genotypes IC 566510 and IC 566513 respectively The *P rosea* genotype recorded a mean number of 36 flowers per inflorescence with IC 566513 recording maximum of 73 and IC 566499 recording a minimum of 11 3 flowers per inflorescence The *P zeylanica* genotype recorded a mean of 19 3 flowers per inflorescence

In *Plumbago rosea* the number of days taken for the opening of first flower bud after visual emergence ranged from 8 to 13 In *P zeylanica* nine days were required for opening of first flower bud in an inflorescence after its visual emergence The number of days for the completion of anthesis in an inflorescence ranged between 15 0 and 45 0 in the *P rosea* genotypes The *P zeylanica* genotype took 19 6 days to complete anthesis in an inflorescence

#### Floral characters

The floral morphology of the two species of *Plumbago* is presented in Table 25 There exists a marked difference among the two species of *Plumbago* for various characters The individual flowers in both the species are complete bisexual and subtended by a bract and two bracteoles The bracts are larger than bracteoles in *P zeylanica* and of equal size in *P rosea* The bracts and bracteoles are covered with sticky glandular hairs

**Table 25 Inflorescence and floral characters of *Plumbago rosea and Plumbago zeylanica***

| Character | P rosea | P zeylanica |
|---|---|---|
| Flowering | Seasonal | Continuous |
| Inflorescence type | Spike | Spike |
| Position of inflorescence | Terminal | Terminal/axillary |
| Length of inflorescence (cm) | 22 9 | 11 8 |
| Number of floral branches | 11 8 | 44 7 |
| Number of flowers /inflorescence | 36 | 19 6 |
| Days for opening of 1s flower in an inflorescence | 11 1 | 9 3 |
| Days for completion of flowering in an inflorescence | 26 2 | 19 6 |
| Sequence of flower opening | Acropetal | Acropetal |
| Flower colour | Scarlet red | White |
| Flower type | Sessile | Sessile |
| Colour of bract | Reddish green | Green |
| Colour of bracteole | Reddish green | Green |
| Number of bracteoles/flower | 2 | 2 |
| Colour of calyx | Red | Green |
| Number of sepals | 5 | 5 |
| Nature of sepals | Gamosepalous sticky and hairy | Gamosepalous sticky ai d hairy |
| Length of calyx (cm) | 0 8 | 1 0 |
| Colour of corolla | Scarlet red | White |
| Aestivat on | Valvate | Valvate |
| Length of corolla tube (cm) | 2 8 | 2 3 |
| Length of stamen | 2 9 | 2 2 |
| Attachment of anther | Dorsifixed | Dorsifixed |
| Structure of stamen | Filantherous | Filantherous |
| Colour of anther | Red | Purple |
| Type of stamen | Ep petalous | Epipetalous |
| Ovary | Superior monocarpellary unilocular | Superior monocarpellary unilocular |
| Placentation | Basal | Basal |
| Type of style | Fimbricate | Terete |
| Length of style (cm) | 2 0 | 2 2 |
| Type of stigma | Pentaf d | Pentafid |
| Fruit type | No fruit set | Dry dehiscent fruit enclosed in pers stent calyx |
| Seed type | | Brown oval seeds with one end pointed |

**Table 26 Mean Performance of 26 accessions of *Plumbago* species for inflorescence and floral characters**

| Accession No | No of floral branches | Length of Inflorescence (cm) | No of buds inflorescence | Days for opening of first flower in an inflorescence | Days for completion of anthesis per inflorescence | Legth of corolla tube(cm) | Length of calyx (cm) | Length of stamen (cm) | Length of style (cm) |
|---|---|---|---|---|---|---|---|---|---|
| IC 566499 | 3 3 | 18 5 | 11 3 | 8 0 | 15 0 | 2 6 | 0 8 | 2 7 | 1 9 |
| IC 566500 | 7 3 | 37 0 | 59 7 | 13 0 | 35 0 | 2 7 | 0 8 | 2 9 | 2 0 |
| IC 566501 | 4 3 | 23 7 | 38 0 | 12 3 | 30 0 | 2 8 | 0 8 | 2 9 | 2 2 |
| IC 566502 | 4 3 | 15 3 | 22 3 | 11 7 | 30 0 | 2 7 | 0 8 | 2 9 | 1 7 |
| IC 566503 | 7 0 | 18 1 | 15 3 | 8 0 | 15 0 | 2 8 | 0 8 | 3 0 | 2 0 |
| IC 566504 | 18 7 | 25 8 | 49 7 | 13 0 | 26 7 | 2 9 | 0 8 | 3 0 | 2 1 |
| IC 566505 | 18 0 | 32 5 | 58 3 | 13 0 | 30 0 | 3 0 | 0 9 | 3 0 | 1 8 |
| IC 566506 | 12 3 | 32 4 | 66 7 | 13 0 | 40 0 | 3 0 | 0 8 | 3 1 | 1 9 |
| IC 566507 | 12 3 | 22 7 | 31 7 | 12 0 | 25 0 | 3 0 | 0 8 | 3 0 | 1 7 |
| IC 566508 | 16 0 | 27 2 | 52 7 | 12 7 | 30 0 | 2 5 | 0 8 | 2 8 | 1 8 |
| IC 566509 | 5 3 | 15 0 | 15 0 | 8 7 | 18 3 | 2 6 | 0 7 | 2 8 | 1 8 |
| IC 566510 | 5 7 | 8 5 | 13 0 | 8 0 | 15 0 | 2 8 | 0 7 | 2 9 | 1 9 |
| IC 566511 | 2 0 | 10 2 | 12 7 | 8 0 | 15 0 | 2 5 | 0 8 | 2 7 | 2 0 |
| IC 566512 | 18 0 | 18 8 | 17 0 | 9 0 | 16 7 | 2 8 | 0 9 | 2 9 | 1 9 |
| IC 566513 | 10 3 | 37 9 | 73 0 | 13 0 | 45 0 | 2 6 | 0 7 | 2 8 | 1 8 |
| IC 566514 | 6 7 | 12 2 | 16 7 | 12 0 | 30 0 | 3 0 | 0 8 | 3 1 | 2 2 |
| IC 566515 | 17 7 | 15 0 | 41 0 | 12 0 | 33 3 | 2 9 | 0 7 | 3 0 | 2 0 |
| IC 566516 | 10 7 | 29 0 | 15 7 | 10 0 | 15 0 | 2 9 | 0 7 | 3 1 | 2 0 |
| IC 566517 | 10 3 | 29 5 | 31 0 | 9 7 | 25 0 | 3 1 | 0 7 | 2 9 | 2 1 |
| IC 566518 | 18 0 | 22 3 | 43 0 | 12 7 | 31 7 | 2 8 | 0 7 | 2 9 | 1 9 |
| IC 566519 | 11 0 | 11 0 | 16 0 | 8 7 | 15 0 | 3 0 | 0 8 | 3 1 | 2 0 |
| IC 566520 | 17 0 | 27 0 | 51 7 | 12 7 | 30 0 | 2 9 | 0 8 | 2 9 | 1 9 |
| IC 566521 | 12 0 | 29 3 | 63 3 | 12 7 | 40 0 | 2 7 | 0 7 | 2 9 | 1 8 |
| IC 566522 | 29 0 | 30 0 | 43 7 | 12 3 | 30 0 | 2 9 | 0 8 | 3 0 | 2 1 |
| IC 566523 | 17 3 | 22 8 | 40 7 | 12 3 | 20 0 | 2 5 | 0 9 | 2 8 | 1 7 |
| Mean( *P rosea)* | 11 8 | 22 9 | 36 0 | 11 1 | 26 2 | 2 8 | 0 8 | 2 9 | 1 9 |
| Mean(Acc no 26 *P zeylanica)* | 44 7 | 8 7 | 19 3 | 9 3 | 19 6 | 2 3 | 1 0 | 2 2 | 2 2 |
| Mean | 13 1 | 22 3 | 35 3 | 11 1 | 25 7 | 2 8 | 0 8 | 2 9 | 1 9 |
| SE | ±4 73 | ±4 1 | ± 0 5 | ±0 4 | ±3 34 | ±0 08 | ±0 03 | ±0 06 | ±0 04 |
| CD 0 05 | 13 4 | 11 6 | 14 86 | 1 14 | 9 49 | 0 24 | 0 09 | 0 18 | 0 13 |
| CV(%) | 62 6 | 31 8 | 51 5 | 6 3 | 22 5 | 5 3 | 7 | 3 7 | 3 8 |

The mean values for the size of corolla calyx stamen and style are presented in Table 26 The corolla in both species forms a slender long tube which is larger than calyx At the end of corolla tube the corolla lobes are free rotate roundish and toothed The androecium of both the species consists of five stamens that are hypogynous The filaments are as long as corolla and connate at base into a lobed nectar secreting disc

The anthers are exerted beyond the throat of corolla tube and they dehisce longitudinally in both the species The gynoecium of both species is monocarpellary and superior In *P rosea* the style is short compared to *P zeylanica* The stigma of *P rosea* is deeply seated inside the corolla tube and the anthers and stigma are held at two levels hindering self pollination However in case of *P zeylanica* the style is held at a same or higher level than that of anther

**4 2 10 Floral Biology**

**4 2 10 1 Anthesis**

A preliminary study revealed that there was no flower opening between 4p m and 4 a m Hence hourly observations on flower opening were taken from 4a m to 4p m The percentage of flower opening in the two species of *Plumbago* at periodic intervals are presented in Table 27 As seen from the table in *P rosea* anthesis started before 7 a m and reached a peak at 8 a m and declined after 9 a m In *P zeylanica* the anthesis started before 5 a m and it reached a peak between 6 a m and 7 a m The anthesis was found to decline after 8 a m

**4 2 10 2 Time of anther dehiscence and stigma receptivity**

Fully mature flower buds in the two species of *Plumbago* were observed for anther dehiscence and stigma receptivity at periodic intervals It was found that only in *Plumbago zeylanica* the anther dehiscence coincides with stigma receptivity In *Plumbago rosea* the anther dehisces three hours after opening of the flower whereas the stigma is receptive one hour after opening of the flower The stigma is receptive only for half an hour in both the species (Table 28) It was also observed that the pollen grains were easily carried off by wind soon after anthesis in the two species

**Table 27 Time of anthesis in *Plumbago* species**

| Time | Number of flower buds observed* | | Per cent flowers opened | |
|---|---|---|---|---|
| | *P rosea* | *P zeylanica* | *P rosea* | *P zeylanica* |
| 4 a m to 5 a m | 96 | 85 | 0 0 | 3 2 |
| 5 a m to 6 a m | | | 0 0 | 10 0 |
| 6 a m to 7a m | | | 3 1 | 50 0 |
| 7 a m to 8 a m | | | 48 8 | 29 0 |
| 8 a m to 9 a m | | | 31 7 | 5 2 |
| 9 a m to 10 a m | | | 16 4 | 2 6 |
| 10 a m to 11 a m | | | 0 0 | 0 0 |
| 11a m to 12 noon | | | 0 0 | 0 0 |
| 12 noon to1 p m | | | 0 0 | 0 0 |
| 1 p m to 2 p m | | | 0 0 | 0 0 |
| 2 p m to 3 p m | | | 0 0 | 0 0 |
| 3 p m to 4 p m | | | 0 0 | 0 0 |

* Number of inflorescence 30

**Table 28 Time of anther dehiscence and stigma receptivity in *Plumbago* species**

| Species | Time of anther dehiscence | Starting time of stigma receptivity | Duration of stigma receptivity (minutes) |
|---|---|---|---|
| *P rosea* | 3 hours after flower opening | 1 hour after flower opening | 30 |
| *P zeylanica* | Coincides with flower opening | Coincides with flower opening | 30 |

**4 2 10 3 Pollen morphology fertility and viability**

The morphology of pollen collected from fully mature flower buds were studied in the two species of *Plumbago* using acetocarmine staining technique which revealed the following results (Table 29) The pollen grains were spherical and tricolpate in both the species The per cent pollen fertility was higher in *P zeylanica*

(94) than in *P rosea* (89 ) The viability of the pollen of the two species of *Plumbago* was assessed under *in vitro* and *in vivo* conditions

**Table 29 Pollen morphology, fertility and viability in *Plumbago* species**

| Species | Pollen fertility (%) | Pollen shape | Pollen type | Germination in sucrose medium | | |
|---|---|---|---|---|---|---|
| | | | | 20% | 40% | 60% |
| *P rosea* | 89 0 | Spherical | Tricolpate | 0 0 | 0 0 | 0 0 |
| *P zeylanica* | 94 0 | Spherical | Tricolpate | 0 0 | 0 0 | 0 0 |

**i) *In vitro* studies**

Pollen germination was tried in the two species of *Plumbago* using three different concentrations of sucrose However no germination occurred in the three concentrations of sucrose under *in vitro* conditions

**ii) *In vivo* pollination studies**

Artificial pollination was attempted within as well as between the two species of *Plumbago* The examination of pistils excised after 24 and 48 hours of pollination under a compound microscope revealed that there was high adhesion of pollens of both the species on the stigma of *P zeylanica* However very little pollen adhered to the stigma of *P rosea*

The data on fruit set m artificially cross pollinated flowers after 24 48 72 and 96 hours of cross pollination are furnished in Table 30 The results showed that in crosses among *P rosea* the calyx was retained only up to 72 hours Only in *P zeylanica* the flowers were retained up to 96 hours Even though there was 8 per cent fruit set in the *P zeylanica* when pollinated with pollen from *P rosea* the seeds were not fully developed

Percentage of fruit set under natural conditions of selfing and open pollination was studied in the two species of *Plumbago* Results are presented in Table 31 Among the two species only *P zeylanica* exhibited fruit set under selfing and open pollinated conditions

Table 30 **Artificial cross pollination in *Plumbago* species**

| Crosses | Number of flowers crossed | Percent of flowers retained after pollination | | | | Fruit set (%) |
|---|---|---|---|---|---|---|
| | | 24 hours | 48 hours | 72 hours | 96 hours | |
| *P rosea* x *P rosea* | 96 | 48 | 36 58 | 14 6 | 0 | 0 |
| *P rosea* x *P zeylanica* | 50 | 68 | 18 75 | 12 5 | 0 | 0 |
| *P zeylanica* x *P rosea* | 25 | 92 | 8 | 8 | 8 | 8 |

Table 31 **Fruit set under natural pollination in *Plumbago* species**

| Species | Fruit set (%) | |
|---|---|---|
| | Selfing | Open pollination |
| *P rosea* | 0 | 0 |
| *P zeylanica* | 40 | 70 |

## 4 3 Induction of variability through *in vitro* regeneration and mutagenesis of *Plumbago* Species

After assessing the variability in accessions of *Plumbago* species the investigation proceeded towards inducing variability in three selected accessions of *Plumbago rosea* and one accession of *Plumbago zeylanica* The experiment was conducted in two phases The first phase consisted of standardization of *in vitro* regeneration and the second phase *in vitro* mutagenesis The detailed results of the two phases are presented under the heads

i) Standardisation of *in vitro* regeneration in *Plumbago* species

ii) *In vitro* mutagenesis

### 4 3 1 Standardisation of *in vitro* regeneration in *Plumbago* species

#### 4 3 1 1 Surface sterilization

All plant materials used for culture are treated with an appropriate sterilisation agent to inactivate the microbes present on their surface The effects of the surface

sterilization treatments on culture contamination in *Plumbago* are presented in Table 32 There was difference between response of leaf disc and the nodal as well as stem segments to surface sterilization treatment The leaf discs were less susceptible to contamination There was high bacterial contamination in the nodal and stem segments The surface sterilisation was effective only against fungal contamination in the nodal as well as stem segments There was no bacterial contamination in leaf discs In leaf discs the most effective sterilisation was achieved by the combinat on treatment of 70 per cent alcohol for two minutes followed by soaking in 0 2 per cent $HgCl_2$ for two minutes where the percentage of live cultures was 90 and no cultures were contaminated In nodal and stem segments the same combmation of ethyl alcohol and $HgCl_2$ gave good results However there was contamination in 20 per cent cultures

Increasing the time of soaking or the concentration of the sterilant adversely affected the survival of the explants The explants did not survive in higher concentration of the sterilant at a longer period of sterilisation Soaking the explant in any chemical for more than five minutes was not desirable It resulted in the browning and death of the explants Even though higher concentration of the chemical sterilants fully controlled the contamination it affected the survival of the explants as well None of the explants survived m the treatment 0 2 percent $HgCl_2$ beyond 3 minutes and beyond six mmutes in 0 1 per cent $HgCl_2$ There was no survival of explants in 100 per cent or 90 per cent ethyl alcohol for 2 minutes

### 4 3 1 2 Standardisation of explants with medium supplements for culture initiation

The effect of basal MS medium with different supplements on the culture establishment of the three explants of *P rosea* and *P zeylanica* is presented in Table 33 The results showed that only nodal segments responded in *P zeylanica* The leaves and nodal segments of *P rosea* responded in the MS medium There was a difference in the response of leaf in dark and light conditions in case of *P rosea* The leaf discs of *P rosea* showed direct generation of shoot in 16 hours photoperiod conditions whereas in complete darkness it showed callusing in T15 containing TDZ Both the species of *Plumbago* did not show any response in all the treatments from T1

**Table 32 Effect of surface sterilants on culture establishment of different explants of *Plumbago* species**

| Sterilant | Concentration (%) | Duration (minutes) | Contamination (%) | Uncontaminated cultures (leaf disc) | | Contamination (%) | Uncontaminated cultures (Nodal & stem segment) | |
|---|---|---|---|---|---|---|---|---|
| | | | Leaf disc | Dead (%) | Live (%) | Nodal & stem segment | Dead (%) | Live (%) |
| $HgCl_2$ | 0 1 | 2 | 10 | 20 | 70 | 40 | 10 | 50 |
| | 0 1 | 4 | Nil | 40 | 60 | 30 | 10 | 60 |
| | 0 1 | 6 | Nil | 50 | 50 | 10 | 30 | 60 |
| | 0 1 | 8 | Nil | 100 | Nil | Nil | 100 | Nil |
| | 0 1 | 10 | Nil | 100 | Nil | Nil | 100 | Nil |
| $HgCl_2$ | 0 2 | 1 | 10 | 30 | 60 | 20 | 20 | 60 |
| | 0 2 | 2 | Nil | 20 | 80 | 20 | 10 | 70 |
| | 0 2 | 3 | Nil | 50 | 50 | Nil | 50 | 50 |
| | 0 2 | 4 | Nil | 100 | Nil | Nil | 100 | Nil |
| | 0 2 | 5 | Nil | 100 | Nil | Nil | 100 | Nil |
| Ethyl Alcohol | 100 | 2 | Nil | 100 | Nil | Nil | 100 | Nil |
| | 90 | 2 | Nil | 100 | Nil | Nil | 100 | Nil |
| | 70 | 2 | 40 | 30 | 30 | 60 | 20 | 20 |
| Ethyl Alcohol & $HgCl_2$ | 70 0 1 | 2 4 | 10 | 30 | 60 | 20 | 20 | 60 |
| | 70 0 1 | 2 6 | Nil | 50 | 50 | Nil | 40 | 60 |
| | 70 0 1 | 2 8 | Nil | 100 | Nil | Nil | 50 | 50 |
| | 70 0 2 | 2 1 | 10 | 10 | 80 | 30 | 10 | 60 |
| | 70 0 2 | 2 2 | Nil | 10 | 90 | 20 | 10 | 70 |
| | 70 0 2 | 2 3 | Nil | 40 | 60 | Nil | 40 | 60 |

Table 33 Standardisat on of explants w th d fferent comb nat ons of gro vth regulators for culture establ shment in *Plu bago* spec es

| T eatmen | Med um | Exp an | Cultures respond ng (° )* | | No of days fo cul ure n a on | | Type of response n 16 hour pho ope od | | Type of esponse n comple e darkness | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *P rosea* | *P zeylan ca* | *P osea* | *P ey a ca* | *P osea* | *P zey an ca* | *P osea* | *P zeylan ca* |
| T1 | MS+ BA 1 0 mg +NAA 1 0 mg | Nodal segment | N l | N l | | | N l | N l | N l | N l |
| | | Stem segment | N l | N | | | N l | N | N | N l |
| | | Leaf d sc | N l | N l | | | N l | N l | N l | N l |
| T2 | MS+ BA 2 0 mg +NAA 0 5 mg l | Noda segment | N l | N l | | | N l | N | N | N l |
| | | Stem segment | N l | N l | | | N | N l | N l | N l |
| | | Leaf d sc | N | N l | | | N l | N l | N | N |
| T3 | MS+ BA 2 5 mg l +NAA 1 5 mg l | Nodal segment | 70 | N l | 10 | | Mult p e shoo | N l | N l | N l |
| | | S em segmen | N l | N l | | | N l | N l | N | N |
| | | Leaf d sc | N l | N | | | N l | N l | N l | N l |
| T4 | MS+ 2 4 D 1 0 mg l +K n 0 5 mg l | Nodal segment | N l | N l | | | N l | N | N | N l |
| | | Stem segment | N l | N l | | | N | N l | N | N |
| | | Leaf d sc | N | N l | | | N l | N | N l | N l |
| T5 | MS+ 2 4 D 2 0 mg l +K n 1 0mg l | Noda segment | N l | N l | | | N | N l | N | N l |
| | | S em segment | N l | N l | | | N l | N l | N l | N l |
| | | Leaf d sc | N l | N | | | N l | N l | N | N |
| T6 | MS+ 2 4 D 3 0 mg +K n 2 0 mg l | Nodal segmen | N l | N l | | | N | N l | N l | N |
| | | S em segment | N l | N l | | | N l | N | N l | N l |
| | | Leaf d sc | N l | N l | | | N l | N l | N | N |
| T7 | MS+ BA 1 0 mg l + Ads 25 mg l | Nodal segment | 80 | 80 | 7 | 5 | Mu ple shoot | Mu t p e shoot | N l | N l |
| | | S em segment | N l | N l | | | N | N l | N | N |
| | | Leaf d sc | N | N | | | N l | N | N l | N l |

| T ea ment | Med um | Exp ant | Cu tures respond ng (%)* | | No of days fo cul u e n t at on | | Type of response n 16 hour photoper od | | Type of response n complete darkness | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *P osea* | *P zey an ca* | *P osea* | *P zeylan ca* | *P osea* | *P zeylan ca* | *P o ea* | *P zeylan ca* |
| T8 | MS+ BA 1 0 mg l + Ads 50 mg l | Nodal segment | 100 | 100 | 7 | 5 | Mul ple shoot | Mu t p e shoot | N l | N l |
| | | S em segmen | N l | N | | | N | | N l | N l |
| | | Leaf d sc | N l | N l | | | N l | | N l | N l |
| T9 | MS+TDZ 0 01 mg | Nodal segment | N l | N l | | | N l | | N l | N l |
| | | Stem segmen | N l | N l | | | N l | | N l | N l |
| | | Leaf d sc | N l | N l | | | N l | | N | N l |
| T10 | MS+TDZ 0 03 mg l | Nodal segment | N l | N | | | N l | | N l | N l |
| | | S em segment | N l | N l | | | N l | | N l | N |
| | | Leaf d sc | N l | N l | | | N l | | N l | N l |
| T11 | MS+TDZ 0 05 mg | Nodal segment | N | N l | | | N | | N l | N |
| | | Stem segmen | N l | N l | | | N l | | N | N l |
| | | Leaf d sc | N l | N l | | | N l | | N l | N l |
| T12 | MS+TDZ 0 07 mg l | Nodal segment | N | N l | | | N | | N l | N |
| | | Stem segment | N l | N | | | N l | | N l | N l |
| | | Leaf d sc | N l | N l | | | N l | | N l | N |
| T13 | MS+TDZ 0 1mg | Noda segmen | N l | N l | | | N l | | N l | N l |
| | | Stem segment | N l | N l | | | N l | | N | N l |
| | | Leaf d sc | N | N l | | | N l | | N l | N |
| T14 | MS+TDZ 0 5 mg l | Noda segn ent | N l | N l | | | N l | | N l | N l |
| | | S em segment | N l | N | | | N l | N | N | N l |
| | | Leaf d sc | N l | N l | | | N l | N l | N l | N l |
| T15 | MS+TDZ 1 0 mg l | Noda segmen | 60 | 50 | 14 | 10 | Mu t ple shoot | Mult ple shoot | N l | N l |
| | | S em segment | N l | N | | | N | N l | N l | N l |
| | | Leaf d sc | N l | 100 | 21 | N l | Mul p e shoot | N | Ca lus ng | N l |

* No of cul ures 10

to T6 havıng auxın cytokının combınatıons except T3 In T3 contaınıng BA (2 5 mg l ¹) and NAA (1 5 mg l ¹) only the nodal segments of *P rosea* responded wıth 70 per cent of the cultures showıng dırect shoot ınductıon ın 10 days The nodal segments of both *P rosea* and *P zeylanıca* showed maxımum response ın the T7 (80 per cent) and T8 (100 per cent) contaınıng adenıne sulphate a cytokının synergıst It took only 5 days for culture ınıtıatıon ın nodal segments *P zeylanıca* and 7 days ın *P rosea* The treatment T8 was consıdered best for dırect regeneratıon ın the nodal segments of both *P rosea* and *P zeylanıca* Hence thıs treatment was carrıed forward for dırect regeneratıon Nodal segment was ıdentıfied as the explant for dırect regeneratıon Leaf dısc was consıdered as best explant for callus ınductıon The treatment T15 was consıdered best for callus medıated organogenesıs ın *P ıosea* and hence was carrıed over for further studıes

**4 3 2 Dırect regeneratıon**

Detaıled studıes were conducted to standardıse a protocol for *ın vıtıo* regeneratıon through dırect organogenesıs ın *Plumbago* specıes The treatment T8 consıstıng of BA (1 0 mg l ¹) and Ads (50 0 mg l ¹) was ıdentıfied as the best medıum for culture ınıtıatıon Thıs treatment was repeated wıth the nodal segments of *P rosea* and *P zeylanıca* whıch showed shoot ınıtıatıon wıthın one week of ınoculatıon The cultures were observed for contamınatıon In both the specıes there was 70 percent survıval The survıvıng cultures were subcultured to multıplıcatıon medıum after two to three weeks growth ın culture ınıtıatıon medıum The cytokının BA was used for shoot prolıferatıon and sl oot elongatıon and the auxın IBA for root development studıes The results of the studıes are as follows

**4 3 2 1 Effect of medıum supplements on shoot multıplıcatıon**

The effects of varıous levels of BA on shoot multıplıcatıon are presented ın Table 34 There was no sıgnıficant effect on shoot multıplıcatıon ın *P zeylanıca* cultures for all the treatment combınatıons Hence ın thıs table shoot multıplıcatıon ın nodal cultures of *P ıosea* ıs presented There was no shoot prolıferatıon ın the treatments T20 and T21 used for culture multıplıcatıon Only one axıllary shoot sprouted ın these medıa combınatıons In T20 consıstıng Ads (50 mg l ¹) about 50 per cent cultures produced at least two shoots In the treatment T17 maxımum cultures

(90 per cent) showed proliferation In all the treatments it took 45 days for shoot proliferation There were significant differences among the treatments for the number of shoots produced per explant The treatments T17 and T18 were at par producing maximum mean number of multiple shoots of 3 0 and 2 8 respectively There were no significant differences among the treatments for the average length of shoot produced per explant The mean length of the shoots induced ranged from 1 14 cm in T 16 to 1 36 cm in T19 The treatment T17 consisting of 2 0 mg $l^{-1}$ BA in half strength MS medium producing an average of 3 0 shoots per culture was identified as the best medium for multiplication of shoots in the nodal segments of *P rosea*

**4 3 2 2 Effect of media supplements on shoot elongation**

The results of second subculture in elongation medium are presented in Table 35 The shoots obtained from all the treatments after culture proliferation were transferred to elongation medium The elongation media consisted of the same treatment combinations used for multiplication There was no significant effect on shoot elongation in *P zeylanica* cultures for all the treatment combinations However there were significant differences among the treatments for shoot elongation in the cultures of *P rosea* The maximum increase in shoot length (4 50 cm) was observed in the treatment T19 followed by T18 (2 96 cm) The number of days required for shoot elongation was 30 The shoots did not show much increase in length in rest of the treatment combinations There was also an increase in the number of leaves produced per shoot in the treatments T18 and T19 The treatment T19 consisting of BA (4 0 mg $l^{-1}$) in basal MS medium was identified as the best medium for shoot elongation

**4 3 2 3 Effect of media supplements on root induction**

As seen from Table 36 *P zeylanica* did not show rooting in any of the treatments There was no root induction in *P rosea* in the treatment T22 having a low concentration of IBA and T26 without any growth regulators Three treatments *viz* T23 T24 and T25 induced rooting in 90 per cent of the cultures There were significant differences among the three treatments for root length The treatment T25 consisting of 1 5 mg $l^{-1}$ IBA showed maximum root length All the *P rosea* cultures

**Table 34 Effect of dıfferent concentratons of cytokının BA on multıple shoot ınductıon from nodal segments ın *Plumbago specıes***

| Treatment | Medıum | Cultures show ng shoot prolıferat on (%)* | | Days for shoot prol ferat on | No of mult ple shoots 1st subculture (*P rosea*) | Length of shoots n 1st subculture (*P rosea*) |
|---|---|---|---|---|---|---|
| | | *P rosea* | *P zeylanıca* | | | |
| T16 | ½ MS+BA 1 0 mg l$^{1}$ | 80 | Nıl | 45 | 2 80±0 08a | 1 14±0 05 |
| **T17** | **½ MS+BA 2 0 mg l$^{1}$** | **90** | **Nıl** | **45** | **3 00±0 14a** | **1 23±0 09** |
| T18 | MS+BA 3 0 mg l | 60 | Nıl | 45 | 2 16±0 17b | 1 14±0 06 |
| T19 | MS+BA 4 0 mg l$^{1}$ | 60 | N l | 45 | 1 58±0 19c | 1 36±0 10 |
| T20 | MS+BA 1 0 mg l$^{1}$+Ads 25mg l$^{1}$ | 50 | 30 | 45 | 1 15±0 10cd | 1 18±0 07 |
| T21 | MS+ BA mg l + Ads 50 mg l$^{1}$ | 15 | 30 | 45 | 1 21±0 11d | 1 36±0 13 |

Fıgures carryıng same alphabets do not dıffer sıgnıf cantly at 5% level based on DMRT

* No of cultures – 20

**Table 35 Effect of dıfferent concentratons of cytokınm BA on elongatıon of shoots on second subculture**

| Treatment | Med um | Cultures show ng shoot elongat on (%)* | | Days for shoot elongatıon | Length of shoots n 2nd subculture | No of leaves per shoot |
|---|---|---|---|---|---|---|
| | | *P rosea* | *P zeylanıca* | | | |
| T16 | ½ MS+BA 1 0 mg l | 90 | Nıl | 30 | 1 46±0 11d | 2 43±0 14c |
| T17 | ½ MS+BA 2 0 mg l$^{1}$ | 80 | Nıl | 30 | 1 79±0 10cd | 2 07± 0 07d |
| T18 | MS+BA 3 0 mg l$^{1}$ | 60 | 15 | 30 | 2 96±0 16b | 4 25±0 03b |
| **T19** | **MS+BA 4 0 mg l$^{1}$** | **60** | **20** | **30** | **4 50±0 17a** | **5 92±0 31a** |
| T20 | MS+BA 1 0 mg l + Ads 25mg l | N l | Nıl | 30 | 2 00±0 22c | 2 50±0 16d |
| T21 | MS+ BA mg l + Ads 50 mg l | Nıl | N l | 30 | 1 80±0 19cd | 2 10±0 1d |

F gures carry ng same alphabets do not d ffer sıgn f cantly at 5% level based on DMRT

* No of cultures 20


110

**Table 36 Effect of dıfferent concentratıons of auxın IBA ın the *ın vıtro* rootıng of shoots from nodal segments of *Plumbago* specıes**

| Treatment | Medıum | Cultures rootıng(%)* | | Days for root n t atıon | Days for completıon of rootıng | Length of roots (cm) |
|---|---|---|---|---|---|---|
| | | *P rosea* | *P zeylanıca* | | | |
| T22 | ½ MS+ IBA 0 25 mg l $^{1}$+ sucrose2% | Nıl | Nıl | Nıl | Nıl | Nıl |
| T23 | ½ MS+ IBA 0 50 mg l $^{1}$ + sucrose2% | 90 | Nıl | 21 | 60 | 2 0±0 09b |
| T24 | ½ MS+ IBA 1 0 mg l $^{1}$+ sucrose3% | 90 | Nıl | 21 | 60 | 1 6±0 16c |
| T25 | ½ MS+IBA 1 5 mg l + sucrose3% | 90 | Nıl | 21 | 60 | 3 4±0 10a |
| T26 | MS+ No growth regulators + sucrose3% | Nıl | Nıl | Nıl | Nıl | Nıl |

Fıgures carryıng same alphabets do not dıffer sıgnıf cantly at 5% level based on DMRT

*No of cultures 20

started rooting in these three treatments in three weeks and took another four to five weeks for completion

### 4 3 2 4 Hardening acclimatization and establishment in field

Eighteen cultures of *Plumbago rosea* showed root induction in 60 days after incubation in each of the three rooting media combinations (T23 T24 and T25) The results of hardening are presented in Table 37 After completion of rooting 66 7 per cent of the plantlets of T23 and 83 3 per cent plantlets in each of the treatments T24 and T25 could be transferred to sterile sand in disposable cups for primary hardening under the laboratory conditions for four weeks The hardened plants were transferred to polythene bags containing potting mixture A maximum of 66 7 per cent plantlets from the treatments T24 and T25 survived in cups The plantlets in bags were placed in net house for eight weeks for secondary hardening None of the plants in the T 23 survived on hardening in the net house In the treatments T24 and T25 22 2 per cent plantlets survived secondary hardening process The *in vitro* plants that had survived the hardening treatment were planted under field conditions Along with the *in vitro* plants three month old rooted cutting of the genotype IC 566508 were planted for comparison

### 4 3 2 5 Establishment in field

*In vitro* plants from treatment T24 named as $TC_1$ and T25 named as $TC_2$ were planted along with the rooted cuttings of IC 566508 as pot culture The data obtained on various morphological traits and biometric traits after harvest were subjected to statistical analysis and the results are presented in Tables 38 and 39

### A) Morphological characters before harvest

The mean values of six morphological traits namely plant height internode length number of branches number of leaves leaf length and leaf breadth taken at quarterly intervals prior to harvest are given in the sub tables of Table 38 There was no significant difference between *in vitro* regenerated plants ($TC_1$ and $TC_2$) and the conventional plants of IC 566508 for the characters such as plant height number of branches and number of leaves during the entire period of growth However during

**Table 37 Effect of hardening and acclimatisation of plantlets obtained from direct regeneration of nodal segments of *Plumbago rosea***

| Treatment | Number of plants rooted | Number of plants planted in cups | Transfer rate (%) | No survived after 4 weeks | Primary hardening (%) | No of plants transferred to bags | No survived in bags | Secondary harden ng (%) |
|---|---|---|---|---|---|---|---|---|
| T23 | 18 | 12 | 66 7 | 8 | 53 3 | 8 | 0 | 0 |
| T24 | 18 | 15 | 83 3 | 8 | 66 6 | 8 | 4 | 22 2 |
| T25 | 18 | 15 | 83 3 | 8 | 66 6 | 8 | 4 | 22 2 |

**Table 38 Mean performance of *in vitro* plants obtained through direct organogenesis for morphological characters**

**Plant Height (cm)**

| Genotype | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $TC_1$ | 15 25 | 24 88 | 31 13 | 40 75 |
| $TC_2$ | 22 75 | 31 25 | 50 13 | 58 50 |
| IC 566508 | 33 00 | 45 50 | 52 25 | 58 25 |
| CD at 5% level | NS | NS | NS | NS |

**Internode length (cm)**

| Genotype | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $TC_1$ | 1 88 | 2 63 | 3 63 | 5 25 |
| $TC_2$ | **2 50** | **3 00** | **4 19** | **5 50** |
| IC 566508 | 1 88 | 2 38 | 3 13 | 2 88 |
| CD at 5% level | NS | NS | NS | 1 91 |

**Number of branches**

| Genotype | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $TC_1$ | 5 25 | 4 75 | 7 00 | 9 75 |
| $TC_2$ | 5 50 | 6 00 | 10 75 | 15 25 |
| IC 566508 | 2 88 | 2 75 | 5 00 | 7 75 |
| CD at 5% level | NS | NS | NS | NS |

**Number of leaves**

| Genotype | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| TC | 14 00 | 25 50 | 53 75 | 72 00 |
| $TC_2$ | 17 25 | 35 00 | 67 75 | 121 50 |
| IC 566508 | 10 50 | 19 25 | 48 50 | 64 50 |
| CD at 5% level | NS | NS | NS | NS |

**Leaf length (cm)**

| Genotype | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $TC_1$ | 8 25 | 8 71 | 9 08 | 9 00 |
| $TC_2$ | **9 83** | **11 25** | **11 71** | **11 71** |
| IC 566508 | 6 67 | 8 46 | 7 28 | 7 75 |
| CD at 5% level | 2 84 | 2 04 | 2 29 | 1 95 |

**Leaf breadth (cm)**

| Genotype | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $TC_1$ | 4 71 | 4 83 | 5 17 | 5 15 |
| $TC_2$ | **5 57** | **6 46** | **6 67** | **6 25** |
| IC 566508 | 3 75 | 3 58 | 4 42 | 4 50 |
| CD at 5% level | 1 64 | 1 13 | 1 07 | 1 19 |

the last season of growth the *in vitro* plants showed significant difference for internode length when compared to conventional plants The TC and $TC_2$ plants had higher average internode length (5 25 5 50cm) than the conventional plants (2 88 cm)

There was significant difference between the conventional plants and the *in vitro* plants for leaf length as well as leaf width The *in vitro* plants produced large sized leaves throughout the growing period The $TC_2$ plants produced leaves of maximum length ranging from 9 83 cm at the end of first quarter to 11 71cm at the end of last quarter and maximum width ranging from 5 57 cm to 6 25 cm

**B) Observation of biometric characters at the time of harvest**

The results of the analysis of variance of the seven biometric traits namely total biomass/fresh weight of plant number of roots per plant root length root girth fresh root weight dry root weight and plumbagin content are presented in Table 39 The *in vitro* plants and the conventional plants did not differ significantly for the root characters such as number of roots per plant root length and root girth/thickness

**Table 39 Observations at the time of harvest on biometric characters of *in vitro* plants obtained through direct organogenesis**

| Genotype | Number of roots /plant | Root length (cm) | Root girth (cm) | Total biomass (g) | Fresh root weight (g) | Dry root weight (g) | Plumbagin content (%) |
|---|---|---|---|---|---|---|---|
| $TC_1$ | 15 50 | 41 00 | 2 25 | 155 00 | 65 00 | 21 75 | 0 66 |
| $TC_2$ | 17 50 | 38 75 | 2 43 | **360 00** | **150 00** | 45 25 | 1 33 |
| IC 566508 | 10 25 | 31 25 | 2 00 | 117 50 | 37 50 | 15 00 | **2 25** |
| CD at 5% | NS | NS | NS | 178 56 | 81 86 | 21 32 | 0 48 |

There was significant difference between the *in vitro* regenerated plants and the conventional plants of *P rosea* for total biomass fresh root weight dry root weight and plumbagin content The $TC_2$ plants produced maximum total biomass (360 0g) fresh root weight (150 0g) and dry root weight (45 25g) The $TC_1$ plants and the conventionally propagated plants were at par producing fresh plant weight of 155 0 g

and 117 5g respectively They were also at par for the characters fresh root weight and dry root weight

**C) Quantity and quality of plumbagin content**

The conventional plants had recorded significantly higher plumbagin content (2 25%) than *in vitro* plants ($TC_1$ 1 33 % and $TC_2$ 0 48 %) However the qualitative analysis of plumbagin by TLC for both *in vitro* and conventional plants showed light red fluorescent bands at Rf value 0 55 These bands were obtained at the same Rf value as that of the standard plumbagin confirming the presence of good plumbagin in both conventional as well as *in vitro* plants

**4 3 3 Regeneration through callus mediated organogenesis**

The best explant for callusing was found to be the leaf Leaves of *P rosea* alone showed callusing in treatment T15 containing TDZ Further study was conducted exclusively for callus induction in leaves of *P rosea* as well as *P zeylanica* The detailed result of the study is as follows

**4 3 3 1 Effect of growth regulators on callus induction and proliferation**

The effects of various media combinations to induce callusing are given in Table 40 The leaf discs of *P zeylanica* did not show callusing in any of the 23 treatment combinations There was callusing only in the leaf discs of *P rosea* Callusing was observed in treatments T27 to T35 consisting auxins *viz* 2 4 D and NAA In both half strength and full strength MS medium supplemented with NAA there was callusing However in the treatments T36 to T40 the auxin synergist phloroglucinol when supplemented with NAA did not show any effect Among the treatments supplemented with cytokinins BA and TDZ respectively only those with TDZ (T46 to T49) could induce callusing

Among the auxins tried for callus induction the treatment containing increasing levels of 2 4 D induced callusing in cultures ranging from 40 per cent in T27 (1 0 $mgl^{-1}$) to 80 per cent T29(3 0 mg $l^{-1}$) In treatments with increasing levels of NAA added to full strength MS medium the percentage of callusing ranged from 30 per cent in T30 (1 0 mg $l^{-1}$) to 90 per cent inT33 (4 0 mg $l^{-1}$) The treatment consisting of half strength MS medium supplemented with NAA (T34 and T35) showed lowest

percentage of callusing (20 0 per cent) Any level of phloroglucinol along with NAA (0 5 mg l $^{-1}$) in the treatments T36 to T40 failed to induce callus

The treatments with varying levels of cytokinin BA (T41 to T45) failed to induce callus in the leaf explants of *P rosea* In the treatments with increasing levels of TDZ (T46 to T49) the treatment T46 with lowest level of TDZ (1 0 mg l $^{-1}$) induced callusing in maximum cultures (90 0 percent) followed by T47 (80 per cent) The treatment T49 containing 2 5 mg l $^{-1}$ TDZ induced callusing in minimum percentage of cultures (30 0 percent)

The maximum spread of the callus in the culture tubes six weeks after incubation was observed for the treatment T33 containing NAA (4 0 mg l $^{-1}$) followed by T48 containing TDZ (2 0 mg l $^{-1}$) and T49 (TDZ 3 0 mg l $^{-1}$) The treatments containing 2 4 D (T27 T28 and T29) NAA (T34 T35) and TDZ (T47) gave the same spread of callus The lowest spread of the callus was noted for T46 containing TDZ (1 0 mg l $^{-1}$)

Callus Index (C I) was highest (270) in medium containing 4 0 mg l $^{-1}$ of NAA (T 33) Callus index in other media combinations varied between 40 in T 34 and 225 in T48 The callus morphology differed in the treatments containing auxin and cytokinin respectively The callus was semi friable to friable soft and wet in texture in case of treatments combination consisting of auxins *viz* 2 4 D (T27 to T29)and NAA (T30 toT35) The treatment combination with TDZ (T46 to T49) had induced hard and compact callus The colour of callus in all the treatments with TDZ was creamy white The colour of callus in treatment combination with 2 4 D varied from white in T27 to light green in T28 and T29 In treatment combinations of full strength MS medium with NAA (T30 to T33) the colour of callus was creamy white The colour of callus in half strength MS medium with NAA (T34 and T35) was brown

On the basis of mode of regeneration the nature of callus differed in treatments containing auxins and cytokinins respectively The callus turned rhizogenic within four weeks of induction in all the treatments consisting of auxin (T27 to T35) The calli produced by the treatments containing TDZ (T46 to T49) alone showed regeneration when transferred medium containing BA The calli showed non embryogenic regeneration turning green with shoot bud initiation

Table 40 Effect of different growth regulator combinations on callus induction from leaves of *Plumbago rosea*

| Treatment | Medium | Days for callusing callus initiation | Callus percentage | Growth score | Callus Index | Callus morphology | Nature of callus |
|---|---|---|---|---|---|---|---|
| | | | Six weeks after incubation | | | | |
| T27 | MS+ 2 4 D 1 0 mg l | 14 | 40 | 2 | 80 | Translucent wet white | Rhizogenic |
| T28 | MS+ 2 4 D 2 0 mg l | 14 | 60 | 2 | 120 | Semi friable wet soft light green | Rhizogenic |
| T29 | MS+ 2 4 D 3 0 mg l | 14 | 80 | 2 | 160 | Semifriable wet soft light green | Rhizogenic |
| T30 | MS+ NAA 1 0 mg l | 7 | 30 | 3 | 90 | Friable wet soft creamy white | Rhizogenic |
| T31 | MS+ NAA 2 0 mg l[1] | 7 | 50 | 3 | 150 | Friable wet, soft creamy white | Rhizogenic |
| T32 | MS+ NAA 3 0 mg l | 7 | 70 | 3 | 210 | Friable wet soft creamy white | Rhizogenic |
| T33 | MS+ NAA 4 0 mg l | 7 | 90 | 4 | 270 | Friable wet soft creamy white | Rhizogenic |
| T34 | ½ MS+ NAA 1 0 mg l | 14 | 20 | 2 | 40 | Friable wet soft brown | Rhizogenic |
| T35 | ½ MS+ NAA 2 0 mg l | 14 | 20 | 2 | 40 | Friable wet soft brown | Rhizogenic |
| T36 | MS + NAA0 5 mg l + Phloroglucinol 50 mg l | | Nil | | | | |
| T37 | MS + NAA0 5 mg l + Phloroglucinol 75 mg l | | Nil | | | | |
| T38 | MS + NAA0 5 mg l + Phloroglucinol 100 mg l | | Nil | | | | |


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| Treatment | Medium | Days for callusing /callus initiation | Callus percentage | Growth score | Callus Index | Callus morphology | Nature of callus |
|---|---|---|---|---|---|---|---|
| | | | Six weeks after incubation | | | | |
| T39 | MS + NAA0 5 mg l + Phloroglucinol 125 mg l | | Nil | | | | |
| T40 | MS + NAA0 5 mg l + Phloroglucinol 150 mg l | | Nil | | | | |
| T41 | MS + BA 0 5mg l | | Nil | | | | |
| T42 | MS + BA 1 0 mg l$^{1}$ | | Nil | | | | |
| T43 | MS + BA 1 5 mg l | | Nil | | | | |
| T44 | MS + BA 2 0 mg l | | Nil | | | | |
| T45 | MS + BA 2 5 mg l | | Nil | | | | |
| T46 | MS+TDZ 1 0 mg l | 21 | 90 | 1 | 90 | Compact hard creamy white | Non embryogenic |
| T47 | MS+TDZ 1 5 mg l | 21 | 80 | 2 | 160 | Compact hard creamy white | Non embryogenic |
| **T48** | **MS+TDZ 2 0 mg l$^{1}$** | **21** | **75** | **3** | **225** | **Compact hard creamy white** | **Non embryogenic** |
| T49 | MS+TDZ 2 5 mg l | 21 | 30 | 3 | 90 | Compact hard creamy white | Non embryogenic |

*No of cultures in each treatment–20

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The treatments also differed in the time taken for callus initiation The treatments with auxins took 1 to 2 weeks for callus induction whereas the treatments with cytokinin TDZ took three weeks The treatments with TDZ alone showed callus regeneration and the treatment T48 with 2 0 mg l TDZ was identified as the best treatment for callus induction showing callusing in 75 per cent cultures with a call is index of 225

**4 3 3 2 Organogenesis/Regeneration from callus**

The calli obtained from the treatments containing TDZ alone were carried over for regeneration studies since the calli obtained from the treatments containing auxin turned rhizogenic after four weeks The effect of growth regulars on the regeneration of callus are g ven in Table 41

Maxim m callus regeneration percentage was observed when the callus was cultured in T 50 containing BA 1 0 mg l and Ads 50 mg l$^{1}$ (100 per cent) There was reduction in regeneration in the treatments with increased levels of the cytokinin BA in both half strength and full strength MS medium In T51 consisting of liquid medium the callus regeneration percentage was 75 while in T52 T53 T54 and T55 it was 50 per cent The days for the proliferation of the callus were least in T50 T52 and T55 (14 days) It took 21 days for callus proliferation in T51 T53 and T54 The creamy wh te callus turned green during proliferat on The treatment T50 induced maximum average number of multiple shoots (6 0) six weeks after proliferation In T53 the average number of shoots was reduced to 2 1

There was no significant effect on the average length of shoot as well as the average number of leaves produced per shoot in all the treatment combinations The length of shoots ranged from 1 14 cm (T50 and T52) to 1 41 cm (T54 and T55) The treatment T50 was found to be the best treatment for callus regeneration with an average of 6 1 shoots per culture

**4 3 3 3 Shoot elongation**

The shoot regeneration medium had produced small shoots Hence for elongation treatments with increasing levels of BA were tried The treatments cons sted of increasing concentration of BA from 5 0 mg l in T56 to 10 0 mg l in

**Table 41 Effect of growth regulator combınatıons on multıple shoot ınductıon and prolıferatıon of callus from leaves of *P rosea***

| Treatment | Med um | No of call plated | No of callı showıng shoot prolıferat on | Callus showıng prol fera t on (%) | Days for prol fera tıon | No of mut ple shoots per culture | Length of the shoots (cm) |
|---|---|---|---|---|---|---|---|
| | | | | Sıx weeks after prol feratıon | | | |
| **T50** | **MS+ BA 1 0 mg l 1+ Ads 50 mg l 1** | **20** | **18** | **100** | **14** | **6 00±0 61a** | **1 14±0 05** |
| T51 | ½ MS + BA 2 0 mg l ( sem solıd) | 20 | 15 | 75 | 21 | 3 33±0 67b | 1 23±0 08 |
| T52 | ½ MS + BA 2 0 mg l (lıquıd) | 20 | 10 | 50 | 14 | 2 10±0 41b | 1 14±0 07 |
| T53 | MS + BA 3 0 mg l | 20 | 10 | 50 | 21 | 2 92±0 34b | 1 23±0 07 |
| T54 | MS + BA 4 0 mg l | 20 | 10 | 50 | 21 | 3 00±0 43b | 1 41±0 14 |
| T55 | MS + BA 0 2 ng l + IAA 0 1 mg l | 20 | 10 | 50 | 14 | 3 00±0 41b | 1 41±0 09 |

Fıgures carryıng same alphabets do not dıffer s gn ficantly at the 5% level based on DMRT

T61 As seen from Table 42 no elongation was observed in all these treatments after four weeks of transfer Instead the calli with small shoots turned brown and later blackened and dried In treatment T56 (5 0 mg l 1 BA) and T57 (6 0 mg l 1) instead of elongation there was shoot proliferation Hence for shoot elongation gibberellic acid ($GA_3$) was used at a concentration of 0 1 mg l in the treatment T62 Gibberellins are generally known to promote shoot elongation But in the present study it not only promoted elongation of shoot but also rooting Ninety percent of the cultures showed elongation as well as rooting in the medium containing $GA_3$

**Table 42 Effects of growth regulators on elongation of shoot produced from callus cultures in *P rosea***

| Treatment | Medium | Cultures showing shoot elongation elongation (%)* | |
|---|---|---|---|
| | | Four weeks from date of subculture | |
| T56 | MS+ BA 5 0 mg l 1 | Nil | Cultures turned brown |
| T57 | MS+ BA 6 0 mg l 1 | Nil | Cultures turned brown |
| T58 | MS+ BA 7 0 mg l 1 | Nil | Cultures turned brown |
| T59 | MS+ BA 8 0 mg l 1 | Nil | Cultures turned brown |
| T60 | MS+ BA 9 0 mg l 1 | Nil | Cultures turned black |
| T61 | MS+ BA 10 0 mg l 1 | Nil | Cultures turned black |
| **T62** | **MS+$GA_3$ 0 1 (semi solid)** | **90** | **Elongation of shoots as well as rooting** |

* No of cultures=20

**4 3 3 4 Root induction**

Further studies on rooting were carried out with increased levels of $GA_3$ The effects of the growth regulators on rooting and shoot elongation are presented in Table 43 To start with lowest concentration of $GA_3$ (0 1 mg l 1) was tried in semisolid MS medium (T62) and liquid MS medium (T63) The concentration of $GA_3$ in semisolid medium was increased to 0 5 mg l 1 in T64 and further increased to 0 7 mg

**Table 43 Effect of different media combination on *in vitro* rooting and elongation of shoots produced from calli of *P rosea* leaves**

| Treatment | Medium | Percentage cultures show ng shoot elongation & root ng* | Days for root nduct on | Length of roots | Shoot length | No of leaves |
|---|---|---|---|---|---|---|
| **T62** | **MS + $GA_3$ 0 1 mg l$^{1}$ (semi solid)** | **90** | **60** | **3 67± 0 13a** | **6 17±0 30b** | **7 28±0 54a** |
| **T63** | **MS + $GA_3$ 0 1 mg l (liquid)** | **75** | **60** | **4 00± 0 33a** | **9 63±0 73a** | **7 00±0 53a** |
| T64 | MS + $GA_3$ 0 5 mg l (sem sol d) | 50 | 60 | 1 87±0 14c | 3 40±0 25cd | 2 70±0 21c |
| T65 | MS + $GA_3$ 0 7 mg l (semi sol d) | 50 | 60 | 1 60±0 14c | 3 20±0 21cd | 2 60±0 22c |
| T66 | MS + $GA_3$ 0 9 mg l (semi solid) | 50 | 60 | 1 25±0 13c | 2 70±0 19d | 2 20±0 13c |
| T67 | MS + IBA 1 5 mg l (sem solid) | 50 | 60 | 2 50±0 24b | 4 45±0 63c | 5 10±0 28b |

Figures carry ng same alphabets do not d ffer s gnificantly at the 5% level based on DMRT

* No of cultures 20


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l in T65 and 0 9 mg l[1] in T66 Besides$GA_3$ the most frequently used rooting hormone IBA was also tried (T67 1 5 mg l )

In all the treatments rooting initiated within 30 days of incubation and was completed after 60 days of incubation The percentage cultures showing root induction differed in all treatments The treatment T62 containing 0 1 mg l $GA_3$ in semisolid medium showed maximum percentage ( 90 per cent cultures) of root induction followed by treatment T63 0 1 mg l[1] $GA_3$ in liquid medium (75 per cent cultures) The treatments from T64 to T67 with increasing concentrat on of $GA_3$ showed root induction in 50 per cent of the cultures

There were significa it differences among the treatments for average length of the roots and shoots as well as number of leaves The treatments T62 and T63 recorded highest mean length of roots (3 67 and 4 00cm) and number of leaves (7 00 and 7 28) All the treatments with higher concentrations of $GA_3$ (T64 to T66) were fou id to be inferior recording lowest length of roots ranging from 1 25 (T66) to 1 87 (T64) with least number of number of leaves The treatment T67 with IBA recorded an average root length of 2 5 cm In the treatments T62 T63 and T67 the roots produced were well developed and white in colour In these treatments the leaves were well ope ied and larger in size The leaves in treatment T64 to T66 were small and not well opened

The treatment T63 recorded longest shoot (9 65cm) followed by treatment T62 (6 17cm) and T67 (4 45cm) The treatment T66 (0 9 mg l[1]$GA_3$) recorded lowest length of shoot (2 70cm) The treatment with lowest concentration of $GA_3$ (0 1 mg l ) *viz* T62 and T63 in semisolid and liquid basal MS medium were identified as best treatment for rooting as well as shoot elongation

**4 3 3 5 Hardening and acclimatisation**

The results of hardening and acclimatisation are presented in Table 44 The number of plants obtained varied from 18 in T62 to 4 in T66 After completion of rooting 100 0 per cent of the rooted plantlets from treatment T62 93 3 rooted plantlets from treatment T63 90 0 per cent rooted plantlets from treatment T67 50 0 per cent rooted plantlets each from treatments T64 and T65 and 40 0 per cent plants

**Table 44 Effect of hardening and acclimatisation of plantlets obtained from callus mediated organogenesis of leaves of *Plumbago rosea***

| Treatment | Number of plants rooted | Number of plants planted in cups | Transfer rate(%) | No survived after 4 weeks | Primary hardening (%) | No of plants transferred to bags | No survived in bags | Secondary hardening (%) |
|---|---|---|---|---|---|---|---|---|
| T62 | 18 | 18 | 100 0 | 12 | 66 7 | 12 | 12 | 66 7 |
| T63 | 15 | 14 | 93 3 | 11 | 71 4 | 11 | 11 | 71 4 |
| T64 | 10 | 5 | 50 0 | 2 | 40 0 | 2 | 0 | 0 0 |
| T65 | 10 | 5 | 50 0 | 0 | 0 0 | 0 | 0 | 0 0 |
| T66 | 10 | 4 | 40 0 | 0 | 0 0 | 0 | 0 | 0 0 |
| T67 | 10 | 9 | 90 0 | 9 | 100 0 | 8 | 8 | 60 0 |

from T66 could be transferred to cups for hardening in the ambient conditions of the laboratory None of the plants from treatments T65 and T66 survived primary hardening whereas 40 0 per cent plants of T64 survived primary hardening On secondary hardening under the net house conditions there was maximum survival of plants from the treatment T62 and T63 (66 7 and 71 4 per cent) followed by T67 (60 0 per cent) None of the plants from T64 survived secondary hardening

**4 3 4 *In vitro* mutagenesis**

*In vitro* mutagenesis was carried out only in one of the species viz *Plumbago rosea* since standardization of *in vitro* regeneration was possible only in this species Mutagenesis was conducted using physical mutagen (gamma rays) and chemical mutagen (EMS) The nodal and callus cultures of the three *Plumbago rosea* accessions (IC 566503 IC 566508 and IC 566514) were subjected to physical and chemical mutagenesis The detailed results of the experiment are presented

**4 3 4 1 Physical mutagenesis**

**4 3 4 1 1 Effect of gamma radiation on culture response in nodal cultures**

The regeneration percentage of two week old nodal cultures of three genotypes of *Plumbago rosea* ((IC 566503 IC 566508 and IC 566514) was used to calculate $LD_{50}$ value of gamma radiation under experimental conditions The data on the effect of different doses of gamma radiation on nodal cultures is presented in Table 45 The cultures of gamma irradiation dose $D_0$ (control 0 Gy) recorded maximum regeneration of shoots (100 0 per cent) while those subjected to $D_4$ 40 Gy and $D_5$ 50 Gy did not record any shoot regeneration The treatment doses $D_2$ 20Gy and$D_3$ 30Gy recorded regeneration in 50 0 percent of the irradiated cultures The percentage shoot elongation in the cultures when transferred to elongation medium is maximum for control ($D_0$ 100 0 per cent) and 50 0 percent for the cultures of $D_2$ (20 Gy) and $D_3$ (30 Gy)

The nodal cultures showing elongation for each of the treatment dose were transferred to rooting medium and observed for root induction There was no root initiation in the cultures of treatment $D_3$ (30 Gy) and $D_4$ (40 Gy) Only 20 per cent cultures of $D_2$ (20 Gy) showed rooting Eighty per cent of the cultures of treatment D

**Table 45 Effect of gamma radiation on culture response in nodal cultures of *P rosea***

| Mutagen dose (Gamma radiation ) | Treatment | No of nodal cultures | No of cultures showing shoot regenaration | Regenerat on percentage | No of cı ltures in shoot medium | No of cultures showing shoot elongat on | Percentage cultures show ng shoot elongation | No of cultures in rooting medium | No of cultures showing root initiation | Percentage cultures showing root induction |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 Gy | $D_0$ | 10 | 10 | 100 0 | 10 | 10 | 100 0 | 10 | 8 | 80 0 |
| 10 Gy | D | 10 | 9 | 90 0 | 10 | 8 | 80 0 | 10 | 8 | 80 0 |
| 20 Gy | $D_2$ | 10 | 5 | 50 0 | 10 | 5 | 50 0 | 10 | 2 | 20 0 |
| 30 Gy | $D_3$ | 10 | 5 | 50 0 | 10 | 5 | 50 0 | 10 | 0 | 0 0 |
| 40 Gy | $D_4$ | 10 | 5 | 0 0 | 10 | 0 | 0 0 | 10 | 0 | 0 0 |
| 50 Gy | $D_5$ | 10 | 0 | 0 0 | 10 | 0 | 0 0 | 10 | 0 | 0 0 |

(10 Gy) and the control treatment $D_0$ (0 Gy) showed rooting Hence the LD $_0$ was fixed as dose 20 Gy Three doses of gamma rays such as 5 Gy 10 Gy and 15 Gy were fixed as optimum doses for nodal cultures

**4 3 4 1 2 Regeneration response of nodal cultures for optimum doses of gamma rays**

Twenty cultures of each *P rosea* accession were treated with respective optimum doses of gamma rays *viz* $D_0$ (0 Gy) $D_1$ (5Gy) $D_2$ (10 Gy) and $D_3$ (15 Gy) There was high rate of bacterial contammation in the nodal cultures when observed for regeneration after two weeks incubation in proliferation medium None of the cultures could be transferred to elongation medium To overcome bacterial contaminat on antibiotic ampicillin @ 1µg/1ml was added to the proliferation medium and elongation medium The gamma irradiation with optimum doses was repeated The effect of irradiation on percentage regeneration was recorded and the results are presented in Table 46 There was contammation in 50 to 55 per cent of the irradiated cultures well as control The surviving cultures were transferred to elongat on medium In this medium also there was bacterial contamination in 50 to 80 per cent irradiated cultures including control belonging to the three accessions The surviving cultures were then transferred to rooting med um In general there was delayed rooting i irradiated cultures as well as control It took 75 90 days for rooting from the date of transfer to rooting medium In some cu ltures only few ill developed roots were produced In accession 566503 the control treatment $D_0$ recorded rooting in maximum cultures (25 0 per cent) and the treatment $D_2$ recorded minimum percentage of rooting (10 0 per cent) In the accession 566508 maximum cultures (45 0 per cent) belonging to treatment $D_2$ induced rooting when transferred from elongat on medium In accession IC 566514 maximum percentage of rooting was observed in control treatment $D_0$ (20 0) and the minimum percentage of rooting was observed in treatment D (10 0 per cent)

**4 3 4 1 3 Plant regeneration percentage after *in vitro* mutagenesis of nodal cultures**

The number of plants produced from the irradiated nodal cultures in each of the treatment dose was recorded and the observations on shoot length root length and

**Table 46 Regeneration response in nodal cultures of *P rosea* for optimum doses of gamma radiation**

| Accession No | Mutagen dose (Gamma rad at on ) | Treatment | No of cultures treated | No of cultures showing mult ple shoot in itation | Regeneration percentage | No of cultures n elongation medium | No of cultures showing elongat on | Percentage elongation | No of cultures n root ng med um | No of cultures show ng root nititation | Percentage root nititation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IC 566503 | 0 Gy | $D_0$ | 20 | 10 | 50 0 | 20 | 8 | 40 0 | 8 | 5 | 25 |
| | 5 Gy | D | 20 | 9 | 45 0 | 20 | 5 | 25 0 | 5 | 3 | 15 0 |
| | 10 Gy | $D_2$ | 20 | 9 | 45 0 | 20 | 5 | 25 0 | 5 | 2 | 10 0 |
| | 15 Gy | $D_3$ | 20 | 9 | 45 0 | 20 | 5 | 25 0 | 5 | 3 | 15 0 |
| IC 566508 | 0 Gy | $D_0$ | 20 | 10 | 50 0 | 20 | 10 | 50 0 | 10 | 5 | 25 0 |
| | 5 Gy | D | 20 | 10 | 50 0 | 20 | 8 | 40 0 | 8 | 6 | 30 0 |
| | 10 Gy | $D_2$ | 20 | 9 | 45 0 | 20 | 9 | 45 0 | 9 | 9 | 45 0 |
| | 15 Gy | $D_3$ | 20 | 9 | 45 0 | 20 | 4 | 20 0 | 4 | 2 | 10 0 |
| IC 566514 | 0 Gy | $D_0$ | 20 | 9 | 45 0 | 20 | 4 | 20 0 | 4 | 4 | 20 0 |
| | 5 Gy | $D_1$ | 20 | 9 | 45 0 | 20 | 8 | 40 0 | 8 | 2 | 10 0 |
| | 10 Gy | $D_2$ | 20 | 10 | 50 0 | 20 | 6 | 30 0 | 6 | 3 | 15 0 |
| | 15 Gy | $D_3$ | 20 | 10 | 50 0 | 20 | 5 | 25 0 | 5 | 2 | 10 0 |

number of leaves are gıven ın Table 47 Due to severe bactenal contamınatıon the percentage plant regenerated from the nodal cultures ırradıated wıth opt mum doses of gamma rays was very low The root development was poor ın the cultures when they were sub cultured ın the medıum contaınıng antıbıotıc In all the accessıons the percentage plants that could be produced from the cultures at all optımum doses ranged from 10 0 to 25 0 per cent except the treatment $D_2$ (20 Gy) of genotype IC 566508 whıch showed 45 0 per cent plant regeneratıon

**4 3 4 1 4 Hardenıng and acclımatısatıon**

The results of hardenıng are presented m Table 48 Fıfteen percent control plants of the accessıons IC 566503 and IC 566508 and 20 0 per cent control plants of the accessıon IC 566514 survıved prımary hardenıng The prımary hardenıng of plants belongıng to treatment D (10 Gy) resulted ın 15 0 per cent survıval of plants ın accessıon IC 566503 20 0 per cent ın IC 566508 and 10 0 per cent ın IC 566514 For the treatment $D_2$ (20 Gy) the percentage survıval of plants was 10 0 ın IC 566503 25 0 per cent ın IC 566508 and 5 0 ın IC 566514 None of the plants produced ın the treatment $D_3$ (30 Gy) of IC 566514 survıved prımary hardenıng whereas 10 0 per cent and 5 0 per cent plants of respectıve accessıons IC 566503 and IC 566508 survıved prımary hardenıng

Under net house cond tıons there was maxımum survıval of plants (20 0 and 25 0 per cent) for the treatments D (10 Gy) and $D_2$ (20 Gy) ın the accessıon IC 566508 For the treatment $D_3$ (30 Gy) of the plants of accessıons IC 566508 and IC 566514 survıved secondary hardenıng ın bags whıle 5 0 per cent survıved ın genotype IC 566503 None of the control plants of genotype IC 566514 survıved secondary hardenıng

The percentage plant regeneratıon was low ın accessıons IC 566503 and IC 566514 and the percentage recovery of plants after hardenıng stıll lower Only ın accessıon IC 566508 the rate of hardenıng was slıghtly hıgher Sınce the recovery of hardened plants followıng *ın vıtro* mutagenesıs was very low from nodal cultures these could not be carrıed over to experıment V for field studıes

**Table 47 Effect of optimum doses of gamma radiation on percentage regeneration and development of plant from nodal culture of *P rosea***

| Access on No | Mutagen Dose | Treatment | No of nodal explants rrad ated | No of plants produced | Percentage plant regenerat on | Shoot length (cm) | Root length (cm) | Number of leaves |
|---|---|---|---|---|---|---|---|---|
| IC 566503 | 0 Gy | $D_0$ | 20 | 5 | 25 00 | 3 30±0 34 | 1 70±0 34 | 5 20±1 11 |
| | 5 Gy | D | 20 | 3 | 15 00 | 3 17±0 44 | 1 00±0 28 | 3 33±0 33 |
| | 10 Gy | $D_2$ | 20 | 2 | 10 00 | 4 50±0 50 | 1 75±0 25 | 4 50±0 50 |
| | 15 Gy | $D_3$ | 20 | 3 | 15 00 | 5 50±0 50 | 1 50±0 41 | 7 00±2 00 |
| IC 566508 | 0 Gy | $D_0$ | 20 | 5 | 25 00 | 3 70±0 51 | 1 80±0 12 | 5 20±0 86 |
| | 5 Gy | D | 20 | 4 | 20 00 | 7 25±1 01 | 2 75±0 59 | 7 50±2 32 |
| | 10 Gy | $D_2$ | 20 | 9 | 45 00 | 4 83±0 86 | 3 06±0 31 | 4 22±0 57 |
| | 15 Gy | $D_3$ | 20 | 2 | 10 00 | 8 00±1 00 | 3 00±0 | 6 50±0 50 |
| IC 566514 | 0 Gy | $D_0$ | 20 | 4 | 20 00 | 6 87±0 66 | 2 38±0 43 | 8 25±1 31 |
| | 5 Gy | D | 20 | 2 | 10 00 | 7 75±0 25 | 3 50±0 50 | 9 00±2 12 |
| | 10 Gy | $D_2$ | 20 | 3 | 15 00 | 4 67±1 30 | 2 17±0 60 | 4 00±0 57 |
| | 15 Gy | $D_3$ | 20 | 2 | 10 00 | 4 25±1 75 | 2 50±1 75 | 5 50±0 50 |


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Table 48 **Effect of hardening and acclimatisation of plantlets obtained from irradiated nodal cultures of *Plumbago rosea***

| Accession No | Mutagen Dose | Treatment | No of nodal explants irradiated | No of plants produced | No survived in cups | Primary hardening (%) | No of plants transferred to bags | No of plants survived in bags | Secondary hardening (%) |
|---|---|---|---|---|---|---|---|---|---|
| IC 566503 | 0 Gy | $D_0$ | 20 | 5 | 3 | 15 0 | 3 | 1 | 5 0 |
| | 5 Gy | D | 20 | 3 | 3 | 15 0 | 3 | 2 | 10 0 |
| | 10 Gy | $D_2$ | 20 | 2 | 2 | 10 0 | 2 | 1 | 5 0 |
| | 15 Gy | $D_3$ | 20 | 3 | 2 | 10 0 | 2 | 1 | 5 0 |
| IC 566508 | 0 Gy | $D_0$ | 20 | 5 | 3 | 15 0 | 3 | 3 | 15 0 |
| | 5 Gy | D | 20 | 4 | 4 | 20 0 | 4 | 4 | 20 0 |
| | 10 Gy | $D_2$ | 20 | 9 | 5 | 25 0 | 5 | 5 | 25 0 |
| | 15 Gy | $D_3$ | 20 | 2 | 1 | 5 0 | 1 | 0 | 0 0 |
| IC 566514 | 0 Gy | $D_0$ | 20 | 4 | 2 | 20 0 | 2 | 0 | 0 0 |
| | 5 Gy | D | 20 | 2 | 2 | 10 0 | 2 | 1 | 5 0 |
| | 10 Gy | $D_2$ | 20 | 3 | 1 | 5 0 | 1 | 1 | 5 0 |
| | 15 Gy | $D_3$ | 20 | 2 | 0 | 0 0 | 0 | 0 | 0 0 |

**4 3 4 1 5 Effect of gamma radiation on culture response in callus cultures**

The regeneration percentage of six weeks old irradiated callus cultures of three genotypes of *Plumbago rosea* was used to calculate LD $_0$ value of gamma radiation under experimental conditions The data on the effect of different doses of gamma radiation on regeneration of callus is presented in Table 49 The gamma irrad ation dose $D_0$ (control) recorded maximum regeneration of multiple shoots (100 0 per cent) followed by $D_1$ 10 Gy (90 per cent) while $D_5$ 50 Gy recorded no shoot regeneration Fifty per cent cultures of $D_4$ (40 Gy) showed multiple shoot bud initiation when transferred to shoot proliferation medium The percentage shoot elongation and root initiation in the cultures when transferred to elongation cum rooting medium as een from tl e table is maximum for control ($D_0$ 100 0 per cent) followed by D and $D_2$ (80 0 per cent) and 70 0 percent of the cultures in $D_3$ None of the callus cultures of treatment $D_4$ (40 Gy) showed shoot elongat on instead tl e e was rooting in 50 0 percent cultures without shoot elongation Hence the $LD_{50}$ was fixed as dose 40 Gy

**4 3 4 1 6 Regeneration response of callus cultures for optimum doses of gamma rays**

Three doses below 40 Gy namely 10Gy 20 Gy and 30Gy were fixed as optimum doses for callus culture Twenty pieces of calli of each of the *P rosea* accession were treated with respective optimum doses of gamma rays *viz* $D_0$ (0 Gy) $D_1$ (10 Gy) $D_2$ (20 Gy) and $D_3$ (30 Gy) The proliferated calli from eacl treatment were sub cultured to shoot elongation cum rooting medium The effect of gamma radiation on regeneration of calli cultures of each *P rosea* accession are presented in Table 50

In the accession IC 566503 100 0 per cent regeneration of callus was recorded in the cultures of control dose $D_0$ (0 Gy) and $D_1$ (10 Gy) and the cultures of $D_3$ (30 Gy) recorded minimum shoot regeneration (60 0 per cent) The treatment $D_1$ (10 Gy) induced maximum percentage of shoot elongation and rooting (88 0) The treatment $D_3$ (30 Gy) induced minimum percentage of shoot elongation and rooting (36 0)

In accession IC 566508 a maximum of 100 0 per cent regeneration of callus was recorded in the cultures of control dose ($D_0$) The treatment $D_3$ (30 Gy) recorded

**Table 49 Effect of gamma radıatıon on culture response ın callus cultures of *P rosea***

| Mutagen dose (Gamma radıat on ) | Treatment | No of callı p eces treated | No showıng shoot regenarat on | Regenerat on percentage | No of cultures n shoot cum elogat on med um | No of cultures show ng shoot elongat on & root ın t tatıon | Percentage cultures show ng elongat on& root ng |
|---|---|---|---|---|---|---|---|
| 0 Gy | $D_0$ | 10 | 10 | 100 0 | 10 | 10 | 100 0 |
| 10 Gy | D | 10 | 9 | 90 0 | 10 | 8 | 80 0 |
| 20 Gy | $D_2$ | 10 | 7 | 70 0 | 10 | 8 | 80 0 |
| 30 Gy | $D_3$ | 10 | 7 | 70 0 | 10 | 7 | 70 0 |
| 40 Gy | $D_4$ | 10 | 5 | 50 0 | 10 | 5* | 50 0* |
| 50 Gy | $D_5$ | 10 | 0 | 0 0 | 10 | 0 | 0 0 |

* Only root ın t atıon & no sl oot elongat on


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**Table 50 Regeneration response in callus culture of *P rosea* for optimum doses of gamma radiation**

| Accession No | Mutagen Dose | Treatment | No of calli pieces treated | No of cultures showing multiple shoot initiation | Regeneration percentage | No of cultures in elongation cum rooting medium* | No of cultures showing elongation & rooting | Percentage elongation & rooting |
|---|---|---|---|---|---|---|---|---|
| IC 566503 | 0 Gy | $D_0$ | 20 | 20 | 100 0 | 25 | 18 | 72 0 |
| | 10 Gy | $D_1$ | 20 | 20 | 100 0 | 25 | 22 | 88 0 |
| | 20 Gy | $D_2$ | 20 | 16 | 80 0 | 25 | 20 | 80 0 |
| | 30 Gy | $D_3$ | 20 | 12 | 60 0 | 25 | 9 | 36 0 |
| IC 566508 | 0 Gy | $D_0$ | 20 | 20 | 100 0 | 25 | 23 | 92 0 |
| | 10 Gy | $D_1$ | 20 | 16 | 80 0 | 25 | 20 | 80 0 |
| | 20 Gy | $D_2$ | 20 | 16 | 80 0 | 25 | 18 | 72 0 |
| | 30 Gy | $D_3$ | 20 | 14 | 60 0 | 25 | 6 | 24 0 |
| IC 566514 | 0 Gy | $D_0$ | 20 | 20 | 100 0 | 25 | 22 | 88 0 |
| | 10 Gy | D | 20 | 16 | 80 0 | 25 | 19 | 76 0 |
| | 20 Gy | $D_2$ | 20 | 16 | 80 0 | 25 | 21 | 84 0 |
| | 30 Gy | $D_3$ | 20 | 12 | 70 0 | 25 | 10 | 24 0 |

*Culture tubes as well as flasks jam bottle were used


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minimum regeneration percentage of 60 0 There was maximum percentage of elongation and rooting in the control treatment $D_0$ (92 0) The treatment $D_3$ recorded minimum percentage of elongation and rooting (24 0)

In accession IC 566514 the contiol treatment ($D_0$) recorded maximum regeneration of callus (100 0 per cent) in proliferation medium The treatment $D_3$ recorded minimum regeneration percentage of 70 0 There was maximum percentage of elongation and rooting in the control treatment $D_0$ (88 0) The treatment $D_3$ recorded minimum percentage of elongation and rooting (24 0)

In general the cultures of treatment $D_3$ (30 Gy) in all the three accessions showed less elongation and rooting in the semi solid rooting medium Hence such cultures were transferred to liquid rooting medium In accession ICC 566514 such a phenomenon was observed in the cultures of treatment D (10Gy) as well Hence these were transferred to liquid rooting medium

**4 3 4 1 7 Plant regeneration percentage after *in vitro* mutagenesis of callus culture**

The effect of gamma radiation on the number of plantlets produced per irradiated callus and the shoot as well as root development in the plantlets is given in Table 51 In accession IC 566503 the treatment D (10 Gy) produced maximum n nber of plantlets (33) with a maximum percentage of plant regeneration per callus (165 0) Tl e treatment $D_3$ (30 Gy) produced least number of plants (9) with min mum plant regeneration percentage (45 0) There was significant difference among the radiation doses for shoot length per plantlet The DMRT at 5 per cent level of signif cance showed the treatments D2 (20 Gy) and $D_2$ (30 Gy) producing maximum average shoot length 9 67cm and 9 61cm respect vely The treatment D (10 Gy) produced plantlets of lowest average shoot length (5 67 cm) There were no significant differences among the treatments for average root length and average number of leaves produced per plantlet

In accession IC 566508 the control treatment (0 Gy) produced maximum number of 40 plants with maximum plant regeneration percentage (200 0) The treatment $D_3$ (30 Gy) produced least number of plants (6) with minimum plant

**Table 51 Effect of optımum doses of gamma radıatıon on percentage regeneratıon and development of plant from callus cultures of *P rosea***

| Access on No | Mutagen Dose | Treatment | No of callus p eces ırrad ated | No of plants produced | Percentage plant regenerat on | Shoot length | Root length | Number of leaves |
|---|---|---|---|---|---|---|---|---|
| IC 566503 | 0 Gy | $D_0$ | 20 | 17 | 85 0 | 7 18ab±1 09 ab | 3 78±0 52 | 6 47±0 88 |
| | 10 Gy | D | 20 | 33 | 165 0 | 5 83±0 34 b | 3 06±0 28 | 5 79±0 49 |
| | 20 Gy | $D_2$ | 20 | 23 | 115 0 | 9 11±0 88 a | 3 20±0 23 | 6 74±0 60 |
| | 30 Gy | $D_3$ | 20 | 9 | 45 0 | 9 67±0 87 a | 2 94±0 91 | 6 44±1 15 |
| Fıgures carry ng same alpl abets do not d ffer s gn f cantly at 5% level based on DMRT | | | | | | | | |
| IC 566508 | 0 Gy | $D_0$ | 20 | 40 | 200 0 | 6 10±0 48 | 3 45±0 18 a | 6 55±0 55 |
| | 10 Gy | D | 20 | 28 | 140 0 | 7 37±0 77 | 2 91±0 19 ab | 6 71±0 55 |
| | 20 Gy | $D_2$ | 20 | 18 | 90 0 | 5 58±0 69 | 2 39±0 37 b | 6 22±0 79 |
| | 30 Gy | $D_3$ | 20 | 6 | 30 0 | 6 25±0 98 | 2 25±0 25 b | 5 67±0 88 |
| F gures carryıng same alphabets do not d ffer s gn ficantly at 5% level based on DMRT | | | | | | | | |
| IC 566514 | 0 Gy | $D_0$ | 20 | 32 | 160 0 | 4 92±0 43b | 3 17±0 17 ab | 5 44±0 40 b |
| | 10 Gy | D | 20 | 25 | 125 0 | 5 448±0 57b | 4 27±0 31 a | 5 64±0 52 b |
| | 20 Gy | $D_2$ | 20 | 30 | 150 0 | 5 95±0 55b | 2 9±0 53 b | 6 37±0 55 b |
| | 30 Gy | $D_3$ | 20 | 10 | 50 0 | 8 95±1 2a | 3 6±0 36 b | 8 10±0 78 a |

Fıgures carry ng same alpl abets do not d ffer s g f cantly at 5% level based on DMRT


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regeneration percentage (30 0) All the treatment doses showed significant difference for the character root length per plantlet The control plantlets had longest root with an average length of 3 45 cm The treatments $D_2$ (10 Gy) and $D_3$ (30 Gy) produced lowest average root length of 2 39cm and 2 25cm respectively There were no significant differences among the treatments for average shoot length and average number of leaves produced per plantlet

In accession IC 566514 the control treatment D0 (0 Gy) produced maximum number of 32 plants with maximum plant regeneration percentage (160 0) The treatment $D_3$ (30 Gy) produced least number of plants (10) with minimum plant regeneration percentage (50 0) All the treatments differed significantly for the three characters *viz* root length shoot length and number of leaves per plantlet The treatment $D_3$ produced plantlets with maximum average shoot length of 8 95cm and number of leaves (8 1) The treatments $D_1$ (10 Gy) and $D_2$ (20 Gy) were at par with the control treatment $D_0$ for average shoot length as well as number of leaves per plantlet The treatment $D_1$ (10 Gy) produced plantlets with longest roots with an average length of 4 27 cm The treatments $D_2$ (20 Gy) and $D_3$ (30 Gy) produced plantlets with shortest roots of average length 2 9 cm and 3 6cm respectively

**4 3 4 1 8 Hardening and acclimatisation**

The survival percentages of plants obtained from irradiated callus cultures of the three *P rosea* accessions on primary and secondary hardening were recorded The results of hardening and acclimatisation are presented in Table 52 In accession IC 566503 there was maximum percentage survival of plants (88 24 per cent) in the control treatment ($D_0$) on primary hardening The treatment $D_2$ (20 Gy) recorded lowest percentage survival (34 78 percent) of plants when transferred to cups Under net house conditions all plants in treatment $D_1$ (10 Gy) survived hardening when transferred to bags (60 61 per cent) while none of the plants in treatment $D_3$ survived hardening when transferred to bags

In accession IC 566508 maximum percentage of plants could be transferred from disposable cups to bags (100 0 per cent) in treatment $D_3$ followed by treatment $D_2$ (83 3 per cent) In the control treatment $D_0$ minimum percentage of plants (52 5) could be transferred from cups to bags Under net house conditions there was

**Table 52 Effect of hardening and acclimatisation of plantlets obtained from irradiated callus cultures of *Plumbago rosea***

| Accession No | Mutagen Dose | Treatment | No of plants produced | No survived in cups | Primary hardening (%) | No of plants transferred to bags | No of plants survived in bags | Secondary hardening (%) |
|---|---|---|---|---|---|---|---|---|
| IC 566503 | 0 Gy | $D_0$ | 17 | 15 | 88 24 | 15 | 10 | 58 82 |
| | 10 Gy | D | 33 | 20 | 60 61 | 20 | 20 | 60 61 |
| | 20 Gy | $D_2$ | 23 | 8 | 34 78 | 8 | 6 | 34 78 |
| | 30 Gy | $D_3$ | 9 | 7 | 77 78 | 7 | 0 | 0 |
| IC 566508 | 0 Gy | $D_0$ | 40 | 21 | 52 5 | 21 | 20 | 52 50 |
| | 10 Gy | $D_1$ | 28 | 19 | 67 86 | 19 | 6 | 21 40 |
| | 20 Gy | $D_2$ | 18 | 15 | 83 33 | 15 | 11 | 61 10 |
| | 30 Gy | $D_3$ | 6 | 6 | 100 | 6 | 0 | 0 00 |
| IC566514 | 0 Gy | $D_0$ | 32 | 23 | 71 88 | 23 | 17 | 53 13 |
| | 10 Gy | D | 25 | 19 | 76 | 19 | 14 | 56 00 |
| | 20 Gy | $D_2$ | 30 | 17 | 56 67 | 17 | 11 | 36 67 |
| | 30 Gy | $D_3$ | 10 | 10 | 100 | 10 | 6 | 60 00 |

maximum percentage survival (61 10) of plants in treatment $D_2$ (20 Gy) There was minimum percentage survival (0 00 per cent) of plants in the treatment $D_3$

In accession IC 566514 the treatment $D_3$ (30 Gy) recorded maximum percentage survival (100 0 per cent) of plants followed by $D_1$ (76 00 per cent) on primary hardening On secondary hardening under the net house conditions treatment $D_1$ (10 Gy) recorded maximum survival of plants (56 00 per cent) followed by control treatment $D_0$ (53 13 per cent) The treatment $D_3$ recorded minimum percentage survival (0 00 percent) of plants in bags under net house conditions

Of the three optimum doses of gamma radiation *viz* 10Gy 20Gy and 30 Gy hardened plants could be obtained from only from the first two doses i e 10 Gy and 20Gy of treatment in all the three accessions of *P rosea* Besides hardened plants were obtained from control treatment as well

**4 3 4 1 9 Mean performance of plants obtained from irradiated callus cultures**

The mean values for five morphological characters in the somaclones of three accessions of *Plumbago rosea* are presented in Table 53

i) Plant height There was significant difference between the treatment doses in two accessions namely IC 566503 and IC 566508 In accession IC 566503 the control plants were found to be superior with maximum height of 15 10 cm The plants obtained from treatment D (10 Gy) were the shortest (8 45 cm) In accession IC 566508 the control plants and plants from 20Gy treatment dose were found to be superior and the plants belonging to treatment $D_1$ (10 Gy) were the shortest

ii) Internode Length In all the accessions there were no significant differences among the treatment doses for this character

iii) Number of leaves The gamma radiation doses showed significant difference for this character in the two accessions namely IC 566508 and IC 566514 In accession IC 566508 the plants from 20 Gy produced maximum number leaves (12 82) The plants from 10 Gy had least number of leaves (6 83) In genotype IC 566514 the treatments $D_1$ (10Gy) and $D_2$ (20 Gy) produced plantlets with maximum number of

**Table 53 Mean performance of plants obtaıned from ırradıated callus cultures**

| Access on No | Mutagen Dose | Treatment | No of plants from secondary harden ng | Plant he ght | Internode length | Numbe of leaves | Leaf length | Leaf breadth |
|---|---|---|---|---|---|---|---|---|
| IC 566503 | 0 Gy | D0 | 10 | 15 10±3 5a | 1 60±0 36 | 12 90±1 8 | 6 31±0 88a | 3 86±0 53a |
| | 10 Gy | D1 | 20 | 8 45b±0 87b | 0 89±0 12 | 9 70±0 74 | 4 18±0 37b | 2 42±0 21b |
| | 20 Gy | D2 | 6 | 10 33±1 25ab | 1 50±0 36 | 8 67±0 95 | 3 86±0 67b | 2 42±0 46b |
| IC 566508 | 0 Gy | D0 | 20 | 8 75±0 67a | 1 16±0 13 | 10 40±0 97ab | 3 51±0 21 | 2 19±0 19 |
| | 10 Gy | D1 | 6 | 5 17±0 91b | 0 52±0 10 | 6 83±0 75b | 3 17±0 32 | 2 08±0 19 |
| | 20 Gy | D2 | 11 | 11 36±1 39a | 1 01±0 19 | 12 82±1 19a | 3 03±0 56 | 1 82±0 35 |
| IC566514 | 0 Gy | D0 | 17 | 8 41±1 57 | 0 88±0 21 | 7 55±1 21b | 3 39±0 56 | 2 08±0 39 |
| | 10 Gy | D1 | 14 | 8 61±1 27 | 1 04±0 14 | 12 71±1 81a | 4 11±0 48 | 2 48±0 35 |
| | 20 Gy | D2 | 11 | 7 73±1 06 | 0 74±0 26 | 12 64±1 05a | 4 13±0 45 | 2 58±0 29 |

F gures carry ng same alphabets do not d ffer s gnıficantly at the 5% level based on DMRT (IC 566503)

F gures carryıng same alphabets do not dıffer s gnıf cantly at the 5% level based on DMRT (IC 566508)

F gures carry ng same alphabets do not d ffer s gn f cantly at the 5% level based on DMRT (IC566514)

leaves (12 71 and 12 64) The control plants ($D_0$) produced least number of leaves (7 55)

iv) Leaf length  For this character there was significant difference among the treatments only in the genotype IC 566503 The control plants produced long leaves (6 31 cm ) The treatment doses 10 Gy and 20 Gy produced leaves of 4 18 and 3 86cm length respectively

v) Leaf breadth  For this character also there were treatment differences in the accession IC 566503 The control plants produced broad leaves (3 86 cm) and the treatment doses 10 Gy and 20 Gy with leaves of lowest mean width (2 42cm)

The analysis of variance in the somaclones (three months old) of the three accessions of *Plumbago rosea* namely IC 566503 IC 566508 and IC 566514 thus revealed significant differences among the gamma radiation doses $D_0$(0 Gy) $D_1$(10 Gy) and $D_2$ (20 Gy) for three morphological characters The somaclones of accession IC 566503 obtained from three doses of mutation showed significant differences for plant height and leaf size In accession IC 566508 the somaclones obtained from *in vitro* mutagenesis differed significantly for plant height and number of leaves The *in vitro* mutated somaclones of genotype IC 566514 showed significant differences for number of leaves The somaclones showing superiority for leaf size and number of leaves were selected from each of the three treatment doses ($D_0$ $D_1$ and $D_2$) of the three *P rosea* accessions and carried over to experiment V for field evaluation

### 4 3 4 1 10 Comparison of somaclones selected from *in vitro* mutagenesis with conventionally rooted cuttings of *P rosea*

Before field establishment the performance of the somaclones were compared with the conventional rooted cuttings of three *P rosea* accessions for five morphological characters and the results are presented Table 54 The somaclones of accessions IC 566503 and IC 566508 showed significant difference for characters whereas in genotype IC 566514 the somaclones did not differ significantly with the conventional clones for any of the characters studied

i) Plant height There was significant difference between the somaclones and conventional clones only in IC 566503 The somaclones of control treatment $D_0$ (0 Gy) were superior with maximum average height of 28 67cm followed by the plants of D (10 Gy) with a mean height of 13 33 cm The plants obtained from treatment $D_2$ (20 Gy) were at par with the conventional clones for plant height

ii) Internode Length For this character also the genotype IC 566503 alone showed significant difference between somaclones and conventional clones The somaclones of $D_0$ (0 Gy) were superior with maximum average internode length of 3 00cm The plants of $D_2$ (20 Gy) with a mean internode length of 2 17cm were at par with the conventional clones (1 50 cm)

iii) Number of leaves The somaclones of the two access ons IC 566503 and IC 566508 showed significant difference for this character when compared with the conventional clones In accession IC 566503 the somaclones from $D_0$ (0 Gy) produced maximum number of leaves (20 0) followed by the somaclones from $D_1$ (10 Gy) with 13 67 leaves The conventional clones of IC 566503 had least number of leaves (5 67) In accession IC 566508 the somaclones from treatments $D_0$ (0 Gy) and $D_2$ (20 Gy) with 16 83 and 18 83 respective number of leaves were at par and superior to somaclones of treatment D (10 Gy) with 8 83 number of leaves and the conventional clones with least number of leaves (4 33)

v) Leaf length For this character there was significant difference between the somaclones and the conventional clones in the accession IC 566503 The somaclones from control treatment D0 (0 Gy) produced leaves with maximum average length of 9 44 cm The conventional clones and the somaclones from treatment doses 10 Gy and 20 Gy were at par for this character In the accessions IC 566508 and IC 566514 the somaclones were at par with the conventional clones for this tra t

vi) Leaf breadth There was significant difference between the somaclones and conventional clones in accessions IC 566503 and IC 566508 In accession IC 566503 the somaclones of controlled treatment produced broad leaves (5 77 cm) whereas the conventional clones and the somaclones of 10 Gy and 20 Gy were at par In accession

**Table 54 Comparison of somaclones selected from *in vitro* mutagenesis and conventional rooted cuttings**

| Accession No | Treatment | *In vitro* Mutagen Dose | Plant height (cm) | Internode length (cm) | Number of leaves | Leaf length | Leaf breadth |
|---|---|---|---|---|---|---|---|
| IC 566503 | somacloneD$_0$ | 0 Gy | 28 67 | 3 00 | 20 00 | 3 68 | 2 20 |
| | somacloneD | 10 Gy | 13 33 | 1 50 | 13 67 | 3 75 | 2 15 |
| | somacloneD$_2$ | 20 Gy | 10 67 | 2 17 | 9 67 | 2 11 | 1 41 |
| | Conventional rooted cutting | | 6 33 | 0 73 | 5 67 | 6 53 | 3 77 |
| CD at 5% level of significance | | | 4 72 | 1 29 | 4 9 | 2 83 | 1 44 |
| IC 566508 | somacloneD$_0$ | 0 Gy | 12 00 | 1 17 | 16 33 | 3 72 | 2 22 |
| | somacloneD | 10 Gy | 8 33 | 0 43 | 8 33 | 4 62 | 2 63 |
| | somacloneD$_2$ | 20 Gy | 14 00 | 1 07 | 18 33 | 4 90 | 2 80 |
| | Convent onal rooted cutt ng | | 9 67 | 0 70 | 4 33 | 7 60 | 4 37 |
| CD at 5% level of signif cance | | | NS | NS | 7 5 | 2 6 | 1 47 |
| IC566514 | somacloneD$_0$ | 0 Gy | 16 67 | 2 08 | 16 00 | 3 77 | 2 27 |
| | somacloneD | 10 Gy | 8 33 | 1 23 | 10 67 | 4 77 | 2 88 |
| | somacloneD$_2$ | 20 Gy | 15 00 | 1 40 | 21 67 | 2 27 | 1 15 |
| | Conventional rooted cutt ng | | 8 67 | 3 00 | 5 00 | 7 22 | 4 13 |
| CD at 5% level of s gn f cance | | | NS | NS | NS | 2 68 | 1 39 |

IC 566508 conventional clones were superior to the somaclones producing broad leaves of 4 27 cm

#### 4 3 4 2 Chemical Mutagenesis

#### 4 3 4 2 1 Effect of varying concentrations of EMS on culture response in nodal cultures

For estimation of $LD_{50}$ of the chemical mutagen EMS in nodal culture two weeks old sprouted explants (20 nos ) were soaked in various concentrations of EMS solution for one hour As seen from Table 55 the concentration of EMS beyond 0 25 per cent resulted in death of cultures In 0 25 percent concentration of EMS only 50 per cent cultures survived Hence the $LD_{50}$ was considered as 0 25 per cent However there was high rate of contamination in cultures especially when subcultured to elongation medium This resulted in loss of cultures The percentage of cultures that could be carried over to rooting medium ranged between 10 and 20

#### 4 3 4 2 2 Regeneration response of nodal cultures for optimum doses of EMS

Three doses below 0 25 per cent namely 0 05 per cent 0 10 per cent and 0 15 per cent were fixed as optimum doses of EMS Twenty cultures of *P rosea* were soaked for one hour in the three concentrations of EMS There was control treatment consisting of 0 0 per cent EMS However within one week of treatment all the cultures were contaminated The treatment was repeated but could not be successfully carried forward due to contamination of the cultures

#### 4 3 4 2 3 Effect of varying concentrations of EMS on culture response in callus cultures

Twenty pieces of six weeks old callus of three accessions of *Plumbago rosea* ((IC 566503 IC 566508 and IC 566514) were used to calculate $LD_{50}$ value of EMS by soaking in various concentrations of EMS for one hour The $LD_{50}$ value could not be calculated as there was 100 per cent contamination of cultures The study could not be carried forward due to complete exhaustion of callus cultures The chemical mutagenesis had to be abandoned for callus cultures not only due to contamination but also for the long period of incubation required for induction of callus in leaf cultures

**Table 55 Effect of different concentrations of EMS on culture response in nodal cultures of *P rosea***

| Treatment | Conc of EMS (%) | No of nodal cultures | No of cultures showing shoot proliferation | Shoot regeneration percentage | No of cultures in shoot elongation medium | No of cultures showing shoot elongation | Percentage cultures showing shoot elongation | No of cultures in rooting medium | No of cultures showing root initiation | Percentage cultures showing root induction |
|---|---|---|---|---|---|---|---|---|---|---|
| $DE_0$ (Control) | 0 00 | 10 | 8 | 80 0 | 8 | 3 | 37 5 | 3 | 2 | 20 0 |
| DE | 0 05 | 10 | 6 | 60 0 | 6 | 2 | 20 0 | 2 | 1 | 10 0 |
| $DE_2$ | 0 10 | 10 | 6 | 60 0 | 6 | 1 | 10 0 | 1 | 1 | 10 0 |
| $DE_3$ | 0 25 | 10 | 5 | 50 0 | 5 | 0 | 0 0 | 0 | 0 | 0 0 |
| $DE_4$ | 0 50 | 10 | 0 | 0 0 | 0 | 0 | 0 0 | 0 | 0 | 0 0 |
| $DE_5$ | 0 75 | 10 | 0 | 0 0 | 0 | 0 | 0 0 | 0 | 0 | 0 0 |


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## 4 4 Induction of polyploidy in the *Plumbago* species

In this experiment a preliminary trial for inducing polyploidy in the rooted stem cuttings of *Plumbago* species was tried The detailed results of the experiment are as follows

### 4 4 1 Preliminary trial to fix the doses of colchicine in *Plumbago* species

The sprouting of axillary bud observed in the preliminary trial in *Plumbago rosea* and *Plumbago zeylanica* are presented in Tables 56 and 57 respectively From the table it can be seen that there was sprouting in more than 50 per cent cuttings of *P rosea* for the concentration ($C_4$) of 1 0 per cent colchicine applied for D (3 hours) and $D_2$ (6 hours) duration for two consecutive days However in *Plumbago zeylanica* the colchicine concentation of 0 75 per cent ($C_3$) applied for the same period of duration resulted in sprouting of more than 50 per cent cuttings The control plants of both species took only one week to sprout As the concentration of colchicine increased from $C_1$(0 25%) to $C_4$ (1 0 %) the number of days for sprouting showed a respective increase from 9 days to 15 days in both the species of *Plumbago* There was no survival of the axillary bud beyond 1 0 per cent concentration of colchicine Hence a dose of 0 75 per cent concentration ($C_3$) of colchicine was fixed for application in both species of *Plumbago*

### 4 4 2 Survival of plants with optimum dose of colchicine in *Plumbago* species

The survival of the treated axillary buds after treatment with 0 75 per cent ($C_3$) of colchicine for $D_1$ (3 hours) and $D_2$ (6 hours) duration for one week is presented in Table 58 In case of *Plumbago rosea* more than 50 per cent plants survived when observed after two weeks of colchicine application In *Plumbago zeylanica* only 4 cuttings (44 4 per cent) survived for the $D_2$ duration of colchicine application Three of the survived cuttings from D (3hours) and $D_2$(6 hours) duration of colchicine application for the concentration ($C_3$ 0 75 per cent) as well as the absolute control i e untreated plants from both the species of *Plumbago* were transferred to pots and maintained in the net house The growth of the treated plants were observed critically in comparison with the untreated control plants

**Table 56 Preliminary trial for fixing doses of colchicine in *Plumbago rosea***

| Treatment | Conc of colchicine (%) | Duration* | Number of cuttings treated | Number of cuttings showing axillary sprouting | Percentage survival | Mean number of days to sprout | Duration* | Number of cuttings treated | Number of cuttings showing axillary sprouting | Percentage survival | Mean number of days to sprout |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $C_0$ | 0 00 | $D_1$ | 9 | 9 | 100 00 | 8 | $D_2$ | 9 | 9 | 100 00 | 9 |
| $C_1$ | 0 25 | 3hours | 9 | 9 | 100 00 | 11 | 6hours | 9 | 9 | 100 00 | 9 |
| $C_2$ | 0 50 | | 9 | 9 | 100 00 | 12 | | 9 | 9 | 100 00 | 12 |
| $C_3$ | 0 75 | | 9 | 8 | 88 80 | 13 | | 9 | 7 | 77 70 | 14 |
| $C_4$ | 1 00 | | 9 | 7 | 77 70 | 13 | | 9 | 5 | 55 50 | 15 |
| $C_5$ | 1 50 | | 9 | all dried | 0 00 | Nil | | 9 | all dried | 0 00 | Nil |
| $C_6$ | 2 00 | | 9 | all dried | 0 00 | Nil | | 9 | all dried | 0 00 | Nil |

* For two consecutive days

**Table 57 Preliminary trial for fixing doses of colchicine in *Plumbago zeylanica***

| Treatment | Conc of colchicine (%) | Duration* | Number of cuttings treated | Number of cuttings showing axillary sprouting | Percentage survival | Mean number of days to sprout | Duration* | Number of cuttings treated | Number of cuttings showing axillary sprouting | Percentage survival | **Mean number of days to sprout** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $C_0$ | 0 00 | D | 9 | 9 | 100 00 | 7 | $D_2$ | 9 | 9 | 100 00 | 7 |
| C | 0 25 | 3hours | 9 | 9 | 100 00 | 9 | 6hours | 9 | 9 | 100 00 | 10 |
| $C_2$ | 0 50 | | 9 | 9 | 100 00 | 12 | | 9 | 9 | 100 00 | 13 |
| $C_3$ | 0 75 | | 9 | 7 | 77 70 | 13 | | 9 | 6 | 66 60 | 14 |
| $C_4$ | 1 00 | | 9 | 5 | 55 50 | 13 | | 9 | 3 | 33 30 | 15 |
| $C_5$ | 1 50 | | 9 | all dried | 0 00 | Nil | | 9 | all dried | 0 00 | Nil |
| $C_6$ | 2 00 | | 9 | all dried | 0 00 | Nil | | 9 | all dried | 0 00 | Nil |

* For two consecutive days

**Table 58 Survival and sprouting of axillary buds with optimum dose of colchicine in *Plumbago* species**

| Conc of colchicine(%) | Duration* | Number of cuttings treated | Number of cuttings showing axillary sprouting | Percentage survival | Mean number of days to sprout | Number of cuttings treated | Number of cuttings showing axillary sprouting | Percentage survival | Mean number of days to sprout |
|---|---|---|---|---|---|---|---|---|---|
| *Plumbago rosea* | | | | | | *Plumbago zeylanica* | | | |
| $C_0$ Absolute control | | 9 | 9 | 100 | 7 | 9 | 9 | 100 | 7 |
| $C_3$ | $D_1$ 3hours | 9 | 6 | 66 6 | 13 | 9 | 5 | 55 5 | 14 |
| $C_3$ | $D_2$ 6 hours | 9 | 5 | 55 5 | 14 | 9 | 4 | 44 4 | 15 |

* For seven consecutive days

### 4 4 2 1 Growth of colchicine treated and control plants in *Plumbago* species

The branch grown from the colchicine treated axillary bud was compared with the branch of the control plant for the four morphological characters namely branch length internode length leaf length and leaf breadth in both the species of *Plumbago* and the results are presented in Tables 59 and 60

**Table 59 Mean performance of colchicine treated axillary branches of *P rosea***

**Branch length**

| Treatment | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $C_0$ | 12 67 | 38 33 | 62 33 | 83 33 |
| $C_3D_1$ | 15 00 | 48 00 | 73 67 | 113 00 |
| $C_3D_2$ | 10 00 | 31 67 | 58 67 | 99 67 |
| CD at 5% level | NS | NS | NS | NS |
| Mean of control | 12 67 | 38 33 | 62 33 | 83 33 |
| Mean of treatments | 12 50 | 39 33 | 66 17 | 106 33 |

**Internode length**

| Treatment | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $C_0$ | 0 83 | 3 33 | 4 00 | 5 50 |
| $C_3D_1$ | 2 17 | 4 50 | 5 83 | 6 50 |
| $C_3D_2$ | 1 67 | 4 33 | 5 67 | 6 17 |
| CD at 5% level | NS | NS | NS | NS |
| Mean of control | 0 83 | 3 33 | 4 00 | 5 50 |
| Mean of treatments | 1 92 | 4 42 | 5 75 | 6 33 |

**Leaf length**

| Treatment | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $C_0$ | 6 50 | 8 13 | 8 32 | 8 60 |
| $C_3D$ | 5 72 | 7 83 | 7 81 | 7 90 |
| $C_3D_2$ | 6 17 | 6 60 | 8 05 | 9 18 |
| CD at 5% level | NS | NS | NS | NS |
| Mean of control | 6 50 | 8 13 | 8 32 | 8 60 |
| Mean of treatments | 5 94 | 7 22 | 7 93 | 8 54 |

**Leaf breadth**

| Treatment | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $C_0$ | 3 44 | 3 55 | 4 44 | 4 83 |
| $C_3D_1$ | 3 17 | 3 71 | 3 99 | 4 15 |
| $C_3D_2$ | 3 33 | 3 43 | 4 05 | 5 24 |
| CD at 5% level | NS | NS | NS | NS |
| Mean of control | 3 44 | 3 55 | 4 44 | 4 83 |
| Mean of treatments | 3 25 | 3 57 | 4 02 | 4 69 |

In *Plumbago rosea* there was no appreciable difference between the treated and the untreated plants for all the characters through out the growing season In *Plumbago zeylanica* during the second quarter the plants of treatment $C_3$ $D_1$ showed an increased branch length (42 7cm) and leaf size (length 11 00cm breadth 6 07cm) compared to control ($C_0$) plants and plants of $C_3D_2$ dose of colchicine treatment

**Table 60 Mean performance of colchicine treated axillary branches of *P zeylanica***

**Branch length**

| Treatment | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $C_0$ | 16 67 | 29 33 | 9ɔ 33 | 157 00 |
| $C_3D$ | 22 00 | 42 17 | 121 33 | 166 33 |
| $C_3D_2$ | 15 67 | 36 67 | 81 67 | 151 67 |
| CD at 5% level | NS | 6 89 | NS | NS |
| Mean of control | 16 67 | 29 33 | 93 33 | 157 00 |
| Mean of treatments | 18 83 | 39 42 | 101 5 | 159 |

**Internode length**

| Treatment | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $C_0$ | 1 33 | 3 33 | 5 67 | 7 63 |
| $C_3D_1$ | 2 17 | 4 67 | 7 00 | 8 17 |
| $C_3D_2$ | 1 50 | 3 97 | 6 33 | 7 33 |
| Mean of control | 1 33 | 3 33 | 5 67 | 7 63 |
| Mean of treatments | 1 83 | 4 32 | 6 67 | 7 75 |

**Leaf length**

| Treatment | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $C_0$ | 6 80 | 8 53 | 8 83 | 8 93 |
| $C_3D_1$ | 7 12 | 11 00 | 9 83 | 9 50 |
| $C_3D_2$ | 6 70 | 9 00 | 9 00 | 8 87 |
| CD at 5% level | NS | 1 39 | NS | NS |
| Mean of control | 6 80 | 8 53 | 8 83 | 8 93 |
| Mean of treatments | 6 91 | 10 00 | 9 42 | 9 18 |

**Leaf breadth**

| Treatment | Season I | Season II | Season III | Season IV |
|---|---|---|---|---|
| $C_0$ | 4 20 | 4 43 | 4 07 | 4 33 |
| $C_3D$ | 3 78 | 6 07 | 4 47 | 4 37 |
| $C_3D_2$ | 4 17 | 4 67 | 3 83 | 4 27 |
| CD at 5% level | NS | 0 59 | NS | NS |
| Mean of control | 4 20 | 4 43 | 4 07 | 4 33 |
| Mean of treatments | 3 98 | 5 37 | 4 15 | 4 32 |

However towards the end of the growing season these plants were more or less like the control plants with normal growth rate

**4 4 2 2 Leaf anatomy of colchicine treated and control plants in *Plumbago* species**

The size of epidermal cells and stomata is found to bear a direct correlation with the ploidy level of plants An increase in the size of stomata and epidermal cells is an indication of increase in the number of chromosomes The results of anatomical studies n leaf of the treated and control plants of both the species of *Plumbago* are presented in Tables 61 62 and 63

i) Size of leaf epidermal cells

As seen in Table 61 the treated plants d d not show any difference for the epider nal cell s ze when compared to control plants in both the species of *Plumbago* In *Plumbago rosea* the length of epidermal a cell ranged between 239 33 µm and 250 63µm when observed under microscopic field of 400 X magnif cation The leaf epidermal cells of treated and untreated plants of *Plumbago zeylanica* recorded a higher length ranging from 270 56 µm to 276 61µm

ii) Number and size of stomata

The results on stomatal studies in *P rosea* are presented n Table 62 All the observat ons for stomata were made under a microscopic field with a magnification of 400X The leaves from treated plants did not sl ow any difference for the mean number of stomata in the tip middle and base when compared to the leaves of control plants The mean number of stomata ranged from 10 67 to 11 4 at the tip 16 87 to 18 13 in the middle and 13 0 to 13 33 in the base of the leaves of control and treated plants of *Plumbago rosea* For stomatal size also the treated plants showed no s gnificant difference when compared to control plants The length of the stomatal opening ranged between 157 25 µm and 162 92 µm whereas the width ranged between 60 21 µm and 63 04 µm

In *Plumbago zeylanica* (Table 63) the mean number and size of stomata of the leaves from treated plants did not show any difference when compared with that of the leaves of control plants The mean number and size of stomata per microscopic

field was slightly less than that of *Plumbago rosea* The mean number of stomata ranged from 8 07 to 8 80 at the tip 14 50 to 15 53 in the middle and 11 33 to 11 90 m the base of the leaves of control and treated plants The length of the stomatal opening ranged from 135 29 µm to 138 13 µm whereas the width ranged from 49 58 µm to 52 77 µm in the leaves of control and treated plants

**Table 61 Size of epidermal cells in the leaf of colchicine treated axillary branch of *Plumbago rosea* and *Plumbago zeylanica***

| | *Plumbago rosea* | | *Plumbago zeylanica* | |
|---|---|---|---|---|
| Treatment | Length (µm) | Breadth(µm) | Length(µm) | Breadth(µm) |
| $C_0$ | 250 63 ± 28 6 | 208 96± 22 0 | 270 59± 10 5 | 201 17± 24 5 |
| $C_3D$ | 239 33± 10 6 | 200 89± 7 0 | 276 61± 11 3 | 205 38± 0 3 |
| $C_3D_2$ | 244 57± 14 2 | 201 87± 8 5 | 270 56± 11 2 | 211 08± 4 2 |
| CD at 5% | NS | NS | NS | NS |

**Table 62 Number and size of stomata in the leaf of colchicine treated axillary branch of *Plumbago rosea***

| | Stomatal number per microscopic field (X 400) | | | Stomatal length(µm) | Stomatal width(µm) |
|---|---|---|---|---|---|
| Treatment | Tip | Middle | Base | (X400) | |
| $C_0$ | 10 6± 0 1 | 16 87± 0 3 | 13 00±0 6 | 157 25±3 7 | 63 04±4 6 |
| $C_3D_1$ | 11 4±0 3 | 17 90±0 7 | 13 03±0 5 | 155 13±2 5 | 62 34±4 6 |
| $C_3D_2$ | 11±0 6 | 18 13±0 6 | 13 33±0 6 | 162 92±2 6 | 60 21±3 1 |
| CD at 5% | NS | NS | NS | NS | NS |

**Table 63 Number and size of stomata in the leaf of colchicine treated axillary branch of *Plumbago zeylanica***

| | Stomatal number per microscopic field(X400) | | | Stomatal length(µm) | Stomatal width(µm) |
|---|---|---|---|---|---|
| Treatment | Tip | Middle | Base | (X400) | |
| $C_0$ | 8 8±0 21 | 15 53±0 34 | 11 90±0 32 | 138 13±4 91 | 49 58±4 96 |
| $C_3D_1$ | 8 50±0 29 | 14 43±0 30 | 11 43±0 30 | 135 29±6 75 | 52 77±4 08 |
| $C_3D_2$ | 8 07±0 64 | 14 50±0 36 | 11 33±0 33 | 135 29±3 75 | 50 29±4 31 |
| CD at 5% | NS | NS | NS | NS | NS |

Figures in columns are mean values ± SEM

### 4 4 2 3 Floral characters of colchicine treated and control plants in *Plumbago* species

All the plants (treated as well as control) started flowering in October 2008 in *Plumbago zeylanica* and November 2008 in *Plumbago rosea* The flowering being seasonal in *Plumbago rosea* was completed by the end of February 2009 whereas in *Plumbago zeylanica* the flowering continued after February The treatment of colchicine did not produce any adverse effect on flowering All the treated axillary branches in both the species of *Plumbago* showed normal flowering The flowers were then observed for the fertility of pollen and the size of pollen

#### 4 4 2 3 1 Size and fertility of pollen in the colchicine treated and untreated plants in *Plumbago* species

The results of the study on pollen produced by the colchicine treated and untreated plants in *Plumbago rosea and Plumbago zeylanica* are presented in Table 64 The matured inflorescence buds of treated and the untreated plants produced fertile pollen in both the species The stainability in acetocarmine revealed pollen fertility ranging from 82 31 per cent to 84 94 per cent in *Plumbago rosea* In *Plumbago zeylanica* the mean percentage value of pollen fertility ranged between 88 72 and 90 62 The treated plants also did not differ for mean pollen size when compared with the control plants in both the species of *Plumbago* The species however differed for pollen size The flowers of *Plumbago zeylanica* produced lager pollen of size ranging from 384 86µm to 396 66µm In *Plumbago rosea* the mean pollen size ranged between 323 47µm and 340 0µm

**Table 64 Pollen fertility and size of the treated as well as control plants of *P rosea* and *P zeylanica***

| Treatment | Pollen fertility (%) | | Pollen size (µm)X400 | |
|---|---|---|---|---|
| | *P rosea* | *P zeylanica* | *P rosea* | *P zeylanica* |
| $C_0$ | 84 24 | 92 11 | 337 62 ± 15 47 | 389 58 ±10 82 |
| $C_3D$ | 84 94 | 88 72 | 340 00 ±17 83 | 384 86 ± 9 44 |
| $C_3D_2$ | 82 31 | 90 62 | 323 47 ±12 49 | 396 66 ± 10 91 |
| CD at 5% | NS | NS | NS | NS |

### 4 4 3 Identification of variants/polyploids

On visual observation no marked phenotypic difference could be noticed between the colchicine treated axillary branch and the branch of control plants in both the species of *Plumbago* Even though in *Plumbago zeylanica* a larger branch length and leaf size was noticed in the second quarter of growth among the treated plants of $C_3D$ (0 75per cent colchicine for three hours for seven consecutive days) it was temporary However towards the end of growing season these plants showed normal rate of growth similar to control plants

For all the characters studied no significant difference was observed between the treated and the control plants when subjected to statistical analysis The cotton swab technique of colchicine application had failed to induce polyploidy in *Plumbago* species Since no variants could be identified in this experiment there were no samples to be carried over to the experiment V for field evaluation

### 4 5 Evaluation of samples of *Plumbago rosea* screened from *in vitro* mutagenesis

The samples of the species *Plumbago rosea* from *in vitro* mutagenesis alone could be obtained for evaluation in field The somaclones showing superiority for plant height leaf size and number of leaves were selected from each of the three treatment doses of gamma radiation ($D_0$ 0 Gy D 10 Gy and $D_2$ 20 Gy) for three *P rosea* accessions (IC 566503 IC 566508 and IC 566514) These were planted in field as pot culture laid in CRD and evaluated for morphological characters

### 4 5 1 Morphology of selected somaclones of *Plumbago rosea* obtained from *in vitro* mutagenesis

The analysis of variance was carried out for six morphological characters namely plant height internode length number of branches number of leaves leaf length and leaf breadth with three replications in the three accessions of *Plumbago rosea* (IC 566503 IC 566508 and IC 566514) at two intervals of growth period (Four and eight months after planting) There were differences between somaclones and conventional clones for the morphological traits in the two intervals of growth period

### 4 5 1 1 Mean performance of somaclones and conventional clones four months after planting

The mean performance of the somaclones and the conventional clones for the following six morphological characters four month after planting is presented in Table 65 After four months of planting the soma clones differed in their mean performance when compared with conventional clones The soma clones differed significantly for plant height and leaf size when compared with conventional clones in all the three accessions The somaclones in general showed dwarfing with smaller leaves and were stunted in appearance The mean performance of the somaclones and the conventional clones for the six morphological characters are as follows

i) Plant height For this character the accession IC 566514 alone showed significant difference between somaclones and convent onal clones The conventional clones were superior and had the maximum height of 32 67cm The somaclones from all treatment doses of mutation ($D_0$ $D_1$ and $D_2$) were at par with average heights of 19 00 cm 12 00 cm and 19 67cm respectively

ii) Internode length There was significant difference between the somaclones and conventional clones only in IC 566508 The somaclones from treatment D2 (20 Gy) and the convent onal clones of IC 566508 were at par for this character with an average value of 2 00cm but higher than the somaclones from treatment D0 (0 Gy) and D1 (10 Gy) having 0 37 and 0 70 cm respective average internode length

iii) Number of branches The somaclones did not show any difference for this character when compared to conventional clones

iv) Number of leaves For this character also the somclones did not show any difference when compared to conventional clones

iv) Leaf length For this character there was significant difference between the somaclones and the conventional clones in all the three accessions In accessions IC 566503 and IC 566508 the convent onal clones had longer leaves compared to the somaclones The conventional clone of accession IC 566508 had leaves of maximum average length of 7 6 cm

**Table 65 Mean performance of somaclones selected from *in vitro* mutagenesis and conventional rooted cuttings four months after planting (four MAP)**

| Accession No | Treatment | *In v tro* Mutagen Dose | Plant he ght (cm) | Internode length (cm) | Number of branches | Number of leaves | Leaf length (cm) | Leaf breadth (cm) |
|---|---|---|---|---|---|---|---|---|
| IC 566503 | somaclone $D_0$ | 0 Gy | 35 67 | 1 03 | 3 33 | 37 00 | 3 68 | 2 20 |
| | somaclone D | 10 Gy | 17 00 | 1 00 | 2 33 | 24 00 | 3 75 | 2 15 |
| | somaclone $D_2$ | 20 Gy | 9 67 | 0 67 | 1 67 | 9 33 | 2 11 | 1 41 |
| | Convent onal rooted cutting | | 31 67 | 1 67 | 4 00 | 39 33 | 6 53 | 3 77 |
| CD at 5% level of signif cance | | | NS | NS | NS | NS | 2 83 | 1 44 |
| IC 566508 | somaclone $D_0$ | 0 Gy | 14 67 | 0 37 | 3 00 | 29 67 | 3 72 | 2 22 |
| | somaclone D | 10 Gy | 25 00 | 0 70 | 2 67 | 42 67 | 4 62 | 2 63 |
| | somaclone $D_2$ | 20 Gy | 21 00 | 2 00 | 2 67 | 38 33 | 4 90 | 2 80 |
| | Conventional rooted cutting | | 27 00 | 2 00 | 2 67 | 24 67 | 7 60 | 4 37 |
| CD at 5% level of s gn f cance | | | NS | 1 08 | NS | NS | 2 6 | 1 47 |
| IC566514 | somaclone $D_0$ | 0 Gy | 19 00 | 1 33 | 3 33 | 19 33 | 3 77 | 2 27 |
| | somaclone D | 10 Gy | 12 00 | 0 70 | 2 00 | 15 33 | 4 77 | 2 88 |
| | somaclone $D_2$ | 20 Gy | 19 67 | 0 10 | 2 00 | 27 67 | 2 27 | 1 15 |
| | Convent onal rooted cutting | | 32 67 | 1 37 | 3 00 | 35 33 | 7 22 | 4 13 |
| CD at 5% level of s gnificance | | | 12 48 | NS | NS | NS | 2 68 | 1 39 |

v) Leaf breadth There was significant difference between the somaclones and conventional clones in all the three accessions for leaf width The conventional clones were superior with broad leaves The conventional clone of accession IC 566508 had leaves of maximum average witdth of 4 37 cm

**4 5 1 2 Flowering in the somaclones and conventional clones of *Plumbago rosea***

The plants in the field experiment were observed for flowering during the period (Aug 2009 Jan 2010) The somaclones from the three treatment doses ($D_0$ 0 Gy $D_1$ 10 Gy and $D_2$ 20 Gy) did not flower However flowering was observed n the conventional clones of all the three accessions Flowering was first observed in the conventional clone of accession IC 566508 in December2009 By the end of January 2010 flowering was observed in the conventional clones of all the three accession of *Plu ibago i osea* None of the somaclones of selected *P rosea* accessions flowered during the entire flowering season

**4 5 1 3 Mean performance of somaclones and conventional clones eight months after planting**

The mean performance of the somaclones and the conventional clones for the following six morphological characters eight months after planting is presented in Table 66 After eight months of planting the soma clones differed considerably in their mean performance when compared with conventional clones The soma clones differed significantly for plant height number of branches number of leaves and leaf size when con pared with conventional clones m all the three accessions The mean performance of the somaclones and the conventional clones for the six morphological characters are as follows

i) Plant height For this character the somaclones of all the three accessions were shorter than conventional clones The conventional clones had the maximum height ranging from 60 0 cm (IC 566508 and IC 566514) to 63 3 cm (IC 566503) The somaclones of control (0 Gy) and 10 Gy were intermediate in all the accessions The somaclones of 20 Gy were the short in all the accessions with shortest (16 67) belonging to accession IC 556514

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ii) Internode length There was no significant difference between the somaclones and conventional clones for this character

iii) Number of branches In all the three accessions *viz* IC 566503 IC 566508 and IC 566514 somaclones of 10 Gy were at par with the corresponding conventional for number of branches clones The somaclones of 20 Gy had produced least number of branches in IC 566503 (2 00)

iv) Number of leaves In accessions IC 566503 and IC 566514 the somaclones of 10 Gy had produced equivalent number of leaves in comparison to their respective conventional clones In accession IC 566508 the somaclones of control treatment as well as 10 Gy irradiation were at par with conventional clone for this character The somaclones of 20 Gy in accession IC 566503 and IC 566514 had produced least number of leaves (21 67) when compared to conventional clones

v) Leaf length The somaclones of 10 Gy in accession IC 566514 had produced leaves of maximum length (15 35 cm) when compared to conventional clones (9 35 cm) In accessions IC 566503 and IC 566508 the somaclones of 10 Gy were at with their corresponding conventional clones The somaclones of 20 Gy of accession IC 566514 had produced smallest leaves (6 47)

vi) Leaf breadth The somaclones of 10 Gy in accession IC 566514 had produced leaves of maximum width (7 67cm) when compared to conventional clones (5 17cm) In all the three accessions the somaclones of 10 Gy were at par with their corresponding conventional clones for this trait The somaclones of 20 Gy in accession IC 566514 had produced leaves of minimum width (3 50cm)

Thus at this stage of growth (eight MAP) *in vitro* mutants having a dwarf plant type with higher number of large leaves could be identified especially from 10 Gy dose of irradiation

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**Table 66 Mean performance of somaclones selected from *in vitro* mutagenesis and conventional rooted cuttings eight months after planting (eight MAP)**

| Access on no | Treatment | *In v t o* Mutagen Dose | Plant height (cm) | Inter ode length (cm) | Number of branches | Number of leaves | Leaf length (cm) | Leaf breadth (cm) |
|---|---|---|---|---|---|---|---|---|
| IC 566503 | somaclone $D_0$ | 0 Gy | 37 00 | 3 00 | 5 00 | 39 33 | 6 67 | 4 17 |
| | somaclone $D_1$ | 10 Gy | 41 67 | 2 50 | 6 33 | 61 67 | 9 67 | 5 67 |
| | somaclone $D_2$ | 20 Gy | 27 33 | 3 50 | 2 00 | 21 67 | 8 17 | 4 33 |
| | Conventional rooted cutt ng | | 63 33 | 4 00 | 10 67 | 76 67 | 12 33 | 5 67 |
| CD at 5% level of s gn f cance | | | 6 99 | | 4 77 | 33 4 | 1 5 | 0 98 |
| IC 566508 | somaclone $D_0$ | 0 Gy | 40 00 | 2 67 | 6 67 | 51 67 | 6 83 | 4 00 |
| | somaclone D | 10 Gy | 41 67 | 2 83 | 9 00 | 68 00 | 8 83 | 5 00 |
| | somaclone $D_2$ | 20 Gy | 25 00 | 3 50 | 5 67 | 53 33 | 7 00 | 4 00 |
| | Convent onal rooted cutting | | 56 67 | 4 33 | 12 33 | 76 67 | 9 17 | 5 67 |
| CD at 5% level of s gnif cance | | | 9 02 | | 5 65 | 28 20 | 1 05 | 1 27 |
| IC566514 | somaclone $D_0$ | 0 Gy | 35 00 | 2 67 | 3 33 | 38 33 | 9 67 | 5 17 |
| | somaclone D | 10 Gy | 48 33 | 3 53 | 6 00 | 46 67 | 13 33 | 7 67 |
| | somaclone $D_2$ | 20 Gy | 16 67 | 2 00 | 2 33 | 21 67 | 6 47 | 3 50 |
| | Conventional rooted cutt ng | | 60 00 | 3 67 | 7 67 | 73 33 | 9 53 | 5 17 |
| CD at 5% level of s gnificance | | | 6 08 | | 2 49 | 24 19 | 1 9 | 0 94 |

# Discussion

# 5. DISCUSSION

Domestication of medicinal plants or wide cultivation in farmer's field is still primitive, especially in southern peninsular India. But demand of medicinal plants is increasing in exponential manner due to its recognition as a natural product to cure almost all diseases occurring in human beings. Primitive way of collection of medicinal plants from wild conditions has led to extinction of many important medicinal plants. Hence, there is an urgent need to domesticate these important medicinal plants for its cultivation. *Plumbago* is one among the important plants which needs both adaptation and domestication in homelands. Even though, *Plumbago* species is vegetatively propagated, notable variability is occurring in this species. To get a clear picture about the extent of variability occurring in this species, an exploration and collection was carried out. In order to widen the available variability in the collected gemplasm, variability was induced through mutation and polyploidy.

An attempt has been made here through studies on variability, character association, genetic diversity and induction of variability through *in vitro* mutagenesis and polyploidy to develop a *Plumbago* plant type with higher plumbagin content and other important agronomic traits. The results obtained are discussed hereunder.

## 5.1 Exploration and collection of different genotypes of *Plumbago* species

The detailed survey of the ecogeographical regions ranging from Western Ghats to coastal areas of Kerala showed wide variability in the geographical distribution of *Plumbago* species. The species *P. rosea* was mainly distributed in the areas of high and medium altitudes especially in midlands of Kerala and hence maximum accessions belonging to this species were collected from districts located in these areas. Twenty five accessions of non-seed setting species *Plumbago rosea* and one accession of seed setting species *Plumbago zeylanica* were collected. During the exploration in naturally occurring areas of *Plumbago* species, apparently rich variability has been noticed with respect to its morphological traits. On preliminary evaluation of these collected accessions, the plumbagin content in *Plumbago rosea* was observed to be higher than the white-flowered *P. zeylanica* (Plate 1). The

*Plumbago* germplasm

*Plumbago rosea* *Plumbago capensis* *Plumbago zeylanica*

**Plate 1. Preliminary evaluation of *Plumbago* germplasm**

passport data of collected accessions were prepared and catalogued. These accessions were assigned IC numbers from 566499 to 566523 by NBPGR, New Delhi.

### 5.2 Assessment of genetic variability in collected accessions of *Plumbago* species

The twenty six accessions which included twenty five from *P. rosea* and one from *P. zeylanica* were raised in replicated trial (Plate 2) and the data on morphological traits and plumbagin content were subjected to statistical analysis. The analysis of variance and covariance was performed and coefficients of variation, heritability, genetic advance, correlation and path coefficient were estimated. The results of the statistical analysis are discussed below.

#### 5.2.1 Genetic Variability, heritability and genetic gain

The 26 accessions of *Plumbago* showed high variability with respect to characters such as number of branches per plant, plant height, number of leaves, leaf length, leaf breadth, fresh plant weight, fresh root weight, dry root weight and plumbagin content(Plate 3). As such it was considered better to more vividly analyse the performance of these accessions through estimation of different components of variation. High genotypic coefficient of variation (GCV) was observed for above ground morphological characters such as number of branches and number of leaves indicating the scope of improvement of these traits. High GCV values indicate little influence of environment on the expression of character. Plant height and internodal length recorded high PCV values and moderate GCV values indicating the influence of environment on these characters. Similarly root characters such as root number, fresh root weight and dry root weight recorded comparatively high PCV values and a moderate GCV values. The biochemical trait plumbagin content in roots also recorded higher PCV value when compared to a moderate GCV value. Most of the characters showed difference between PCV and GCV suggesting that these characters were affected by environment. The various components of variability have been shown in Fig.1.

The magnitude of variability present in a crop species is of utmost importance as it is a key factor, which determines the amount of progress expected from selection. The extent to which the variability of a quantitative character is transferable to the progeny is referred to as heritability for that particular character. Genetic advance

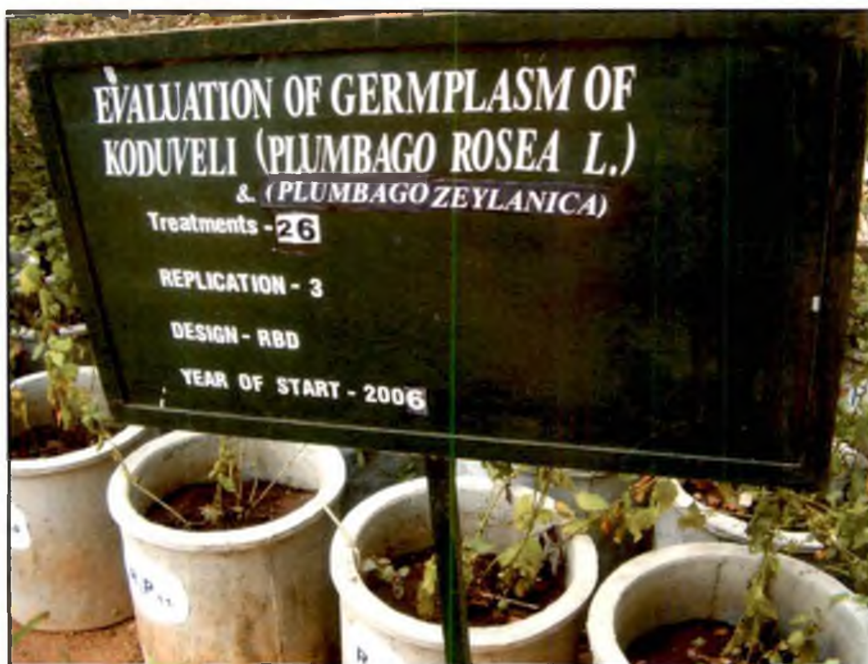


**Plate 2. Evaluation of *Plumbago* accessions in replicated trial**

P-26 *P.zeylanica*

IC-566504

IC-566511

IC- 566510

IC-566520

IC-566500

IC-566508

IC-566522

**Plate 3. Variability in *Plumbago* accessions for morphological and biometric traits**

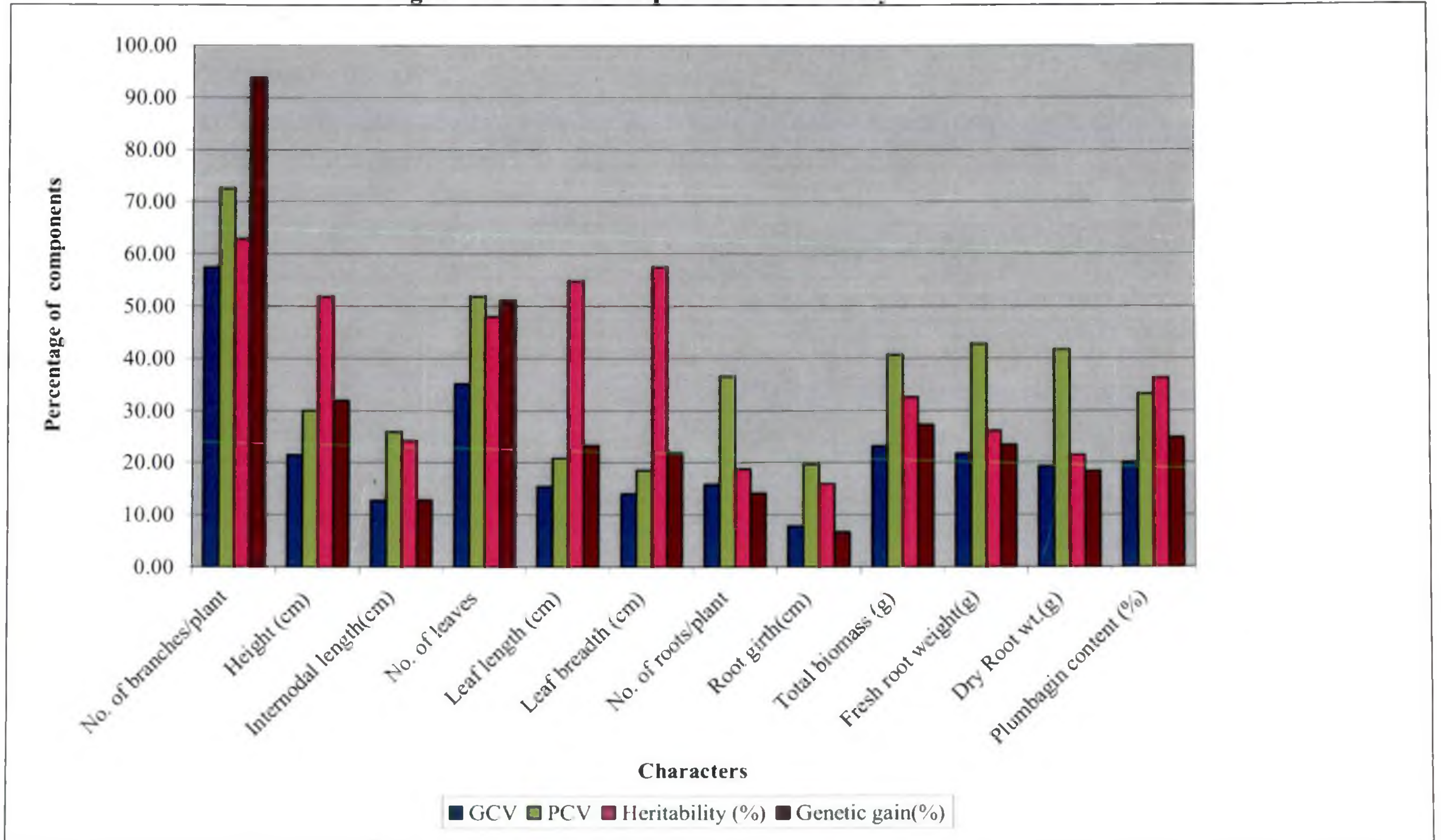


Fig.1 Estimation of components of variability
Percentage of components
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
No. of branches/plant
Height (cm)
Internodal length(cm)
No. of leaves
Leaf length (cm)
Leaf breadth (cm)
No. of roots/plant
Root girth(cm)
Total biomass (g)
Fresh root weight(g)
Dry Root wt.(g)
Plumbagin content (%)
Characters
GCV
PCV
Heritability (%)
Genetic gain(%)

refers to the improvement in genotypic value of new population as compared with the base population and when it is expressed in percentage of mean, the new parameter is termed as genetic gain. So to have an effective selection, along with genetic variability, heritability and genetic gain measurements are also important. Heritability values for various morphological and biometric characters ranged from 16.00 per cent to 62.90 per cent. Among all the morphological traits, number of branches alone exhibited high heritability. The remaining above ground morphological characters as well as plumbagin content exhibited moderate heritability values. All the root characters and internodal length exhibited low heritability values. Among the morphological traits maximum genetic gain was expressed by number of branches and number of leaves. Plant height, leaf length, leaf breadth, fresh plant weight, fresh root weight and plumbagin content showed more than 20 per cent genetic gain. The characters such as internodal length, root number, root girth and dry root weight had shown very low genetic gain. Singh and Narayanan (1993) suggested that estimates of heritability and genetic advance when considered together are more useful than heritability alone. High heritability accompanied with high genetic advance indicates that most likely the heritability is due to additive gene effects and selection may be effective. In the present study, number of branches and number of leaves were the only two characters that exhibited high heritability along with high genetic gain. So selection is effective only for these characters. Kanakamony (1998) reported high heritability along with genetic gain for number of leaves and rhizome number in *Kaempferia galanga.*

The most important trait dry root weight showed low genetic gain indicating the ineffectiveness of selection based on yield alone. Abraham (2002) reported low heritability and genetic gain estimates for tuber yield in *Coleus.* Arya (1999) reported very low genetic gain for plumbagin content. It may be concluded that dry root yield and plumbagin content have to be monitored through appropriate agronomic practices than being realized through selection alone.

**5.2.2 Association of characters**

A study on the association of characters is an important plant breeding activity, since it helps to determine the relationship of yield with its components which in turn helps to select superior genotypes from diverse genetic populations. Genotypic

correlations provide a reliable measure of genetic association between characters and help to differentiate the vital associations useful in breeding from non vital ones (Falconer, 1981).

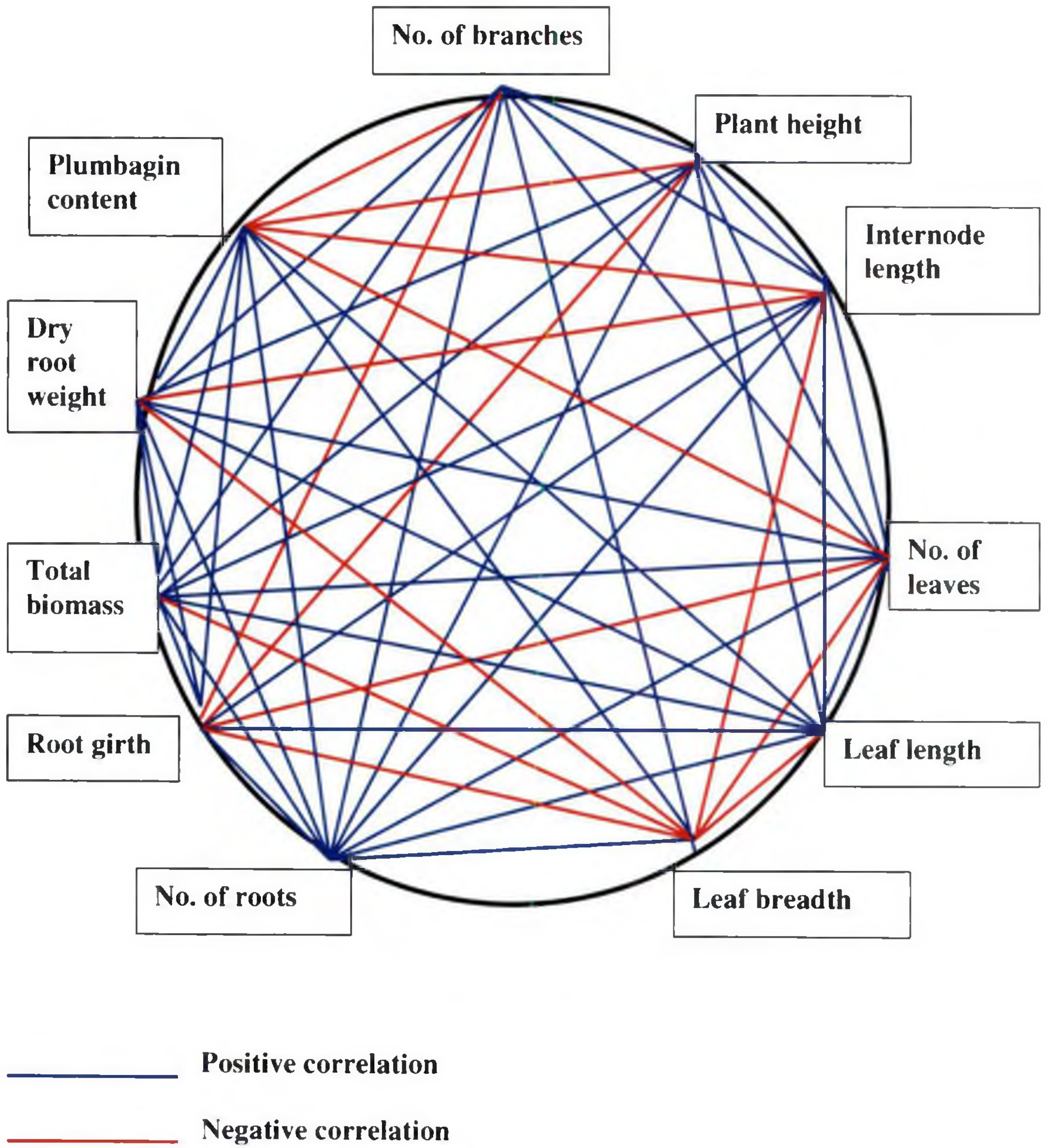
In the present investigation, the correlation studies revealed significant positive genotypic correlation of all the characters with dry root yield except internode length and leaf breadth (Fig.2). This was in accordance with Arya (1999) who reported significant positive association of yield with other component characters such as plant height, number of leaves, internode length, number of roots, fresh weight of shoot and roots. Similar positive association between yield and its component characters were reported in vegetatively propagated crops like ginger (Tiwari, 2003) and *Chlorophytum borivilianum* (Kumar *et al.*, 2008).

The association of plumbagin content with the component characters showed that number of branches, plant height, internode length and number of leaves had a negative impact on plumbagin content. The root characters such as root girth, number of roots and dry root yield had positive ssociation with plumbagin content. Arya (1999) also reported a negative genotypic correlation of plant height with plumbagin content but a positive association of dry root yield with the same. This indicates that excess vegetative growth leads to poor fixation of plumbagin in the roots. There may be many other factors that lead to this phenomenon. One of the factors might be the delayed root initiation coupled with slow growth and development of roots that necessitates for such a luscious vegetative growth. Thus, a plant having lesser number of branches with diminished height may be selected for propagation as this will lead to a better performance of the root characteristics.

### 5.2.3 Path coefficient analysis

Correlation studies are helpful in discovering the association between yield and yield components but they do not provide a clear picture of direct and indirect causes of such associations. However, this can be obtained through path analysis. Path coefficient analysis is very much useful in identifying the important yield components which can be utilized for formulation of selection parameters. In the present investigation path analysis revealed high and positive direct effects of number of leaves, leaf length, leaf breadth and dry root yield on plumbagin content (Fig. 3).

Fig. 2 Genotypic correlations between eleven characters of *Plumbago* accessions



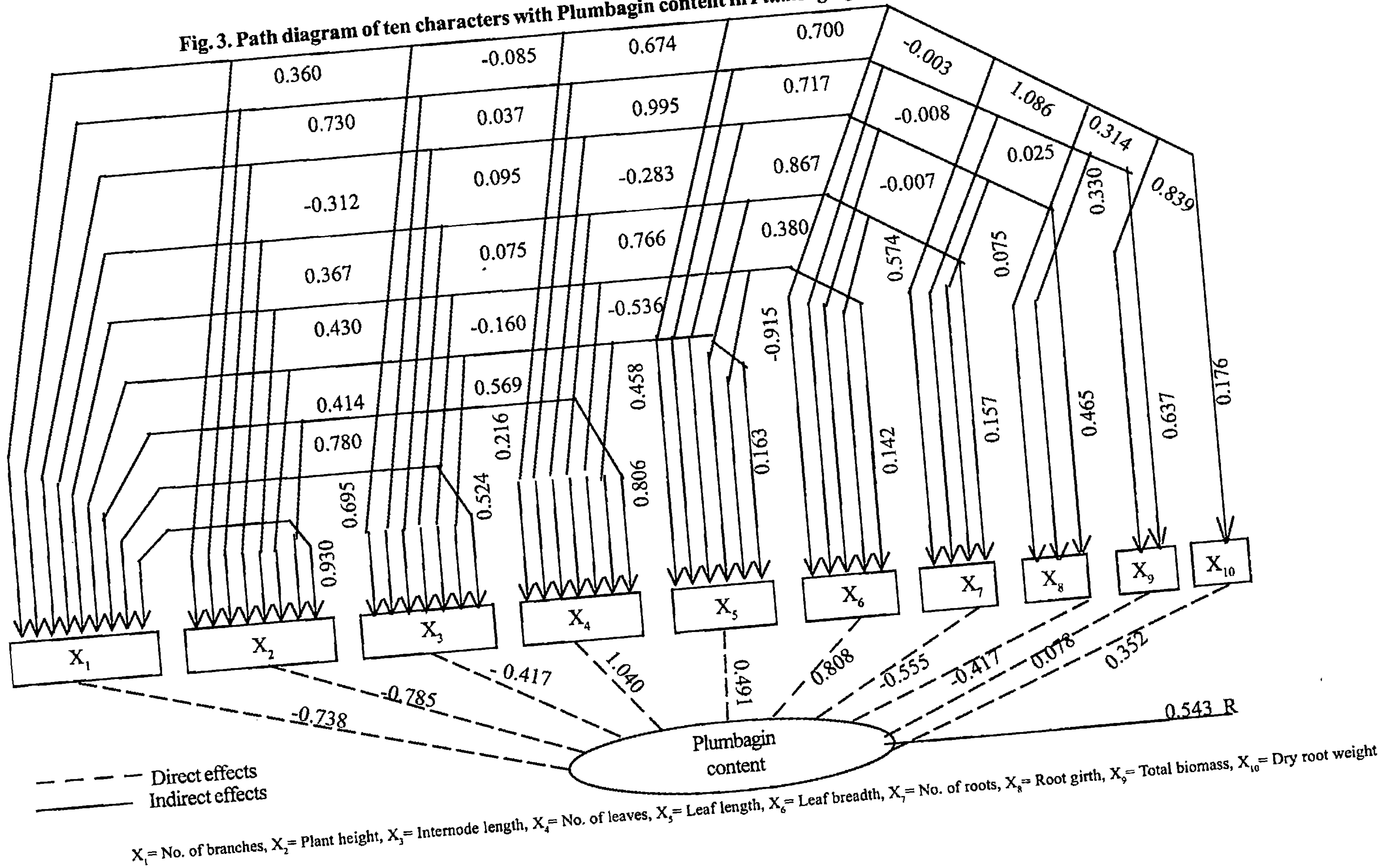



**Fig. 3. Path diagram of ten characters with Plumbagin content in *Plumbago* genotypes**

Similar observation was also made by Arya (1999) who reported dry root yield as a positive and direct contributing factor of plumbagin content.

The negative direct effects of number of branches, plant height and internode length are due to negative genotypic correlations of the same with plumbagin content. Even though number of leaves had high positive direct effect it had a negative genotypic correlation with plumbagin content. Similarly, leaf length and leaf breadth had very insignificant genotypic correlation. These correlations may be the result of the indirect influence of these characters through the component characters such plant height, number of branches and internode length. Singh and Narayanan (1993) suggested that in such a situation direct selection for such traits can be practised to reduce undesirable indirect effects. Dry root weight was the only root character which exhibited a high positive direct effect along with a significant genotypic correlation with plumbagin content thus, revealing a true genetic association of these characters.

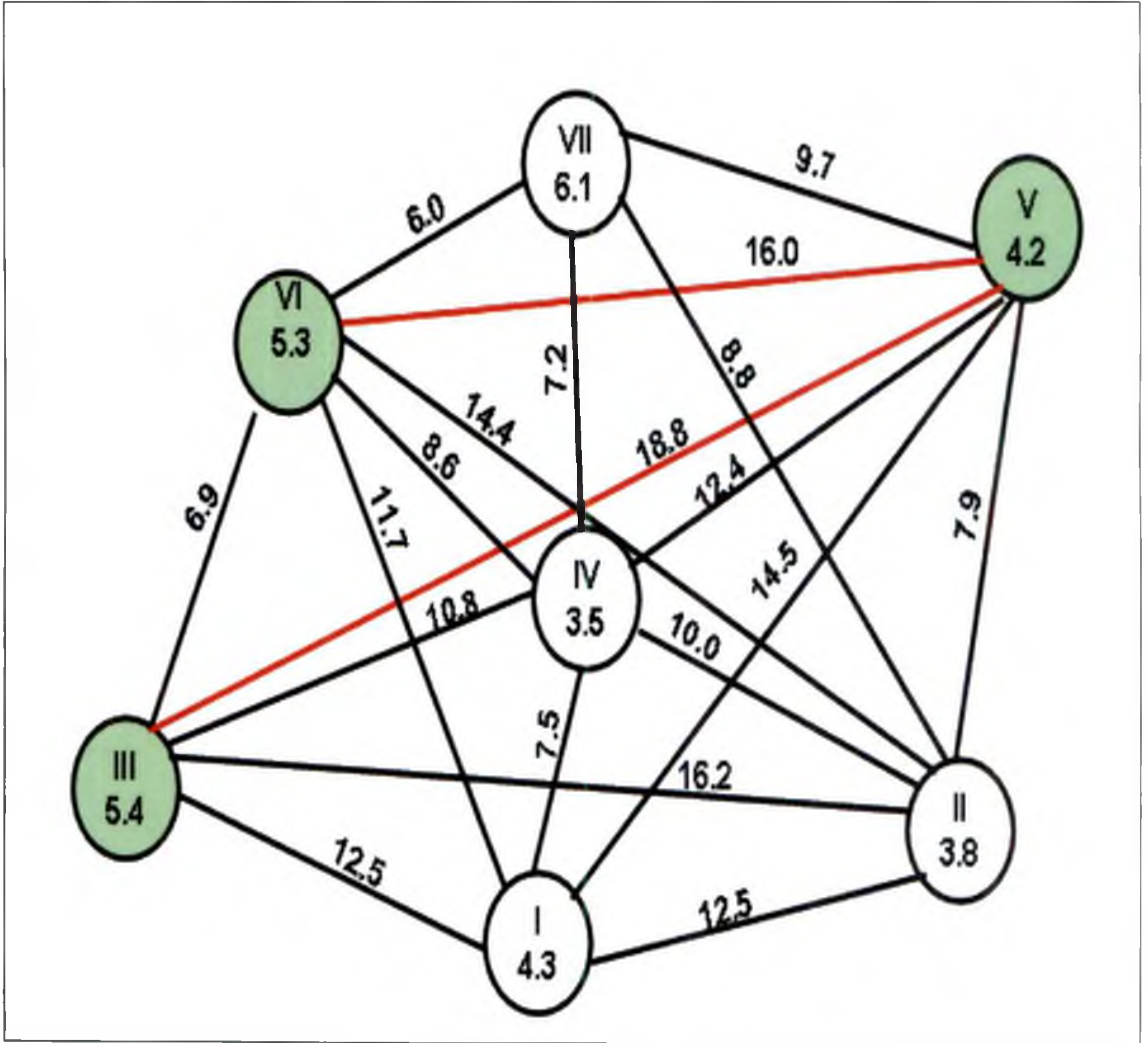
Path coefficient analysis thus identified number of leaves, leaf length, leaf breadth and dry root yield as the most important selection parameters for *Plumbago* genotypes. Thus, a dwarf and bushy plant with large leaves and tuberous roots will yield high plumbagin content.

**5.2.4 Cluster analysis**

Mahalonobis $D^2$ statistics is found to be powerful tool in the hands of plant breeder to assess the range of dissimilarity among genotypes consequently group them based on phenotypic expression. The variability can be further expanded in the representative samples taken from divergent clusters through hybridization in case of seed propagated crops or special breeding techniques like mutation in case of vegetatively propagated crops.

The diversity of collected accessions of *Plumbago* species was assessed by cluster analysis based on Mahalanobis $D^2$ statistics and most divergent clusters were identified for inducing variability. The 26 accessions of *Plumbago* species were grouped into seven clusters. The spatial diagram of clusters and their relative distance are represented in the cluster diagram (Fig. 4). The grouping pattern indicated that

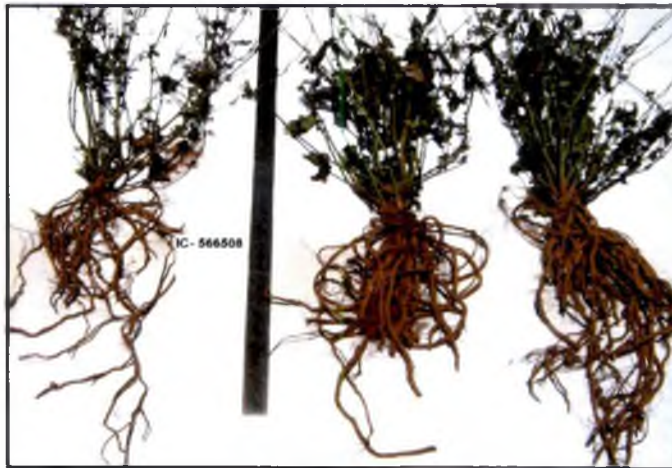
**Fig. 4 Cluster diagram**



there was no parallelism between geographic distribution and genetic diversity. These results are in confirmation with Abraham (2002), Kumar *et al.* (2008) and Radhakrishnan *et al.* (2008).

The clusters III, V and VI were identified as highly divergent clusters. So as to identify promising accessions within these clusters that could yield better plumbagin content in consonance with dry root weight, ranking based on these attributes was performed for the cluster members. Three superior ranking accessions of *P. rosea* from highly divergent clusters (Plate 4) and one accession of *P. zeylanica* were selected for inducing variability through *in vitro* mutagenesis and polyploidy The accessions IC-566508 and IC-566514 were selected because these accessions had very high dry root weight along with high plumbagin content and a further enhancement of plumbagin content might be useful for large-scale extraction of plumbagin to supplement the colchicine supply from pharmaceutical industry. Plumbagin is well known for its colchicine like activity. Besides, the anti-cancer properties of plumbagin make it a potential candidate for drug development against this malignant disease. The Acc. no. 26 of *P. zeylanica* was selected because of its poorest plumbagin content. Further investigation using this accession might be useful for an optimal plumbagin. This species could also be co-opted for large scale cultivation especially in peninsular India. The accession IC-566503 was selected so that further enhancement of plumbagin content of this accession might be useful in having optimal plumbagin content recovery especially for ayurvedic preparations. The roots are usually cured in lime water and dried or simply dried before being used in ayurvedic preparations (Pandey, 1960).The main objective behind this practice might have been reduction of plumbagin content. However, Menon (1999) reported presence of additional compounds such as steroids and flavanoids with low plumbagin content in cured root samples. This finding was reinforced by the very negligible presence of plumbagin in the leachate obtained after curing of roots. This in turn suggested the conversion of plumbagin to a compound having medicinal properties. Thus, the selection of accessions of *Plumbago* species to induce of variability for plumbagin content was done based on the use of plumbagin in various fields. Thus, the *P. zeylanica* accession with lowest plumbagin content of 0.87 per cent, the accession IC-566503 with an intermediate level of plumbagin content of 2.79 per cent and the

IC-566503




IC-566508

IC-566514

**Plate 4.** ***Plumbago rosea*** **accessions selected for induction of variability**

accessions IC-566508 and IC-566514 with high plumbagin content of 4.11 and 4.62 per cent were selected from cluster analysis.

**5. 2. 5 Reproductive biology of *Plumbago* species**

Flowering is observed in the two species of *Plumbago* but seed set occurs only in *P. zeylanica*. In order to find out the causes of failure of seed set in *P. rosea*, studies on reproductive biology in *P. rosea* and *P. zeylanica* were taken up .The results of floral studies are discussed below.

**5.2.5.1 Floral morphology**

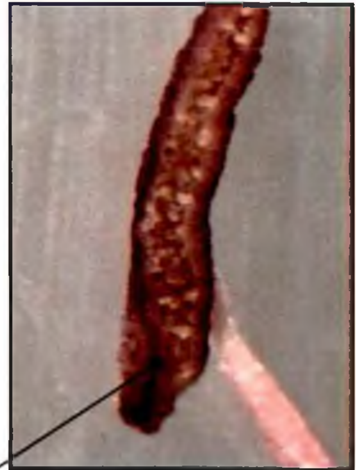
Even though the two species of *Plumbago* showed general characters of Plumbaginaceae, they could be identified by specific morphological characters. The inflorescence is a spike in both the species. It is borne either terminal or axillary position in *P. zeylanica* but was observed only in terminal position in *P. rosea*. *P. rosea* could be easily distinguished by red flowers with red calyx tube, fimbricate style and stamens with red anthers (Plate 5) *P. zeylanica* is characterized by white flowers with green calyx tube, terete style and purple anthers (Plate 6). The level of anthers and stigma are different in both species. Even though anthers in both the species are exerted beyond a tube, the stigma in *P. rosea* is deeply seated inside corolla tube due to short style. In *P. zeylanica* the stigma is held either at the same level or slightly higher level than anthers.

The *P. rosea* accessions produced lesser floral branches than *P. zeylanica*. The inflorescence of *P. rosea* was longer with higher number of floral buds per inflorescence than that of *P. zeylanica*. Hence, *P. rosea* took a longer time (26.2 days) for completion of flowering per inflorescence in comparison to *P. zeylanica* (19.6 days). The mean performance of the *Plumbago* species for various floral characters is shown in Fig. 5.

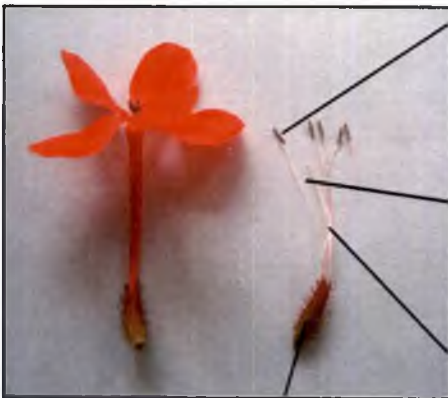
**5.2.5.2 Anthesis**

Study of anthesis conducted at hourly intervals revealed that there is variation between the two species in peak period of anthesis. In *P. rosea* the peak period of anthesis was between 7 a.m. and 8 a.m. However, in *P. zeylanica* it was observed one hour earlier.

Inflorescence



Anther



Stigma

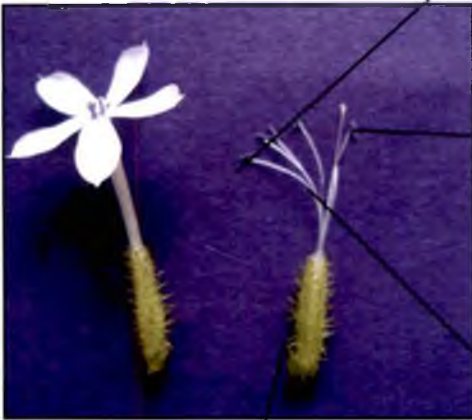
Ovary

Style

Plate 5. Floral morphology of *Plumbago rosea*

Inflorescence

Anther



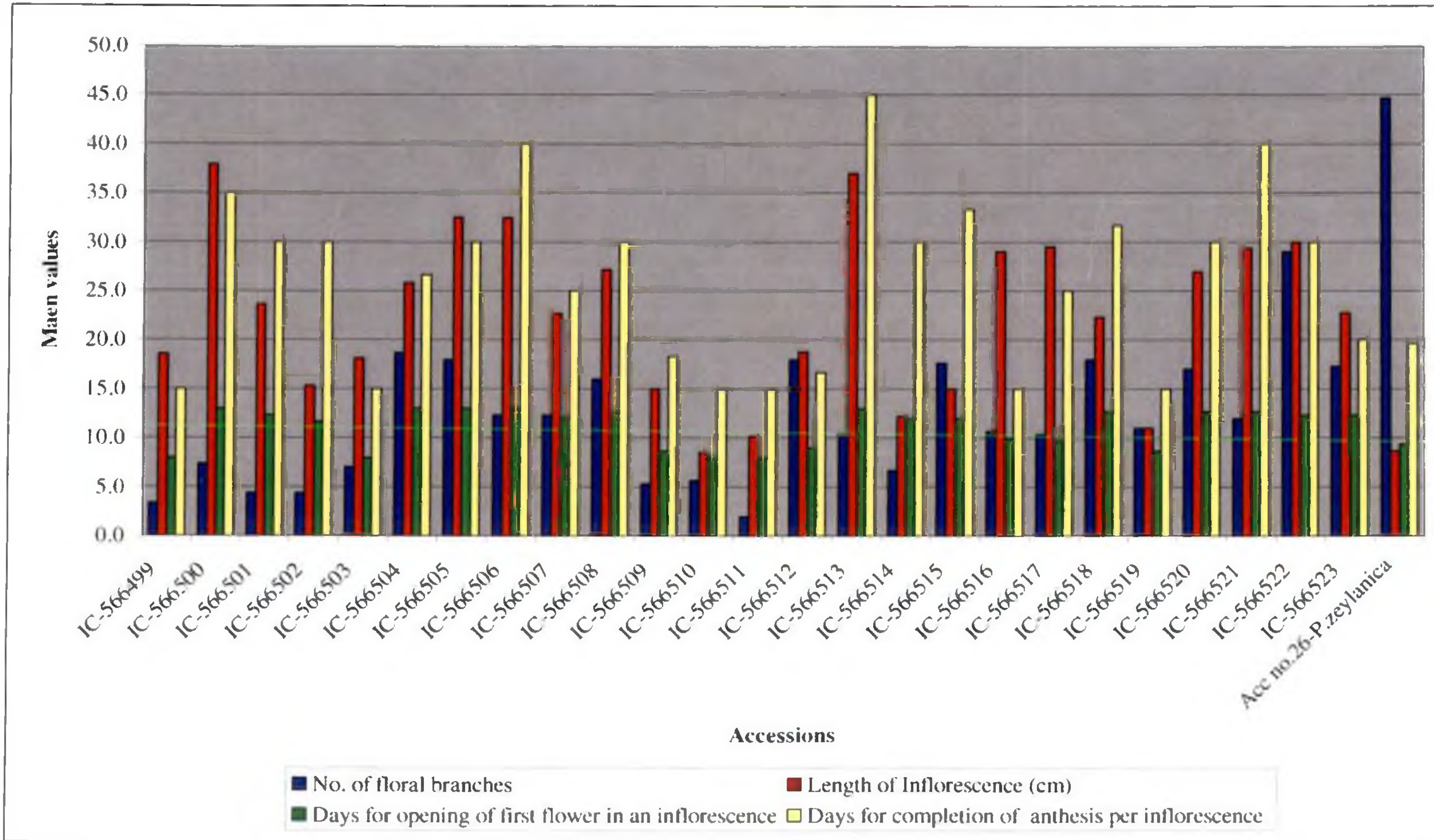
Stigma

Ovary

Style

**Plate 6. Floral morphology of *Plumbago zeylanica***

**Fig. 5 Mean performance of *Plumbago* species for floral characters**



The flower opening started earlier and continued for a longer period in *P. zeyalnica*. The anthesis started at 4 a.m. and continued up to 10 a.m. Flower opening started much later in *P. rosea* at 6a.m. and continued for a short period of 4 hours only.

**5.2.5.3 Anther dehiscence and stigma receptivity**

Anther dehiscence was found to occur after flower opening in *P. rosea* whereas the stigma became receptive before dehiscence of anthers indicating the presence of protogyny (Plate7). However, in *P. zeylanica* anther dehiscence coincided with stigma receptivity (Plate 8). This observation was in confirmation with study conducted by Subha (2000).

**5.2.5.4 Pollen fertility and viability**

Very high pollen fertility was observed in the two species in acetocarmine staining technique (Plates 9 and 10). Subha (2000) also reported a similar result based on acetocarmine staining technique. However, Menon (1999) had earlier reported very low pollen fertility in *P. rosea* using the same staining procedure. There was no pollen germination under *in vitro* conditions in both the species. Arya (1999) and Menon (1999) also observed lack of pollen germination under *in vitro* conditions.

The artificial pollination studies revealed very high adhesion of pollens of the two species of *P. rosea* as well as *P. zeylanica* on the stigma of *P. zeylanica* (Plate 11). However, very little pollen adhered to the stigma of *P. rosea* when pollinated with pollen from *P. rosea* (Plate 12) and *P. zeylanica* (Plate 13). Similar observations were also made by Arya (1999).Even though there was a negligible seed set in *P. zeylanica* when pollinated with pollen from *P. rosea* the viability of pollen of *P. rosea* could not be confirmed due to the non viable seed in *P. zeylanica*. This is in confirmation to the observation made by Menon (1999) who noticed lack of germination of pollen from *P. rosea* on the stigma of *P. zeylanica*.

When pollination was under taken in natural conditions, there was 40 per cent seed set in *P. zeylanica* under selfing and 70 per cent seed set under open pollinated conditions. In this species stigma receptivity was found to coincide with pollen dehiscence. Besides, stamen and style were of equal length. Thus simultaneous

Receptive stigma

Stigma losing receptivity

Undehisced anther

Completely dehisced anther

**Plate 7. Anther dehiscence and stigma receptivity in *Plumbago rosea***

Receptive stigma

Stigma losing receptivity

Just dehisced anther

Completely dehisced anther

Plate 8. Anther dehiscence and stigma receptivity in *Plumbago zeylanica*

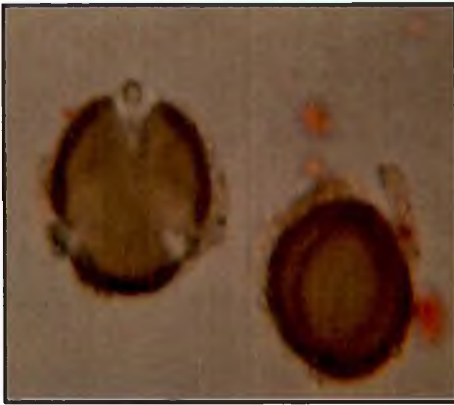


**Plate 9. Pollen grains of *P. rosea* (X 400)**

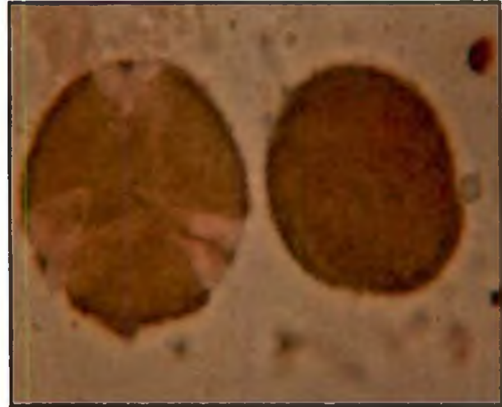


**Plate 10. Pollen grains of P.zeylanica (X 400)**

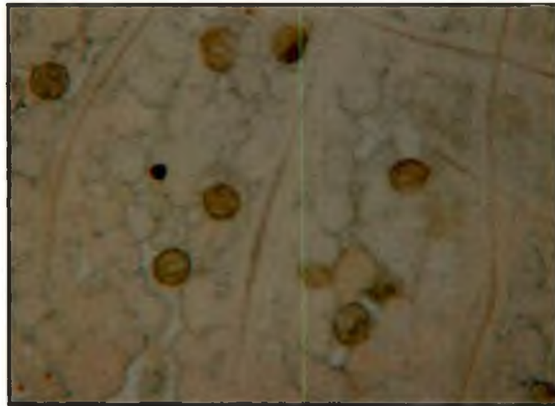


**Plate 11. High adhesion of pollen grains of *P. rosea* on stigma of *P. zeylanica* (X100)**

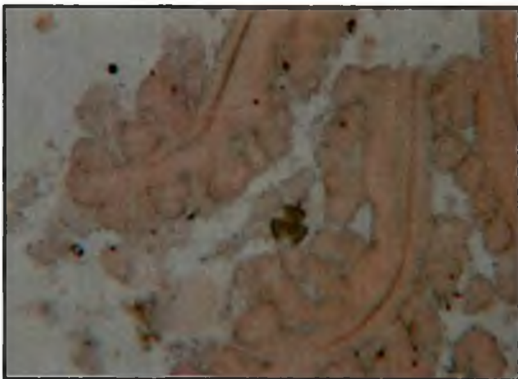
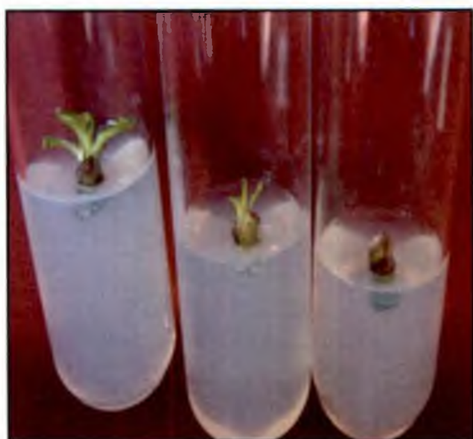


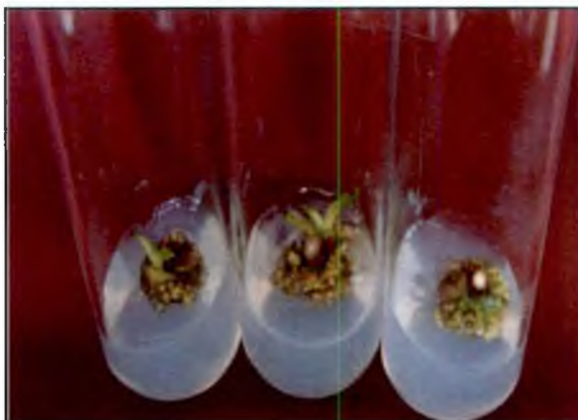
**Plate 12. Pollen grains of *P. rosea* on stigma of *P. rosea* (X100)**

**Plate 13. Pollen grains of *P. zeylanica* on stigma of *P. rosea* (X100)**



*P. rosea* in MS+ BA (2.5 mg $l^{-1}$) + NAA (1.5 mg $l^{-1}$)

*P. rosea* in MS+ BA (1.0 mg $l^{-1}$) +Ads (50 mg $l^{-1}$)

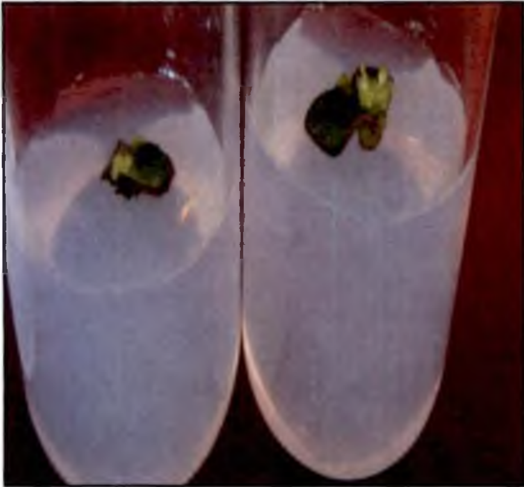


*P. rosea* in MS+ TDZ (1.0 mg $l^{-1}$)

*P. zeylanica* in MS+ BA (1.0 mg $l^{-1}$) +Ads (50 mg $l^{-1}$)

*P.zeylanica* in MS+ TDZ (1.0 mg $l^{-1}$)

**Plate 14. Culture initiation in nodal segments of *Plumbago* species**



**Direct initiation of shoots under light in *P. rosea***

**Callusing under dark conditions in in *P. rosea***

**No response in *P. zeylanica***

**Plate 15. Culture initiation in leaf discs of *Plumbago* species**

explants. In the present investigation under a 16-hour photoperiod, TDZ not only produced axillary shoot in the nodal segments but also promoted adventitious formation of shoots in the nodal segments and leaf discs of *Plumbago rosea* in a period of three weeks (Plate 14 and 15). Faisal *et al.* (2005) reported similar effect of TDZ on *in vitro* shoot proliferation from nodal explants of *Rauvolfia tetraphylla.* Adventitious induction of shoots on leaf explants cultured on MS medium containing TDZ have been reported in woody plant species *Hydrangia quercifolia* (Ledbetter and Preece, 2004) and *Pawlonia tomentosa* ( Corredoira *et al.*, 2008).

The nodal segments were therefore, carried forward for direct organogenesis and leaf discs for callus mediated organogenesis.

**5.3.2 Direct organogenesis of nodal segments**

A protocol for direct organogenesis was standardised in *P. rosea* (Plate 16). The nodal segments of *P. rosea* as well as *P. zeylanica* were transferred from best culture initiation medium (MS + BA 1.0 mg $l^{-1}$ + adenine sulphate 50 mg $l^{-1}$) after two weeks to shoot multiplication and elongation medium containing increased concentrations of BA from 1.0 mg $l^{-1}$ to 4.0 mg $l^{-1}$. The nodal cultures of *P. zeylanica* showed no response in any of the treatment combinations. The nodal cultures of *P. rosea* showed maximum shoot multiplication (3.0 ± 0.14) in half strength MS medium containing 2.0 mg $l^{-1}$ BA in 45 days of first subculture. Shoot multiplication was low in culture initiation medium with adenine sulphate as well as in increased concentrations of BA. The cytokinin precursor adenine sulphate was ineffective in further multiplication of shoots in subsequent subcultures. This is in concurrence with the observation of Sankar *et al.* (2007) in Trichosanthes cucumerina but contrary to the report in *Plumbago* species. Rout et al. (2002) reported high frequency shoot-bud regeneration in the leaves of *P. zeylanica* and *P. rosea* in the medium containing BA along with adenine sulphate. Thus, in the present study a concentration of 2.0 mg $l^{-1}$ BA was optimum for shoot multiplication in nodal explants. Similar observations were made in the *in vitro* nodal cultures of *Bacopa monieri* (Mohapatra and Rath, 2005) and *Tylophora indica* (Nadha, 2006).

Elongation of multiplied shoots required subculture in increased concentrations of BA (3.0 - 4.0 mg $l^{-1}$) with maximum elongation of 4.5 ± 0.17 cm being obtained at

**Shoot proliferation in ½ MS+BA (2.0 mg $l^{-1}$)**

**Shoot elongation in MS+ BA (4.0 mg $l^{-1}$)**

**Rooting in ½ MS + IBA**

**½ MS+ IBA (1.0 mg $l^{-1}$)**

**½ MS+ IBA (1.5 mg $l^{-1}$)**

**Primary hardening**

**Secondary hardening**

**Plate 16. Protocol for direct organogenesis in nodal cultures of *P.rosea***

a concentration 4.0 mg $l^{-1}$ BA in a period of 30 days. Even though BA is known for high cytokinin activity, its increased concentration suppressed shoot proliferation and promoted shoot elongation in the nodal cultures of *P. rosea*.

No rooting was observed in *P. zeylanica* cultures. However, rooting of elongated shoots of *P. rosea* was achieved in half strength MS medium with three concentrations of IBA such as 0.5, 1.0 and 1.5 mg $l^{-1}$ (Fig. 6). Maximum cultures showed initiation of rooting in 21 days and completion of rooting in 60 days from inoculation in medium. Maximum root length of was obtained with 1.5 mg $l^{-1}$ IBA. This is in conformity with Satheeshkumar and Bhavanandan (1988), and Chaplot *et al.* (2006) and Preetha *et al.* (2007) who also reported successful rooting of *in vitro* shoots of *P. rosea* in 1.0 to1.5 mg $l^{-1}$ IBA.

There was no rooting in *P. rosea* and *P. zeylanica* cultures in medium without IBA as well as in medium with low concentration of IBA and sucrose. However, this is in contrast to the following reports. Rout *et al.* (1999) reported successful rooting of *in vitro* generated shoots from nodal cultures of *P. zeylanica* in half strength MS medium with 0.25 mg $l^{-1}$ IBA and 2.0 % sucrose. Satheesh and Seeni (2003) also reported successful rooting of *in vitro* shoots of *P. rosea* in low IBA concentration (0.1 mg $l^{-1}$). Gopalakrishnan *et al.* (2009) reported rooting of *in vitro* shoots from leaf explants transferred to half strength MS basal medium without any plant growth regulator.

#### 5.3.2.1 Hardening and acclimatisation of *in vitro* plants from direct organogenesis

The *in vitro* plants of *P. rosea* from direct organogenesis showed a maximum of 66.6 per cent survival in first four weeks of primary hardening in sterile sand. However, the rate of hardening reduced to 22.2 per cent in secondary hardening stage in potting mixture in the net house with only eight plants available for field planting (Fig. 7).

Hardening and acclimatization is often critical stage in overall tissue culture (Jagannathan, 1985). The success of tissue culture protocol is not only dependent on the maximum multiplication under *in vitro* conditions but also eventually on the

**Fig. 6 Effect of IBA on *in vitro* rooting of shoots regenerated from nodal explants of *P. rosea***

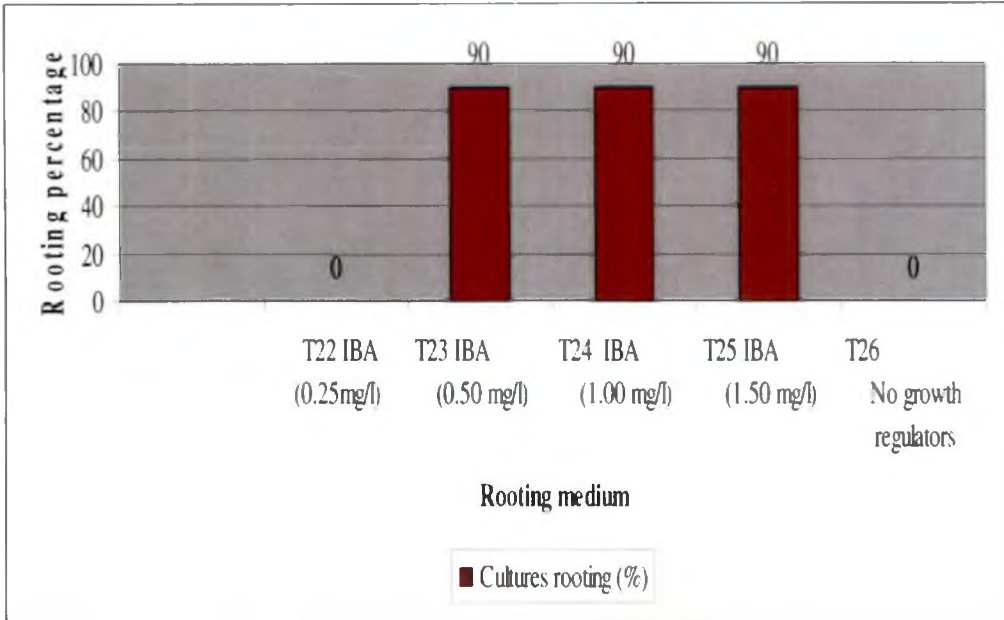
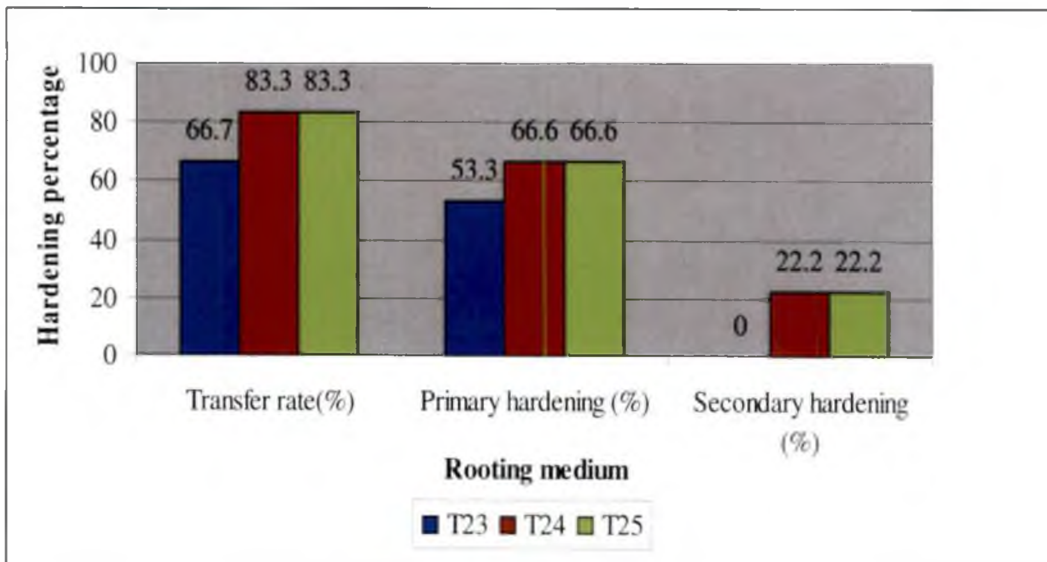



**Fig. 7 Hardening percentage of *in vitro* plants from *P. rosea* nodal explants**



maximum survival of plants under *ex vitro* conditions. In the present investigation the nodal cultures of *P. rosea* showed high rate of contamination under *in vitro* conditions and the plants obtained from these cultures showed a poor survival under ex-vitro conditions. However, there are reports of 95 per cent recovery of plantlets obtained from direct *in vitro* organogenesis after hardening in *P. zeylanica* (Rout *et al.* (1999) and (Chaplot *et al.*,2006).

**5.3.2.2 Field evaluation of *in vitro* plants from direct organogenesis**

The *in vitro* plants were evaluated in pot culture for six morphological characters namely plant height, number of branches, internodal length, number of leaves, leaf length and leaf breadth at quarterly intervals for one year. After one year, the plants were harvested and evaluated for seven biometric traits including plumbagin content. The performance of the *in vitro* plants was compared with the conventional rooted cutting of the accession of IC-566508 for all the traits.

The morphological evaluation revealed no significant difference between tissue cultured plants and the plants from conventional rooted cutting for all the characters except for leaf size and intermodal length. The *in vitro* plants ($TC_1$ and $TC_2$) had produced large sized leaves during the entire period of growth and during the last season of growth had longer internodes compared to conventional plants (Plate 17) Similar observations were made in palmarosa grass (Patnaik *et al.* 1999), in black pepper by Sanchu (2000), Sujatha (2001) and Rathy *et al.* (2007) and in rose-scented geranium (Ravindra *et al.*, 2004).

The evaluation of biometric traits at the time of harvest, revealed significant variation for total biomass, fresh root weight, dry root weight and plumbagin content (Plate 18). The $TC_2$ plants had produced comparatively higher biomass (360.0 g total biomass/ plant) as well as tuberous and heavy roots (150.0 g fresh root weight /plant) when compared to conventional rooted plants of accession IC-566508 (117.5 g total biomass and 37.5 g fresh root weight).The dry root weight (45.25 g/plant)) recovered from $TC_2$ plants was also comparatively higher than conventional plants (15.0 g/plant). The concentration of root specific compound plumbagin was higher in conventional plants (2.25 per cent) compared to the *in vitro* plants (TC1-0.66 per cent, TC2-1.33 per cent).

**Evaluation of morphological traits of *in vitro* plants and rooted cuttings of *P.rosea***

***In vitro*  plant (TC2)**

**Rooted cutting of accession IC-566508**

**Plate 17. Field evaluation of *in vitro* plants from direct regeneration of *P.rosea* nodal segments**

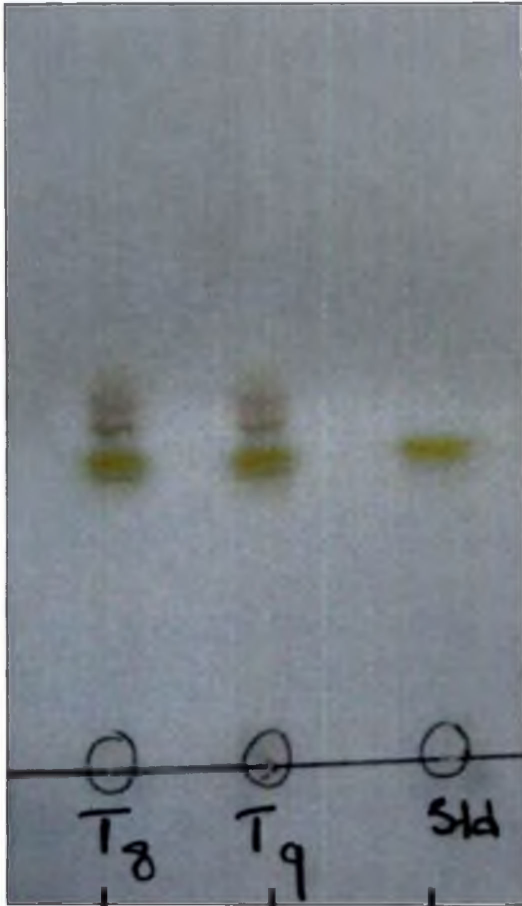
**Plate 18. Evaluation of *in vitro* and conventional plants of *P.rosea* at harvest**

The quality analysis of plumbagin obtained from roots of *in vitro* plants and conventional plants showed similar banding pattern (Plate19). The bands produced by root samples from *in vitro* plants and conventional plants were having the same Rf value (0.55) as that of standard plumbagin. This reveals that the quality of plumbagin from *in vitro* regenerated plants is comparable to that from conventional plants. There are only few instances of evaluating *in vitro* derived plants against conventionally propagated plants especially medicinal plants. Satheeshkumar and Seeni (2003) reported a similar superior performance of the micropropagated plants of *P. rosea* showing significant increase in growth, biomass production, tuberisation of roots as well as plumbagin content as against conventionally propagated plants. In the present study, the quality of plumbagin was analysed in crude acetone extracts of root samples. The quantity of plumbagin could not be estimated, as it would require a more sophisticated procedure like HPLC (High Performance Liquid Chromatography).

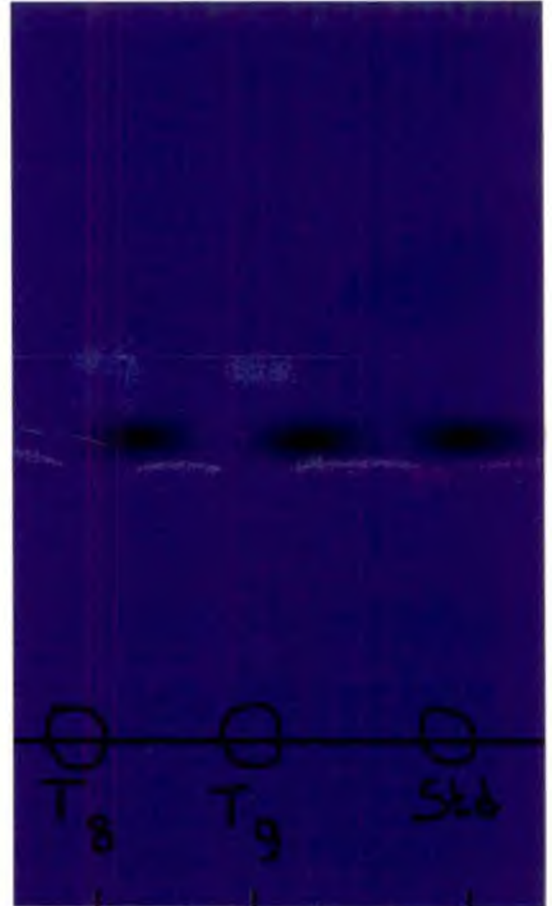
**5.3.3 Callus -mediated organogenesis**

Only leaf discs were used for callus induction studies, as leaf explants alone had responded during standardisation. Further studies on callus induction were carried out using auxins (2, 4-D, NAA), phloroglucinol and cytokinins (BA, TDZ) individually in the basal medium. Callusing was observed in all the treatment combinations containing auxins. However, phloroglucinol was ineffective in inducing callus when used along with auxins. Among cytokinins only TDZ induced callusing in leaf cultures of *P. rosea*. Callusing was observed only under complete dark conditions. In *Taxus* species, the auxins 2, 4-D and NAA had induced higher amount and rate of callus production in complete dark conditions compared to a 16h light/8-h dark day/night regime (Wickremsinhe and Arteca, 1998).

Callusing was initiated in treatments with varying concentrations of auxins (2, 4-D, NAA) in one to two weeks and the texture of the callus was soft and friability varied from wet to semi-friable. However, the callus started initiating small white roots after six weeks of incubation (Plate 20). Similar morphogenic response of callus cultures maintained in MS medium containing 2,4-D (2-4 mg $l^{-1}$) under dark conditions was reported by Paul (2006) and Suma and Keshavachandran (2007) in




TC Conventional Standard




TC Conventional Standard

TLC plate under visible light

TLC plate under UV

Plate 19. TLC comparison of plumbagin from *in vitro* regenerated plants and conventional plants of *P. rosea vs* standard plumbagin

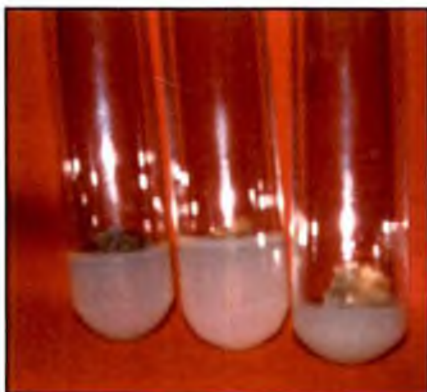
**Four weeks old cultures** | **Six weeks old cultures**

**MS+ 2, 4-D (1.0, 2.0, 3.0 mg $l^{-1}$)**

**Rhizogenesis**

**MS+ NAA (1.0, 2.0, 3.0, 4.0 mg $l^{-1}$)**

**Rhizogenesis**



**½ MS+ NAA (1.0, 2.0 mg $l^{-1}$)**

**Rhizogenesis**

**Plate 20. Callus induction and regeneration in varying levels of auxins**

ginger. Rhizogenesis of callus produced in increased concentrations of NAA has been reported in leaf explants of *Andrographis alata* (Nagaraja *et al.*, 2003) and in callus cultures *Withania somnifera* (Govindraju *et al.*, 2003). Nikam and Sawant (2007) also reported formation of hairy mass on the surface of callus of *Ceropegia sahyadrica* produced on medium containing IAA and NAA.

The best treatment for callus induction was found to be the medium containing TDZ. Of the four concentrations of TDZ (1.0, 1.5, 2.0, 2.5 mg $l^{-1}$) the treatment with 2.0 mg $l^{-1}$ produced maximum callus index.TDZ induced compact and nodular callus which turned green in regeneration medium containing BA (Plate 21). In the present investigation among all the growth regulators used individually or in combination, TDZ was identified as the best regulator for inducing organogenic callus. This is in accordance with a report of Tawfik *et al.* (1998) in *Rosmarinus officinalis* wherein high concentrations of TDZ (upto 2.0 mg $l^{-1}$) induced dark green compact callus in leaf, stem and shoot tip explants cultured on MS medium. The difference in the caulogenic response of the same explants in auxins and cytokinin may be due to the varying concentration of endogenous hormones and their interaction with the exogenously supplemented hormones (Singh, 2006).

When the compact callus was subdivided and transferred to medium supplemented with different concentrations of BA (0.2, 1.0, 2.0, 3.0, 4.0 mg l-1) multiple shoots were produced (Plate 22). The best medium for regeneration was identified as the one with BA 1.0 mg $l^{-1}$ and adenine sulphate 50 mg $l^{-1}$ which showed maximum proliferation of callus within 14 days and generated six shoots per callus in a single subculture after six weeks of incubation. The presence of Adenine sulfate was found favourable for shoot proliferation in callus cultures. Similar favourable response of adenine sulfate was reported by Chaplot *et al.* (2006) in leaf callus cultures of *P. zeylanica*. However, the shoots produced in concentrations of BA up to 4.0 mg $l^{-1}$ were short. On subsequent subcultures in increased concentrations of BA (5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 mg $l^{-1}$) lead to complete suppression of shoot multiplication as well as elongation (Plate 23) Preetha *et al.* (2007) made similar observation in callus culture from nodal explants of *P. rosea* which produced maximum shoots in MS medium supplemented with BA (2.0 mg $l^{-1}$) and higher concentrations of BA were found to suppress the growth of shoots Huettemann and

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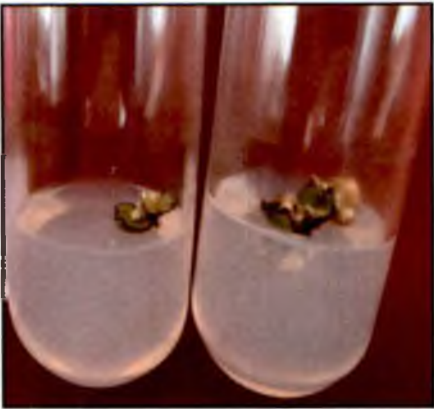


MS+ TDZ (1.0 mg $l^{-1}$)

MS+ TDZ (1.5 mg $l^{-1}$)

MS+ TDZ (2.0 mg $l^{-1}$)

MS+ TDZ (2.5 mg $l^{-1}$)

Shoot bud initiation in BA

**Plate 21. Callus induction and regeneration in varying levels of cytokinins**

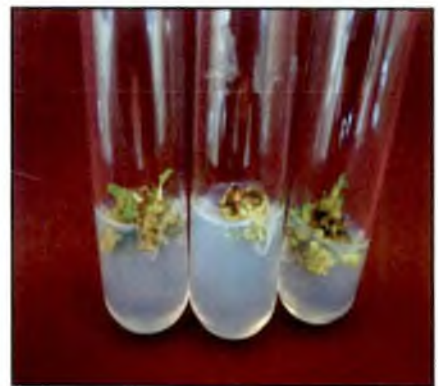
MS+ BA (1.0 mg $l^{-1}$) + Ads (1.5 mg $l^{-1}$) (50 mg $l^{-1}$)

½ MS+BA (2.0 mg $l^{-1}$) semi-solid medium

½ MS+BA (2.0 mg $l^{-1}$) liquid medium

MS+BA (3.0 mg $l^{-1}$)

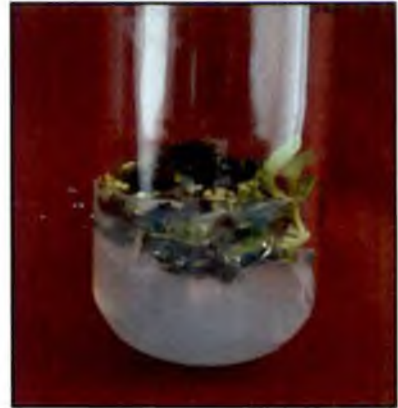
MS+BA (4.0 mg $l^{-1}$)



MS+BA (0.2 mg $l^{-1}$) +IAA (0.1 mg $l^{-1}$)

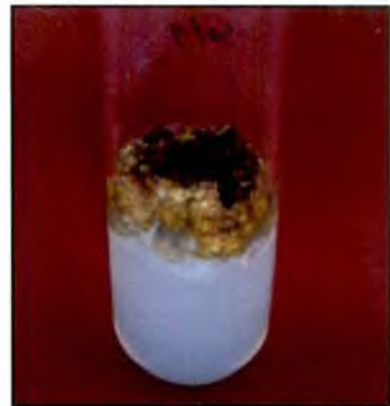
Plate 22. Multiple shoot initiation in callus cultures with varying levels of BA

MS+ BA (5.0 mg $l^{-1}$)



MS+ BA (6.0 mg $l^{-1}$)

MS+ BA (7.0 mg $l^{-1}$)



MS+ BA (8.0 mg $l^{-1}$)

MS+ BA (9.0 mg $l^{-1}$)

MS+ BA (10.0 mg $l^{-1}$)

**Plate 23. Suppression of shoot elongation in increasing levels of BA**

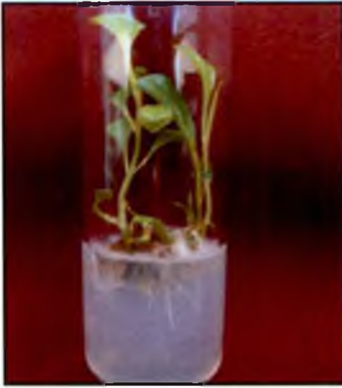
Preece (1993) have reported thidiazuron to lead to formation of short shoots *in vitro* in several woody species. Similar reports were also made by Ledbetter and Preece (2004) and Corredoira *et al.*, 2008. Cytokinins commonly stimulate shoot proliferation and inhibit their elongation. Therefore, inhibition of shoot elongation by TDZ may be consistent with its high cytokinin activity.

Elongation of shoots was achieved only in the medium containing $GA_3$ (0.1 mg $l^{-1}$). In the present case, $GA_3$ not only promoted shoot elongation but also rooting. Huettemann and Preece (1993) suggested transfer of shoot cultures to a secondary medium lacking TDZ or with a different balance of growth regulators for overcoming the problem of shoot elongation. In general $GA_3$ promotes elongation but often prevents shoot and root formation. So, in the present investigation the medium containing $GA_3$ might have served as secondary medium suppressing the 'carry over' effect of cytokinins from the shoot proliferation medium leading to elongation and ultimately to rooting of shoot cultures as well. The effective concentration of $GA_3$ for shoot elongation and rooting was 0.1 mg $l^{-1}$ in liquid as well as semi-solid MS medium (Plate 24). Even though $GA_3$ is rarely known to induce morphogenesis, Ochatt *et al.*, (1988) reported successful rooting of shoots regenerated from internodal callus cultures Oxalis species in half strength liquid MS medium containing 0.1 mg $l^{-1}$ $GA_3$.

#### 5.3.3.1 Hardening and acclimatisation of *in vitro* plants obtained through callus mediated organogenesis

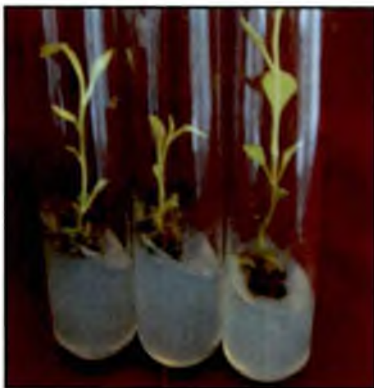
In a period of eight weeks, maximum percentage of *in vitro* plants (66.7 to 71.4 per cent) could be obtained from the medium containing 0.1 mg $l^{-1}$ $GA_3$ after two stage hardening (Fig.8). The hardening rate was higher in callus mediated organogenesis when compared to direct organogenesis form nodal cultures. Since there was very low microbial contamination of callus cultures, it produced maximum number of plants. With increased rate of successful hardening, maximum recovery of hardened plants could be achieved through callus mediated organogenesis.

Satheeshkumar and Bhavananadan (1988) reported 60 per cent survival of plants regenerated from callus mediated organogenesis in stem cultures of *Plumbago rosea*. However, later on Satheeshkumar and Seeni (2003) reported 95 to 98 per cent



**MS+ $GA_3$ (0.1 mg $l^{-1}$) semi-solid medium**

**MS+ $GA_3$ (0.1 mg $l^{-1}$) liquid medium**

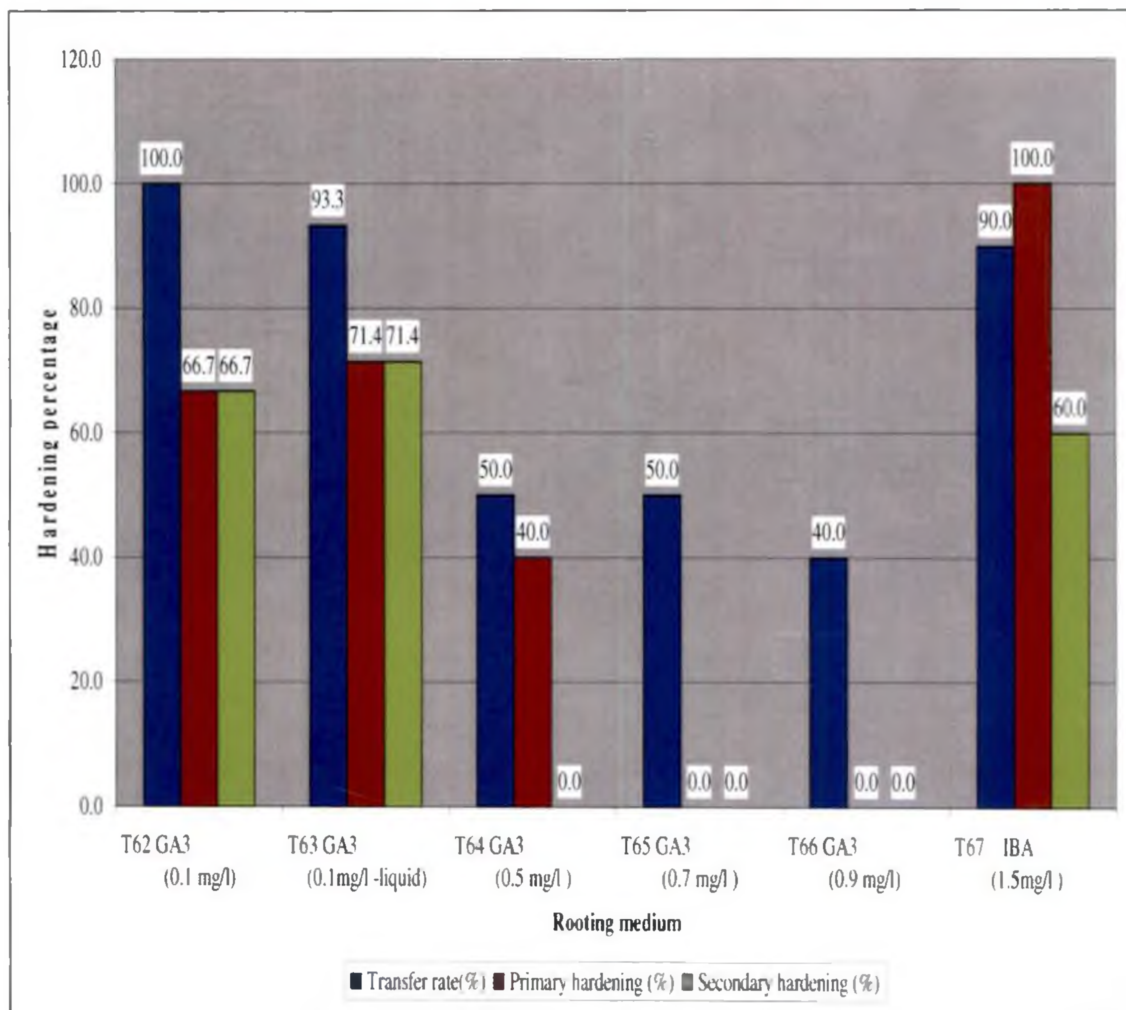


**MS+$GA_3$ (0.3, 0.5, 0.7, 0.9 mg $l^{-1}$)**

**MS + IBA (1.5 mg $l^{-1}$l)**

**Plate 24. Shoot elongation and rooting in callus cultures of *P.rosea***

**Fig. 8 Hardening percentage of callus regenerated plants from *P. rosea***



establishment of rooted plants obtained from indirect organogenesis in young stem, leaf and root of *P. rosea* that too without hardening in a period of four weeks.

### 5.3.4 *In vitro* mutagenesis

Mutations are defined as heritable changes in DNA segment that are not produced from genetic segregation or recombination (Van Harten, 1998). Genetic variation can be induced either by specific treatment with physical and chemical mutagens or by tissue culture. It can also be achieved by combining the techniques of mutation and tissue culture. Physical or chemical mutagens can be used on undifferentiated tissues and organs without preformed axillary buds either prior to regeneration or in different stages of adventitious meristem differentiation; thus adding somaclonal variation source to the mutagen effects (Predieri, 2001).

The probability that the technique of mutation induction will lead to obtaining useful material is highly dependent on the initial choice of plant material and on its handling throughout the whole selection process. When the aim of a breeding program is to improve only one or few traits of an existing cultivar without undesired additional variations, the most suitable method may be the treatment and propagation of *in vitro* axillary shoots without passage through undifferentiated growth. However, the frequency of variants obtained is low. Mutation frequency is expected to increase considerably in mutagenic treatments coupled with passages through undifferentiated growth phases. The probability of unwanted additional variations in the selected material also increases (Predieri, 2001). Hence, taking into consideration the above mentioned facts, in the present investigation both nodal and callus cultures were subjected to mutagenesis. Since the major objective of this study was improvement of plumbagin content, selected accessions of *P. rosea* having improved root characteristics namely IC-566503, IC-566508 and IC-566514 were used. The mutation of nodal cultures was aimed at inducing variability specifically for plumbagin content. Callus cultures were used for obtaining maximium frequency of mutation. Both physical and chemical mutagens were used for *in vitro* mutation. Ionizing radiation penetrates deeper into the tissue and can induce a great number of chemical changes. Gamma rays, the most widely used ionizing radiation were used for physical mutagenesis. As compared with physical mutagens, chemical mutagens may give rise to relatively more gene mutations rather than chromosomal changes.

Alkylating agents are considered to be most useful for mutation induction in plants. Ethyl methane sulphonate (EMS) the most commonly used alkylating agent was used for the present investigation. The results of both physical and chemical mutagenesis are discussed below

**5.3.4.1 Physical mutagenesis**

**5.3.4.1.1 Estimation of $LD_{50}$ for nodal and callus cultures**

One of the first steps in mutagenic treatments is to identify appropriate dose to apply. It involves in the determination of dose that can cause 50 per cent reduction of vegetative growth of treated material ($LD_{50}$) when compared to the control (Gaul, 1977). Fixing of the appropriate dose of mutagen is done by estimation of $LD_{50}$ values. Optimum dose is chosen from the subjected doses of each mutagen below $LD_{50}$ value (Abraham, 2002).Optimum dose is the one that gives maximum number of desirable mutants with minimum killing.

Based on the shoot and root regeneration percentage in the irradiated nodal and callus cultures LD50 dose was fixed for gamma rays. There was shoot regeneration and root induction in eighty per cent nodal cultures subjected to 10 Gy and 0 Gy (control) irradiation (Fig. 9). Fifty percent nodal cultures showed shoot regeneration up to 30 Gy but very poor or delayed rooting for the dose 20 Gy and no rooting for the dose 30 Gy (Plate 25). Hence, the LD50 for nodal cultures was 20 Gy and three doses below 20Gy, such as 5, 10 and 15 Gy were fixed as optimum doses.

In case of callus culture, shoot regeneration was observed in the order of 100 per cent, 90 per cent, 80 per cent and 70 per cent for cultures subjected to 0Gy (control), 10 Gy, 20 Gy and 30 Gy radiations (Fig. 10). The shoots regenerated showed elongation as well as rooting. However, 50 per cent of the cultures subjected to 40 Gy irradiation showed rooting without shoot elongation (Plate 26). Hence, the $LD_{50}$ was fixed as 40 Gy for callus cultures and three doses such as 10, 20 and 30 Gy were fixed as optimum doses. Thus, the nodal and callus cultures differed in radiosensitivity. Such an observation was also made by Walther and Saucer (1985) in the *in vitro* cultures of Prunus avium. The sensitivity found for shoot apices (22 Gy) was different to the one observed in basal parts (dose 29 Gy), indicating that the physiological factors can induce a difference in sensitivity even among the various

**Fig. 9 Estimation of $LD_{50}$ for nodal cultures**

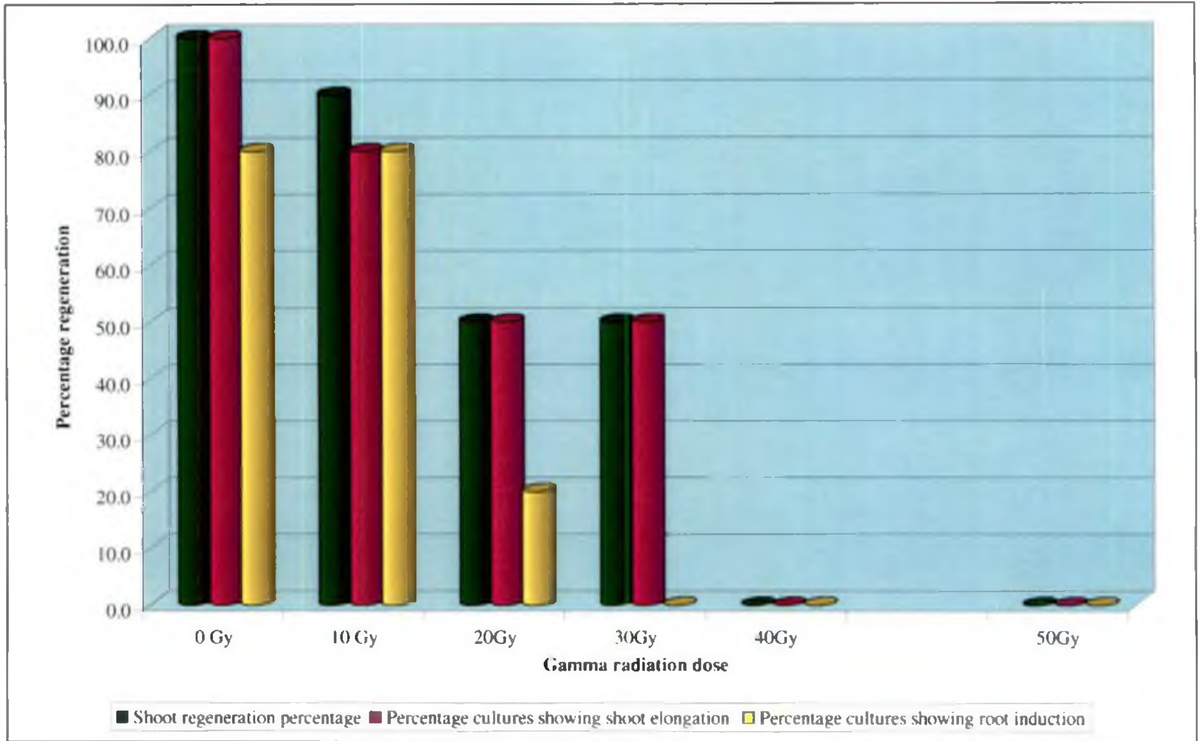



**Fig.10 Estimation of $LD_{50}$ for callus cultures**

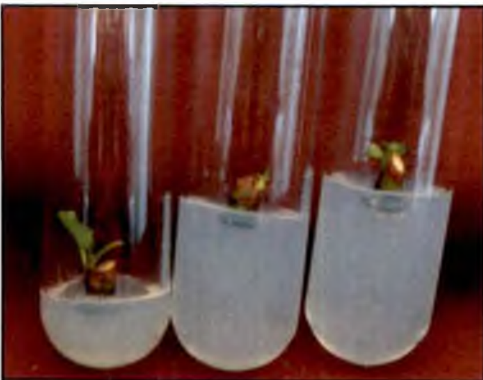
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Regeneration percentage
0 Gy
10 Gy
20Gy
30Gy
40Gy
50Gy
Gamma radiation dose
Shoot regeneration percentage
Percentage cultures showing elongation& rooting

$D_0$- 0Gy (control)

$D_1$- 10Gy

$D_2$ - 20Gy

$D_3$ - 30Gy



$D_4$ - 40Gy

$D_5$ - 50Gy

**Plate 25. Regeneration in nodal cultures for estimation of $LD_{50}$ dose of gamma radiation**

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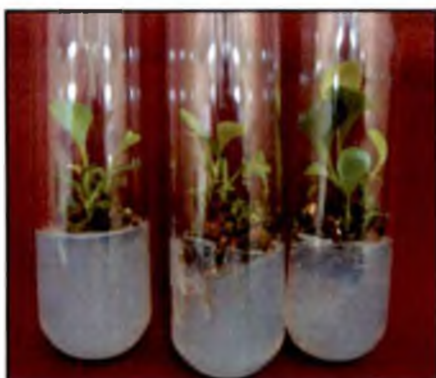
$D_0$- 0Gy (control)

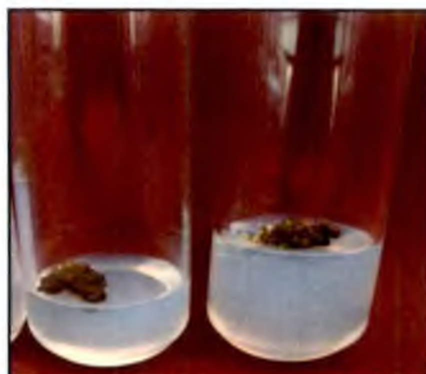
$D_1$- 10Gy



$D_2$ - 20Gy

$D_3$ - 30Gy

$D_4$ . 40Gy



$D_5$ . 50Gy

**Plate 26. Regeneration in callus cultures for estimation of $LD_{50}$ dose of gamma radiation**

axillary buds of a single microcutting. Radiosensitvity varies with the species and cultivar, with physiological condition of the plant and organs, and with the manipulation of the irradiated material before and after mutagenic treatment (Briggs and Konzak, 1977; D'Amato, 1992).

In the present study, lower doses of radiation stimulated culture growth and higher doses inhibited growth of cultures. In experiments of irradiation carried out with other species the effects of gamma radiation ($LD_{50}$ on the basis of survival, growth, proliferation rate, etc.) were very variable depending not only on the kind of explants and genotype utilized for each species but also developmental stage of the cultures. In *Datura innoxia* growth of callus cultures was stimulated at 0.2 kR dose of gamma-irradiation but it decreased as radiation dose increased with complete inhibiton of growth at 5 kR (Jain *et al*., 1984). Shylaja (1996) reported inhibition of callus growth at higher doses of 40 and 50 Gy on gamma irradiation of callus cultures in black pepper. Gavidia and Bermudez (1999) assessed the radiosensitivity of *in vitro* generated shoot tips of *Digitalis obscura* and determined the $LD_{50}$ around 60 Gy. KAU (2001) reported the highest dose of gamma radiation that *in vitro* ginger sprouts could withstand as 20 Gy.

**5.3.4.1.2 Regeneration of nodal and callus cultures at optimum doses of radiation**

**Nodal cultures**

The plant regeneration percentage from irradiated nodal cultures was very low due to high contamination following transfer to the proliferation medium after irradiation. The contamination was compounded on further subculture to shoot elongation and rooting medium. The percentage cultures showing shoot regeneration ranged from 45 to 50 for the optimum doses in callus cultures of the three accessions. The percentage cultures showing shoot elongation ranged from 25 to 40 per cent in accession IC-566503, 20 to 50 per cent in accession IC-566508 and 20 to 40 per cent in accession IC-566514 (Fig. 11).The number of rooted plants obtained for hardening, ranged from 10 to 15 per cent in accession IC-566503, 20 to 45 per cent in accession IC-566508 and 10 to 20 per cent in accession IC-566514. There was further reduction in the recovery of plants on two stage hardening (Fig. 12). Loss of cultures after mutagenic treatment due to contamination is occasionally reported (Shen *et al*., 1990),

**Fig. 11 Regeneration of nodal cultures for optimum doses of irradiation**

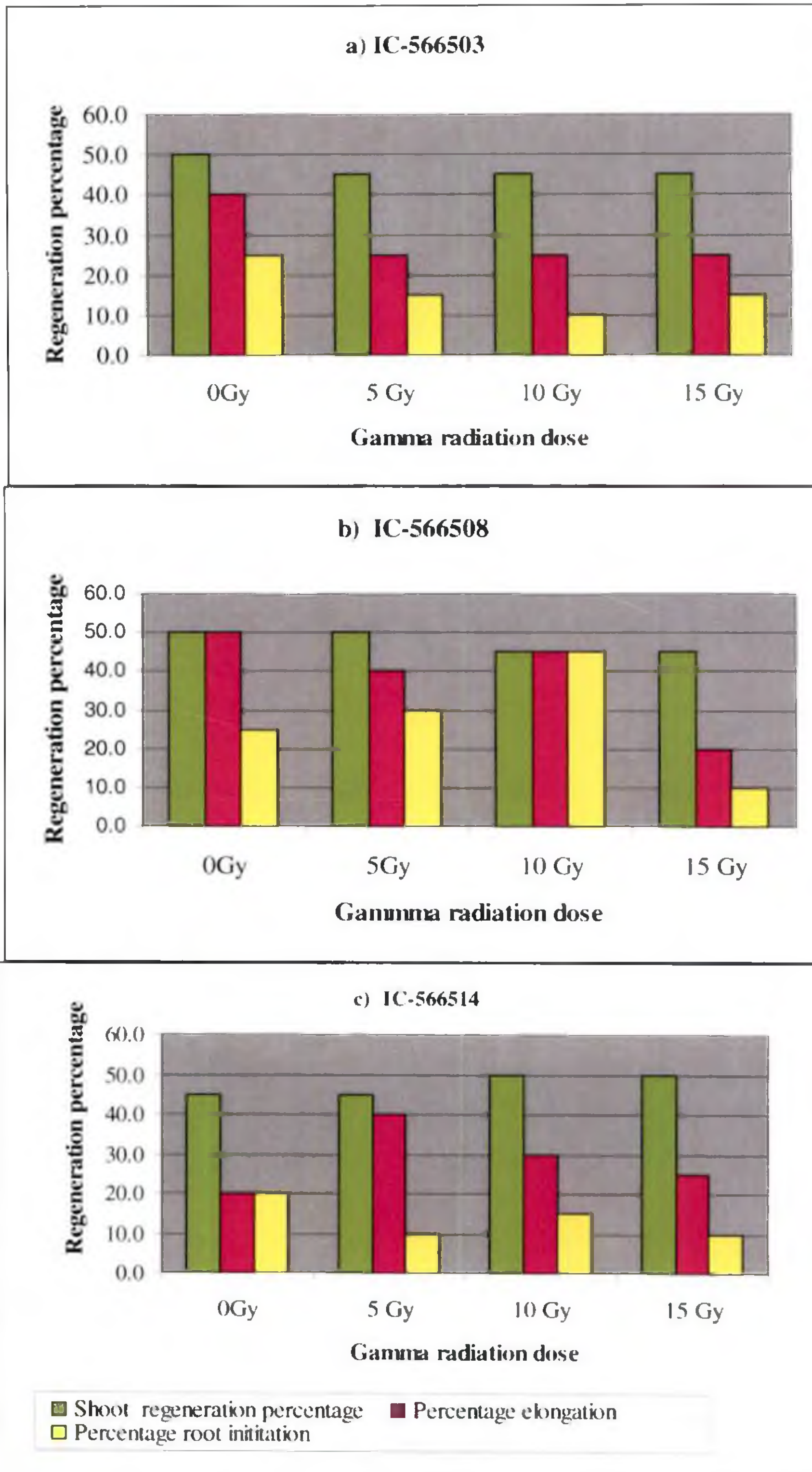
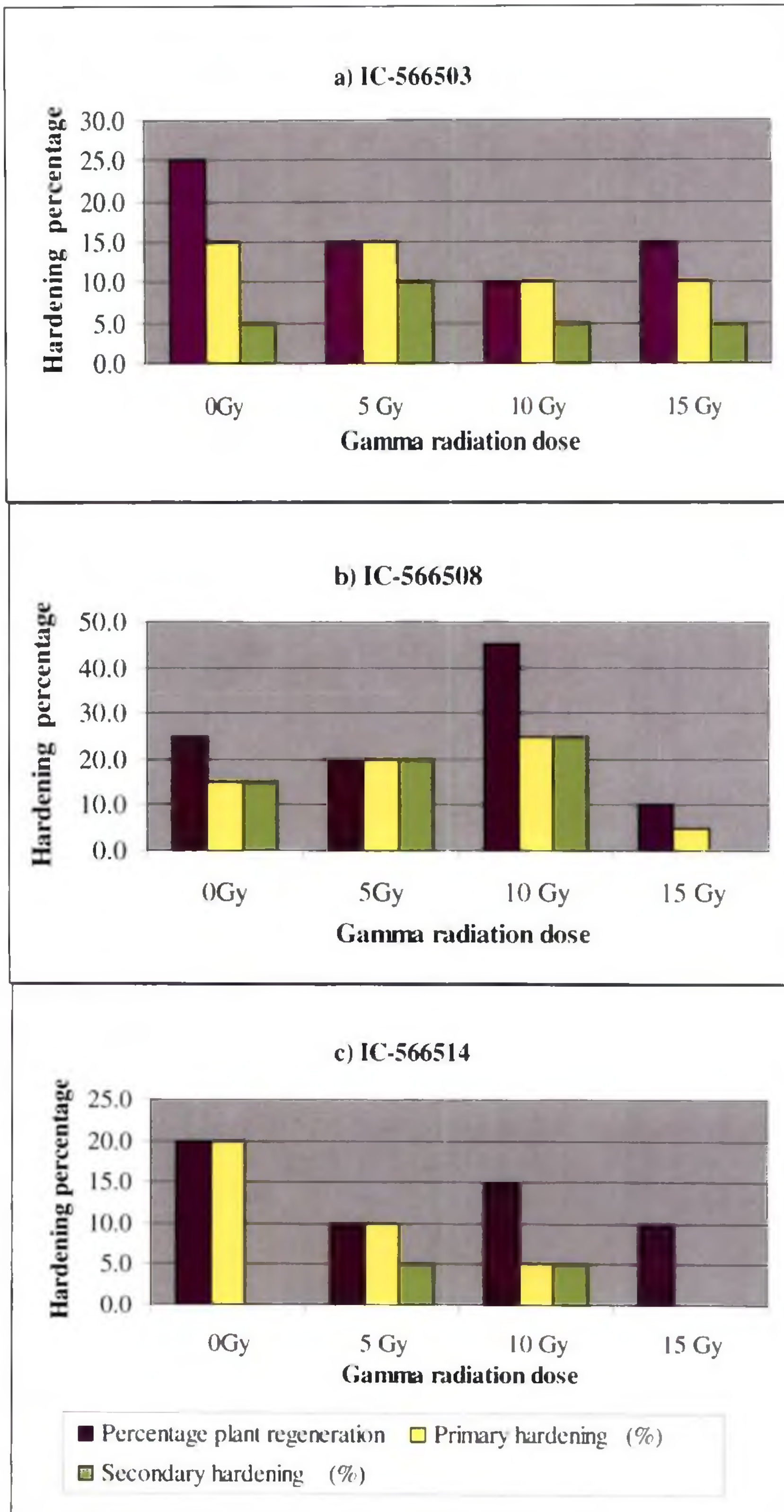


**Fig. 12 Hardening recovery from irradiation of nodal cultures**



but should be considered as a frequent cause of the perturbation of the tissue cultured material's physiological responses.

**Callus cultures**

The irradiated callus cultures of all the three accessions showed multiple shoot regeneration for the optimum doses (10, 20, 30 Gy) of gamma radiation. The percentage of shoot regeneration for optimum doses of radiation ranged from 60 to 100 in accessions IC-566503 and IC-566508. In the accession IC-566514, 70 to 100 per cent cultures showed shoot regeneration for optimum doses of radiation. The regenerated shoots from 10 Gy and 20 Gy irradiation showed elongation as well as rooting in more than 70 per cent cultures of the three accessions. The cultures of 30 Gy irradiation showed a decrease in shoot elongation as well as rooting with less than 40 per cent cultures exhibiting elongation along with rooting. (Fig.13). However, cultures with short shoots and poor rooting when transferred to liquid basal medium with $GA_3$ (0.1 mg $l^{-1}$) showed improvement in shoot elongation and rooting.

The accessions differed for the number of plantlets produced per dose of radiation (Fig. 14.). In the accession IC-566503, callus cultures of 10 Gy irradiation regenerated maximum number of plantlets (33 ) and callus cultures of 30 Gy produced minimum (9).The percentage of plant regeneration from irradiated callus piece ranged from 45 ( 30 Gy) to 165 (10 Gy). In accession IC-566508 the maximum number of plantlets was produced by control treatment (40) and the minimum by cultures of 30 Gy (6) .The percentage of plantlets produced from callus irradiated ranged between 30 (30 Gy) and 200 (0 Gy). In accession IC-566514, the cultures of control treatment (0 Gy) produced maximum number of plantlets (32) and the cultures of 30Gy irradiation produced minimum number of plantlets (10). The plant regeneration percentage ranged from 50 (30 Gy) to 160 (0 Gy).

The effect of radiation on the morphology of the *in vitro* regenerated plantlets varied in each accession. The plantlets produced by callus cultures of the accession IC-566503 showed variation shoot length (Plate 27).The plants of 30 Gy had maximum shoot length. The callus cultures of IC-566508 (Plate 28) produced plantlets with varying root length per dose of radiation. In accession IC-566508 the control plantlets of IC-566508 had maximum root length. In accession IC-566514, the

**Fig. 13 Regeneration of callus cultures at optimum doses of irradiation**

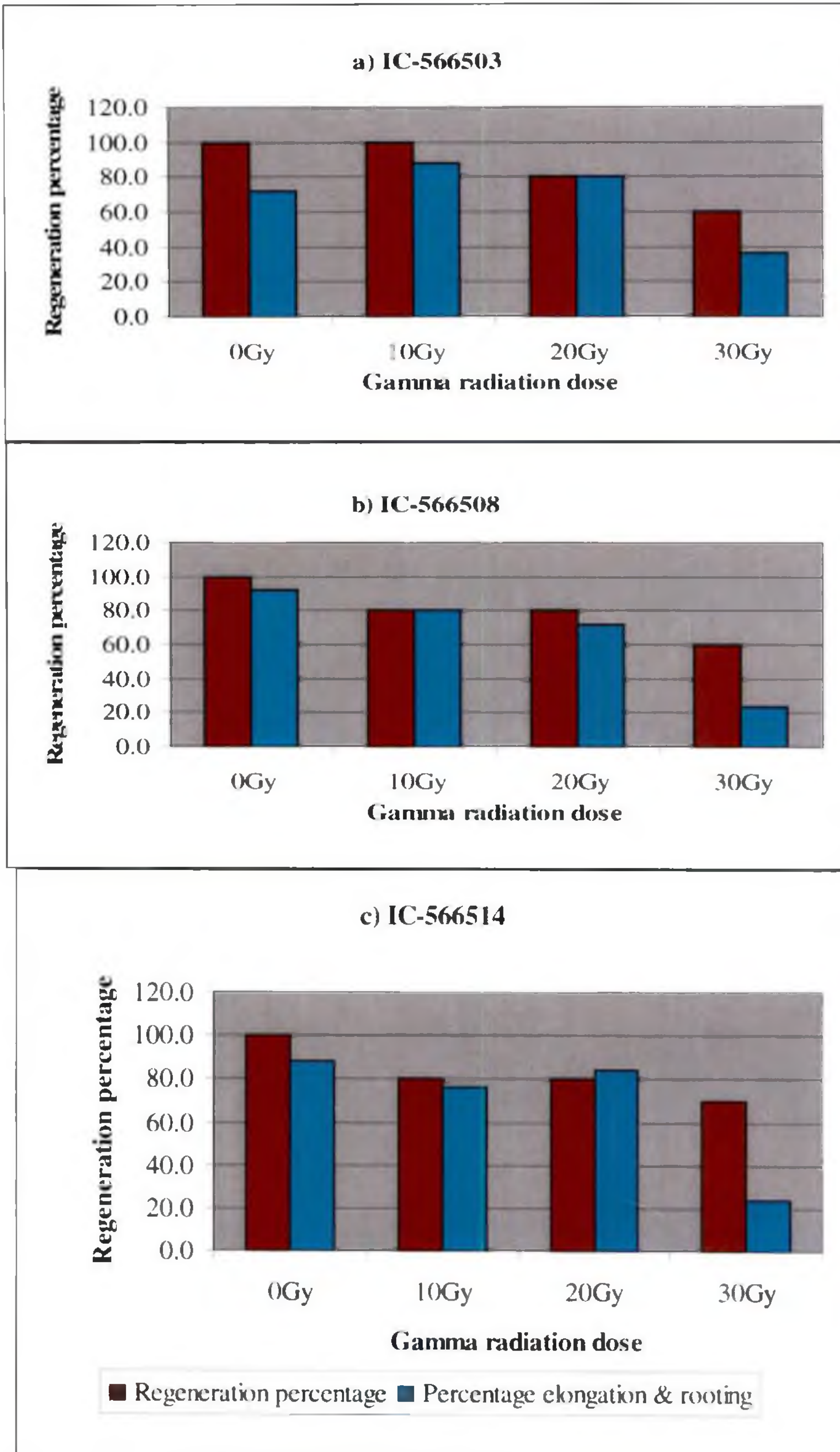
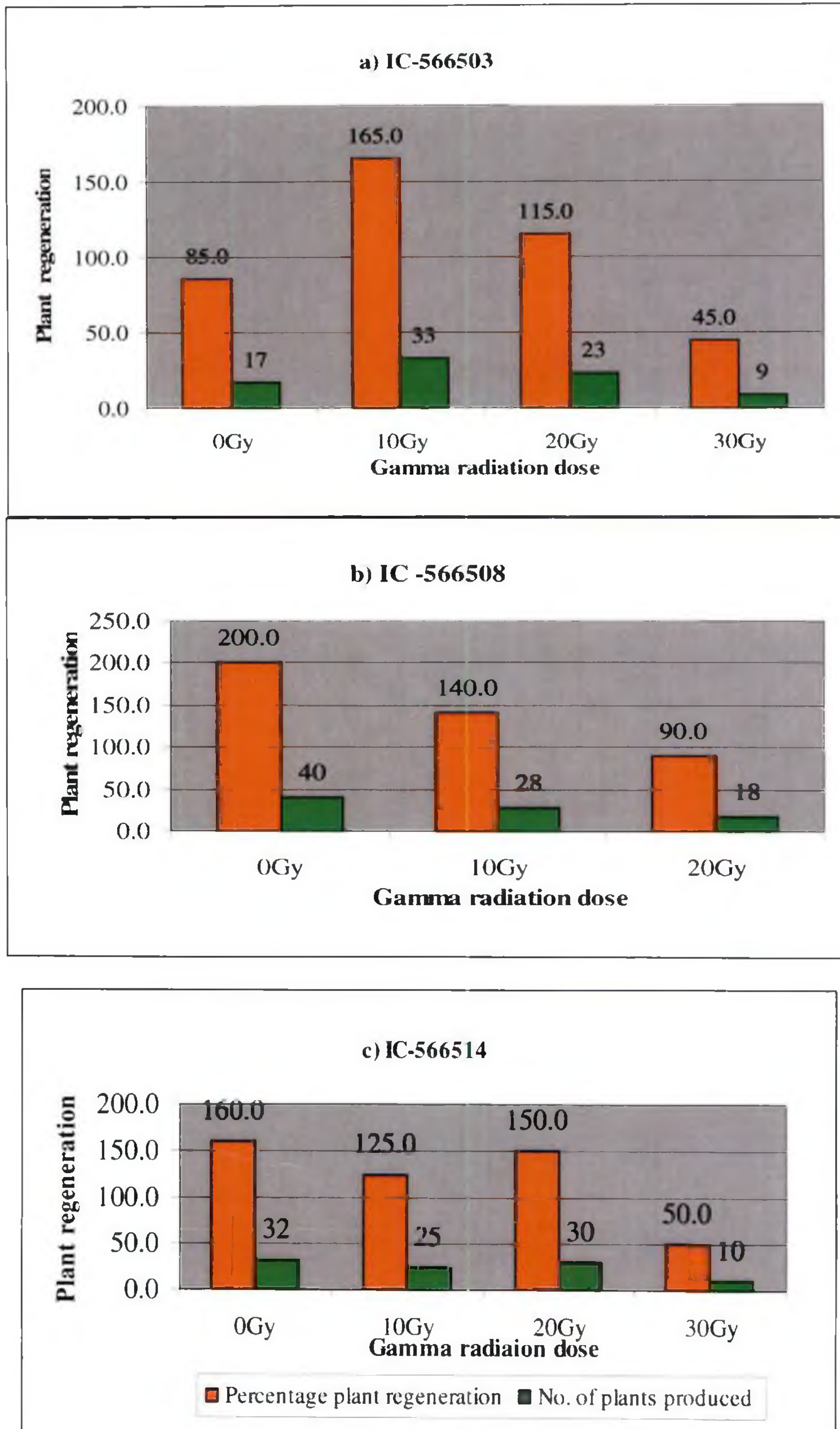
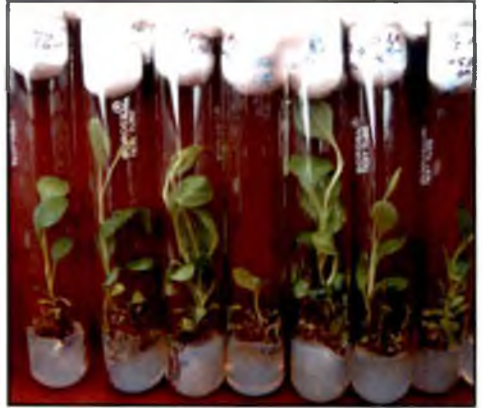


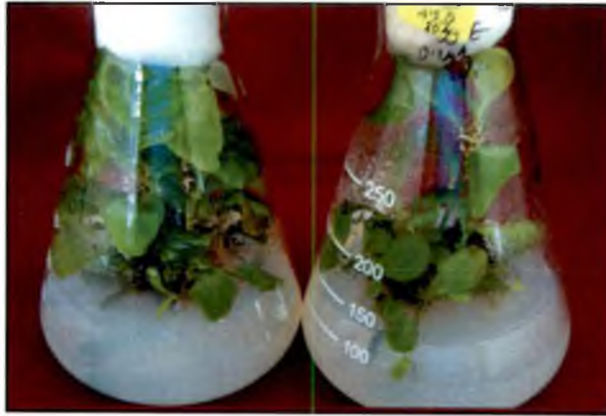
**Fig. 14 Plants obtained from callus cultures at optimum doses of irradiation**



$D_0$- 0Gy (control)



$D_1$- 10Gy



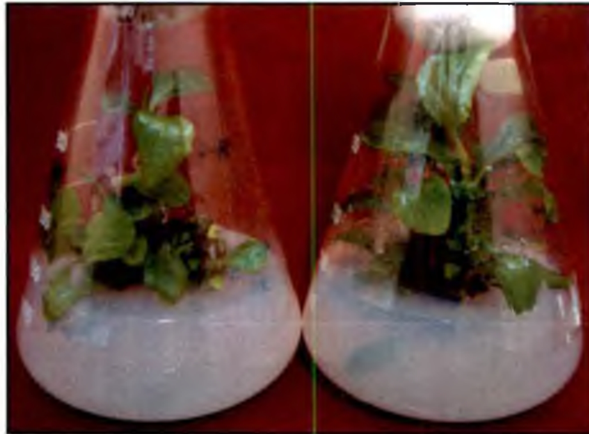
$D_2$ - 20Gy

$D_3$ - 30Gy

**Plate 27. Plants regenerated from callus cultures of IC-566503 at optimum doses of gamma radiation**

$D_0$- 0Gy (control)

$D_1$- 10Gy



$D_2$- 20Gy

D3-30Gy

**Plate 28. Plants regenerated from callus cultures of IC-566508 at optimum doses of gamma radiation**

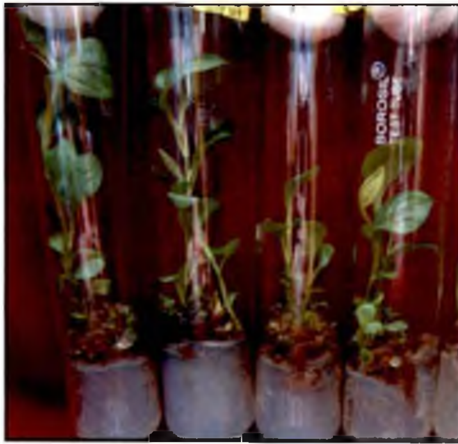
plantlets produced by irradiated callus cultures showed variation for shoot length, root length and number of leaves (Plate 29).

The callus cultures of the two accessions IC-566503 and IC-566514 had regenerated short shoots at 30 Gy. These shoots were transferred to liquid basal medium which resulted in maximum elongation. In accession IC-566514 similar phenomenon was observed in cultures of 10 Gy as well which were also transferred to liquid rooting medium. Similar difference in radio sensitivity and post radiation recovery were observed by Novak *et al.* (1990), (Karmarkar *et al*., 2001 and Obeidy *et al.* (2002) in the *in vitro* cultures of banana cultivars. The differences in the effect of gamma radiation on the development of plantlets of various accessions indicate that mutation is genotype dependent.

**5.3.4.1.3 *In vivo* selection of somaclones obtained from *in vitro* mutagenesis**

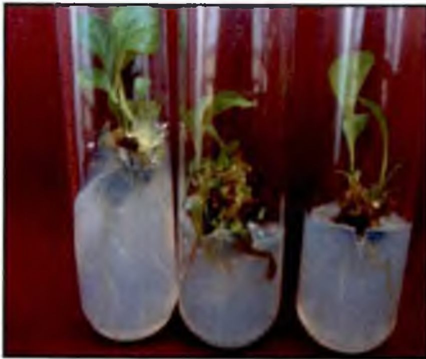
In general the plants obtained from *in vitro* regeneration are referred to as somaclones. The somaclones from *in vitro* mutagenesis of the three accessions were hardened in two stages, under laboratory (primary hardening) and net house conditions (secondary hardening) respectively (Plate 30). None of the somaclones of 30 Gy radiation survived after secondary hardening in all the three *P. rosea* accessions. The percentage survival of plants after secondary hardening in the three accessions ranged from 34.78 to 60.61 (IC-566503), 21.40 to 61.10 (IC-566508) and 36.67 to 56.00 (IC-566514) for 0, 10 and 20 Gy of radiation doses (Fig. 15).

The somaclones recovered from hardening were evaluated for five morphological characters namely, plant height, internodal length, number of leaves, leaf length and leaf breadth. Since it was difficult to carry over all the plants for field studies, somaclonal selection based on morphological evaluation was carried out in the net house. The somaclones per dose of radiation from three accessions showed variation for plant height, number of leaves and leaf size. The selection of somaclones was done on the basis of three characters namely, number of leaves, leaf length and leaf breadth. The main objective of this study being development of *Plumbago* types with high plumbagin content, the somaclonal selection was hence based on these three traits identified as selection parameters for high plumbagin content by path analysis. From accession IC-566503, which showed variation for plant height as well as leaf



$D_0$- 0Gy (control)

$D_1$- 10Gy



$D_1$- 10Gy

$D_2$ - 20Gy

$D_3$ - 30Gy

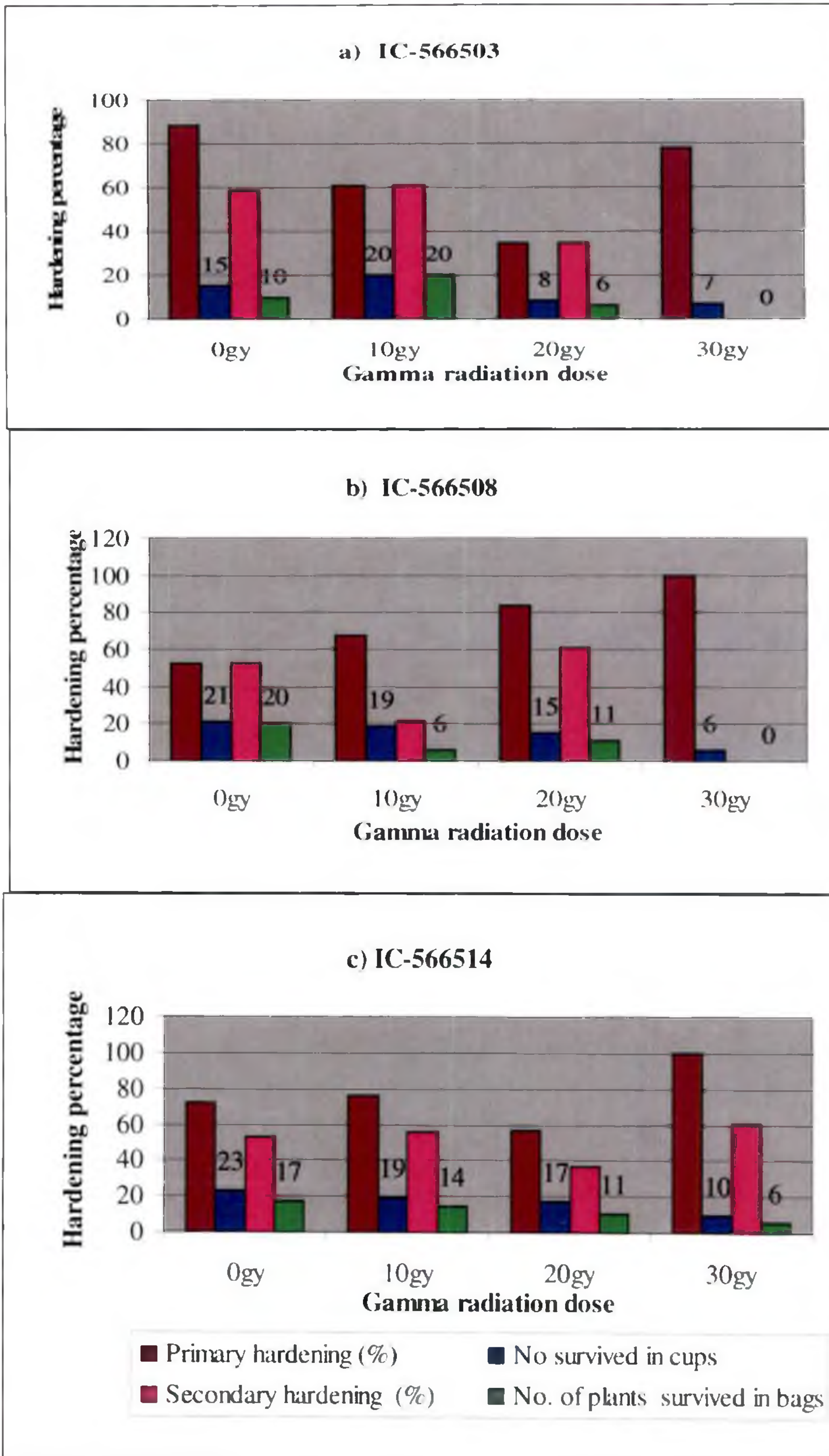
**Plate 29. Plants regenerated from callus cultures of IC-566514 at optimum doses of gamma radiation**

**Primary hardening**

**Secondary hardening**

**Plate 30. Hardening of plants obtained from *in vitro* mutagenesis**

**Fig.15 Hardening recovery from irradiation of callus cultures**



size, three plants were selected from three doses of radiation namely, $D_0$ (control), $D_1$(10 Gy) and $D_2$ (20 Gy) based on the superiority for these characters Similarly from accession IC-566508 and IC-566514 somaclones were selected based on superiority for number of leaves. The selected somaclones were carried over for replicated trial in field. Since the formation root specific compound plumbagin takes considerable time, evaluation of somaclones for plumbagin content must be entirely postponed until harvest stage. Although it is not possible to select among populations as large as those easily handled in tissue culture experiments, the responses observed *in vivo* are more reliable, since plants are grown in a setting that resembles more closely the actual field conditions. Since in the present investigation unequal number of plants were obtained from each dose of radiation in each accession, a strategy of somaclonal selection was adopted based on the morphological evaluation. Green house selection for tolerance to *Fusariumm oxysporium* f.sp. cubense has been reported following an *in vitro* treatment with gamma -ray radiation in *Musa* spp. AAA group (Bhagwat and Duncan, 1998).

#### 5.3.4.1.4 Comparison of somaclones obtained from *in vitro* mutagenesis with conventional rooted cuttings

*Plumbago* species when propagated through rooted cuttings require eighteen months of cultivation for evaluation of root characters and plumbagin content. Hence, as a part of preliminary investigation prior to main field trial, three month-old somaclones of three *Plumbago rosea* accessions selected for field evaluation studies were compared with the conventional rooted cuttings of the corresponding accessions for morphological characters. The somaclones showed significant variation for plant height, internode length, number of leaves and leaf size. The somaclones of control treatment and 10 Gy radiation dose in accession IC-566503 (Plate 31) were superior to conventional rooted cuttings for the four mentioned characters. The somaclones of control treatment and 20 Gy in accession IC-566508 (Plate 32) were superior to conventional rooted cuttings for number of leaves. However, the somaclones of accession IC-566514 (Plate 33) were at par with conventional rooted cuttings for all the morphological characters except leaf size.

$D_0$- 0Gy (control)

$D_1$- 10Gy

$D_2$ - 20Gy

Rooted cuttings

**Plate 31. Somaclonal variants and rooted cuttings of IC-566503**

$D_0$- 0Gy (control)

$D_1$- 10Gy

$D_2$ - 20Gy

Rooted cuttings

**Plate 32. Somaclonal variants and rooted cuttings of IC-566508**

$D_0$- 0Gy (control)

$D_1$- 10Gy

$D_2$ - 20Gy

Rooted cuttings

**Plate 33. Somaclonal variants and rooted cuttings of IC-566514**

#### 5.3.4.2 Chemical mutagenesis

*In vitro* mutagenesis using the chemical mutagen EMS could not be successfully conducted due to frequent contamination of cultures during the entire period of study. There was severe contamination in both nodal as well as callus cultures following treatment with EMS. The method of handling mutagen treated material may be the cause of contamination. Hence, further studies are required to standardise the procedure for *in vitro* application of chemical mutagen.

### 5.4 Induction of polyploidy in *Plumbago* species

Genetic improvement through conventional recombination breeding has limitations in *Plumbago* species because of complete absence of seed set in *P. rosea* and the difficult germination of seeds in *P. zeylanica*. In such situations induced mutation and polyploidy are the alternate methods for induction of variability. In experiment III variability in *Plumbago* species was induced through *in vitro* mutagenesis. Hence, in experiment IV induction of polyploidy in *Plumbago* species was attempted by in vivo application of colchicine.

#### 5.4.1 Methods of colchicine application

The method of colchicine application varies with species and also the mode of propagation. Since colchicine affects only dividing cells, it is applied when the tissues are actively dividing. In case of seed propagated crops, treating germinating seeds, treating whole seedlings or apical bud treatment of young seedlings using cotton swab are commonly followed. In case of vegetatively propagated and woody plants colchicine is generally applied directly or by soaking cotton swab placed on growing shoot apices or axillary /apical shoot buds. Cotton swab method of colchicine application was adopted in the present investigation wherein the terminal bud was removed and cotton swab placed in the immediate leaf axil was soaked with colchicine (0.75%) for fixed duration continuously for seven days (Plate 34.) Ajithmohan (1995) induced polyploidy in *Kaempferia galanga* wherein cotton swab was one of the methods used for colchicine application. Similarly, in *Hevea brasiliensis*, aqueous solution of colchicine (0.75 %) was applied to young bud sprouts using cotton swab method continuously for seven days (Sankariammal and Saraswathyamma, 2004).

*Plumbago rosea*

*Plumbago zeylanica*

**Plate 34. Cotton swab method of colchicine application**

A concentration of 0.75 % of colchicine was arrived at from preliminary trial. In the preliminary trial the survival rate of treated buds reduced with increasing concentration of colchicine as well as duration of treatment. In other words high lethality was noted with high dose of colchicine. Similar trend was reported by Dhawan and Tyagi (1989) in Hyoscyamus muticus and Ajithmohan (1995) in *Kaempferia galanga*.

**5.4.2 Morphological evaluation of colchicine-treated axillary branches**

The growth of colchicine- treated axillary branch was similar to control plants with no appreciable difference (Plate 35.). There was plant neither with gigas character nor reduced vigour in both the species of *Plumbago*. There was no appreciable difference between the size leaf epidermal cells as well as stomata of colchicine treated plants and control plants. There was normal flowering in colchicine-treated as well as control plants. Ajithmohan (1995) made a similar report of no appreciable difference between control plants and plants obtained from cotton swab method of colchicine treatment in *Kaempferia galanga*. In the present investigation also cotton swab method was ineffective in inducing polyploidy in *Plumbago* species. This may be due to less penetration of colchicine to the inner tissues of the axillary bud. Singh (1993) has suggested repeated treatment of colchicine at brief intervals to double the chromosome in large number of cells of shoot apex or growing axillary bud, since at a given time only a small proportion of cells would be in division. The polyploid and diploid cells present in a treated shoot-tip or axillary bud compete with each other and diploid cells may often out compete the polyploid ones. The degree of competition varies from species to species and may be more pronounced in perennial species like *Plumbago*.

There are different methods in vogue for application of colchicine. In the present investigation only cotton swab method was tried. Hence, further studies based on various other methods of colchicine application is required .At present no reports are available on colchicine induced polyploidy in *Plumbago* species. There are also no reports confirming the ploidy level of chromosome in the two species of *Plumbago* namely, *P. rosea* and *P. zeylanica*. For the deeper understanding of absence of seed

*Plumbago rosea*

*Plumbago zeylanica*

**Plate 35.Growth of colchicine treated buds in *Plumbago* species**

set in *Plumbago rosea*, there is need to establish the ploidy level of this species through meiotic studies.

**5.5 Field evaluation of somaclonal variants of *Plumbago rosea* obtained from *in vitro* mutagenesis**

The somaclonal variants obtained from *in vitro* gamma radiation of callus cultures of the three *P. rosea* accessions were evaluated in a replicated pot culture experiment (Plate 36). The variants had shown superiority over conventional clones at nursery stage but did not maintain this superiority on field establishment in the first season (four MAP). Mutation had induced drastic change in the plant stature and other morphological traits of these variants. When compared to conventional clones the *in vitro* mutants of the three accessions namely, IC-566503, IC-566508 and IC-566514 were dwarf with short internodes and small leaves (Plates 37, 38 and 39). The floral biology as well as ploidy of somaclones could not be studied since none of these flowered during the entire flowering season whereas the conventional rooted cuttings planted along with this flowered. The initial phenotypic observation of somaclones indicated a dwarf plant stature.

However, in the second season (eight MAP), the somaclones had shown significant difference for number of leaves and leaf size. Three *in vitro* mutants of 10 Gy irradiation belonging to three respective accessions *viz.*, IC-566503, IC-566508 and IC-566514 (Plate 40) could be identified. These were plant types of dwarf and bushy stature with increased number of large leaves. The major objective of this investigation being induction of variability for plumbagin content, these mutants were having plant type for high plumbagin content as identified by path analysis. However, the initial morphological evaluation needs to be correlated with the plumbagin production in the final harvest stage.This will be possible only when plants reach maturity. The assessment of plumbagin content in somaclones will thus suggest the efficiency of *in vitro* mutagenesis in inducing variation for this trait in *Plumbago rosea.*

The present study was undertaken with the objective of developing *Plumbago* genotypes for high plumbagin content as well as other desirable agronomic traits. With this goal in view mutation breeding under *in vitro* conditions and

**Four MAP**

**Eight MAP**

**Plate 36. Field evaluation of *in vitro* mutants and conventional plants of selected accessions**

polyploidization under in vivo conditions was carried out to induce variability for these traits. The plants obtained from selected accessions through *in vitro* mutagenesis showed significant variation for leaf number and size. When these mutants were raised in field showed wide variability with respect to plant type, stature and other morphological traits. The plumbagin content of these *in vitro* mutants have to be evaluated for commercial exploitation.

Based on information gathered from the present investigation, the following future line of work has been suggested:

i. Multiplication of the *in vitro* mutants for further evaluation for plumbagin content and other desirable traits.

ii. Evaluation of the *in vitro* mutants for floral traits

iii. Detailed morphological and molecular characterisation of existing genotypes and mutants

**Conventional rooted cutting**

**$D_0$- 0Gy (control)**

**$D_1$- 10Gy**

**$D_2$ - 20Gy**

**Plate 37. *In vitro* mutants and conventional rooted cutting of IC-566503 (Four MAP)**

Conventional rooted cutting

$D_0$- 0Gy (control)

$D_1$- 10Gy

$D_2$ - 20Gy

**Plate 38.** ***In vitro*** **mutants and conventional rooted cutting of IC-566508 (Four MAP)**

**Conventional plant**

**$D_0$- 0Gy (control)**

**$D_1$- 10Gy**

**$D_2$ - 20Gy**

**Plate 39.** ***In vitro*** **mutants and conventional rooted cutting of IC-566514(Four MAP)**

Rooted cutting of IC-566503

IC-566503 $D_1$ (10Gy)

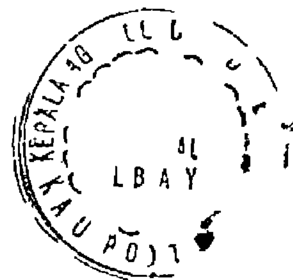
Rooted cutting of IC-566508

IC-566508 $D_1$ (10Gy)

Rooted cutting of IC-566514

IC-566514 $D_1$ (10Gy)

**Plate 40. *In vitro* mutants identified for superior plant type (Eight MAP)**



# Summary

# 6 SUMMARY

The present study entitled Assessment and induction of variability in *Plumbago* species for high plumbagin content was carried out in the Department of Plant Breeding and Genetics College of Horticulture Vellanikkara during 2005 2009

The programme envisaged exploration and collection of different genotypes of *Plumbago* species evaluation of genetic variability for various morphological and biometric traits including plumbagin content in the collected accessions and selection of accessions for inducing variability Induction of variability in the selected *Plumbago* accessions was attempted through *in vitro* mutagenesis and *in vivo* polyploidy The mutants obtained from *in vitro* mutagenesis were evaluated in the field The other aspects included in the study were studies on reproductive behavior of the two species of *Plumbago* and standardisation of *in vitro* regeneration The salient findings are presented below

1 Twenty five accessions of *Plumbago rosea* were collected from different districts of Kerala one accession of *Plumbago zeylanica* and one accession from of *Plumbago* capensis from Thrissur

2 Based on the passport data of collected materials IC (indigenous collection) numbers were assigned to 25 accessions of *P rosea* by NBPGR New Delhi

3 The analysis of variance in the 26 accessions of *Plumbago* revealed significant differences for all the characters except root length and root girth The *P zeylanica* accession had a maximum plant height with maximum number of branches

4 For all biometric traits including plumbagin content the *P rosea* accessions had higher values than *P zeylanica* Plumbagin content was significantly low in *P zeylanica* when compared to *P rosea*

5 High heritability coupled with high genetic gain was observed only for the character number of branches per plant

6 Correlation coefficient between plumbagin content and other traits exhibited significant negative genotypic association with the morphological traits such as number of branches plant height and number of leaves but a significant positive association with the biometric traits such as number of roots root girth and dry root weight

7 Path coefficient analysis of important yield and morphological traits indicated maximum positive direct effect of number of leaves followed by leaf size and dry root weight on plumbagin content

8 Based on cluster analysis three accessions *viz* IC 566503 IC 566508 and IC 566514 from the highly divergent clusters V VI and III respectively were selected for inducing variability based on their s iperior ranking for dry root weigl t and plumbagin content

9 Floral morphology and seed setting were studied in the two species of *Plumbago*

10 Peak period of anthesis was between 6 00 to 7 00 a m in *P zeylanica* whereas it was between 7 00 to 8 00 a m in *P rosea*

11 Anther dehiscence and stigma receptivity was found to coincide with flowei opening in *P zeylanica* In *P ı osea* the stigma became receptive only after flower opening and lost its receptivity before anther dehiscence which took place three hours after flower opening

12 Pollen grains of both the species did not germinate under *in vitı o* conditions

13 On artificial pollination the pollen grains of both the species showed least adhesion on the stigmatic surface of *P rosea* whereas abundant adhesion was noticed on the stigma of *P zeylanica*

14 The fertility of pollen of *P rosea* could not be confirmed as the seeds obtained from *P zeylanica* after artificial pollination with *P rosea* were poorly developed

15 *In vitro* regeneration could be standardised only in *Plumbago rosea*

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16 Among the different explants tried the nodal segments were found to be best for direct organogenesis and rapid multiplication and the leaf discs were suited for callus mediated organogenesis

17 The basal MS medium with 1 0 mg l $^{1}$ BA and 50 mg l $^{1}$ adenine sulphate considered best for direct shoot initiation in the nodal segments of *P rosea* seven days

18 The treatment consisting of 2 0 mg l $^{1}$ BA in half strength MS medium producing on an average of three shoots per culture in 45 days was identified as the best for multiplication of shoots from the nodal segments of *P rosea* The treatment consisting of BA (4 0 mg l $^{1}$) in basal MS medium was identified as the best for shoot elongation up to 5 0 cm in 30 days

19 The treatments consisting of 1 0 and 1 5 mg l $^{1}$ IBA respectively in half strength MS medium were identified as best rooting media for nodal segments

20 The *in vitro* regenerated plants of *P rosea* required two stage hardening for a period of eight weeks The percentage success of hardening was 22 2 per cent in case of plantlets regenerated from nodal segments

21 The *in vitro* regenerated plants had larger leaves and recorded high fresh and dry root weight than conventional plants of *P rosea* in field condition The qualitative analysis through thin layer chromatography emulated the presence of plumbagin on the silica gel plate with Rf 0 55 in both *in vitro* regenerated and conventional plants

22 Out of 23 treatments tried for callus induction in leaf discs of *P rosea* the treatments containing auxins such as NAA and 2 4 D produced rhizogenic callus Non embryogenic compact callus was produced in the medium containing TDZ (Thidiazuron)

23 The treatment with 2 0 mg l $^{1}$ TDZ was identified as the best treatment for callus induction showing callusing in 75 per cent cultures with a callus index of 225 in six weeks

24 The treatment consisting basal MS medium BA (1 0 mg l$^{-1}$) and AdS 50 mg l induced maximum average number of multiple shoots (6 0) six weeks after proliferation The multiple shoot regeneration medium had produced small shoots

25 The treatments with increasing concentration of BA from 5 0 mg l$^{-1}$ to 10 0 mg l$^{-1}$ suppressed shoot proliferation and elongation

26 The treatment with $GA_3$ (0 1 mg l$^{-1}$) both solid and liquid basal MS medium produced maximum shoot elongation and rooting

27 The percentage success of hardening after two stage hardening ranged betv een 60 0 to 70 0 per cent m case of callus regenerated plants

28 Induction of variability was attempted in *Plumbago* species through *in vitro* mutagenesis

29 *In vitro* mutagenesis using gamma radiation alone could be standardized in the three accessions of *P rosea*

30 Chemical mutagenesis could not be standardized due to high rate of contamination both in callus and nodal cultures

31 In case of physical mutagenesis the nodal and callus cultures differed in radio sensitivity The $LD_{50}$ for nodal cultures was 20 Gy and three doses below 20 Gy such as 5 10 and 15 Gy were fixed as optimum doses In case of callus culture the $LD_{50}$ was fixed as 40 Gy as cultures treated with this dose showed rooting without shoot elongation and three doses such as 10 20 and 30 Gy were fixed as optimum doses

32 The callus cultures of 30 Gy irradiation showed a decrease in shoot elongation as well as rooting with less than 40 % cultures exhibiting elongation along with rooting

33 The accessions differed for the number of plantlets produced per dose of radiation Only plantlets from mutated callus cultures of 10 Gy and 20 Gy dose could be successfully hardened

34 *In vivo* selection of somaclones obtained from *in vitro* mutagenesis was carried out after secondary hardening based on number of leaves and leaf size from each accession(IC 566503 IC 566508 and IC 566514) and three doses of radiation namely $D_0$ (control) $D_1$(10 Gy) and $D_2$ (20Gy) for field evaluation

35 Under field conditions the somaclonal variants in general showed dwarf stature and none flowered during the entire flowering season in contrast to the conventional rooted cuttings

36 Three *in vitro* mutants of *P rosea* showing significant variation for morphological traits were identified based on the plant type for high plumbagin content

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# ASSESSMENT AND INDUCTION OF VARIABILITY IN *PLUMBAGO* SPECIES FOR HIGH PLUMBAGIN CONTENT

By

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## ABSTRACT OF THE THESIS

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# ABSTRACT

The study entitled Assessment and induction of variability in *Plumbago* species for high plumbagin content was carried out in the Department of Plant Breeding and Genetics College of Horticulture Vellanikkara during 2005 2009 The main objective of the study was to induce variability in *Plumbago* species for high plumbagin content through *in vitro* mutagenesis A detailed survey was conducted throughout Kerala and accessions of *Plumbago* were collected from different ecogeographical regions ranging from Western Ghats to coastal regions of Kerala The species *Plumbago rosea* seemed to be mainly distributed in highlands and medium altitude especially in the midlands of Kerala The passport data of the collected accessions were prepared and indigenous collection(IC) numbers were obtained from NBPGR New Delhi Twenty five accessions of *Plumbago rosea* and one accession of *Plumbago zeylanica* were catalogued

Twenty six accessions thus collected were evaluated in replicated trial for genetic variability with respect to their biometrical traits and quality To know the causes of failure of seed set in *Plumbago rosea* floral biology of the two species of *Plumbago* was studied The data were subjected to statistical analysis and results interpreted The twenty six accessions were grouped into seven clusters which showed that there is no parallelism between the geographical distribution and clustering pattern The path coefficient analysis indicated that large sized leaves and increased dry root weight contributed maximum to plumbagin content Based on the ranking for dry root weight and plumbagin content three accessions were subjected to *in vitro* mutagenesis

*In vitro* regeneration through direct organogenesis and indirect organogenesis was standardised in *Plumbago rosea* in MS medium Nodal segments were identified as best explants showing direct regeneration The *in vitro* regenerated plants from nodal cultures on cultivation produced significantly large tuberous roots with moderate plumbagin content compared to conventional rooted cuttings The callus derived from leaves regenerated shoots and roots Thus mass multiplication of *Plumbago rosea* through tissue culture was standardised

*In vitro* mutagenesis of selected accessions of *P rosea* was successfully carried out Based on percentage of regeneration $LD_{50}$ values of 20 Gy and 40 Gy were fixed for nodal cultures and callus cultures respectively The hardened plants obtained from selected accessions through *in vitro* mutagenesis showed significant variation for leaf number and size Based on these characters variants were selected and carried over for field establishment in pot culture The tissue culture variants on field establishment showed wide variability with respect to its stature plant type and other morphological traits Three *in vitro* mutants having plant type with large leaves and short stature for high plumbagin content were identified The plumbagin content of these *in vitro* mutants have to be evaluated for its commercial exploitation